Morphological and phylogenetic study of *Hyalomma anatolicum* in Al-Najaf, Iraq

M.A.A. AL-Fatlawi, M.J. Ali and H.H. Albayati

Department of Parasitology & Microbiology, College of Veterinary Medicine, University of Al-Qadisiyah, Al-Diwaniyah, Iraq
Email: monyerr.abd@qu.edu.iq

(Received March 23, 2018; Accepted April 22, 2018)

Abstract

Studies had been previously conducted to genetically identify species of ticks in Iraq. Therefore, the current investigational study was conducted to recognize the species of 50 ticks collected from infested skin of cattle. The current study defined the ticks to be from *Hyalomma* genus depending on their morphological features. Using mitochondrial cytochrome oxidase subunit I (*Cox*I) gene, 16 ticks were further confirmed using polymerase chain reaction (PCR). Two PCR products were subjected to DNA sequencing to name the species of the ticks and compare them to some other known ticks in neighbor and world countries. The sequencing results identified the ticks to be *Hyalomma anatolicum.* One isolate is closely similar to Indian and Iranian isolates, and the other isolate is clustered alone by itself. The results indicated that *H. anatolicum* is one of the wide-spread ticks that affect cattle in Al Najaf province, Iraq.

Keywords: DNA sequencing, *Hyalomma anatolicum*, Iraq
Available online at http://www.vetmedmosul.com

دراسة مظهرية والنشوء والتطور للـ *Hyalomma anatolicum* في النجف العراق

منير عبد الامير عبد الفتلاوي، منصور جدعان علي و هديل هادي البياتي

فرع الطفيليات والاحياء المجهرية, كلية الطب البيطري, جامعة القادسية, الديوانية, العراق

الخلاصة

دراسات قليلة قد اجريت سابقا لتحديد انواع القراد في العراق جينيا. لذلك, اجريت الدراسة التحرية الحالية لتمييز انواع 50 قرادة مجموعة من جلد ماشية مصاب. الدراسة الحالية عرفت القراد ليكون من جنس *Hyalomma* اعتمادا على السمات المظهرية. باستخدام الانزيم الفرعي المؤكسد السيتوكرومي التابع لبيوت الطاقة (*Cox*1), 16 قرادة اثبتت اكثر باستخدام اختبار تفاعل البلمرة المتسلسل (PCR). اثنان من نواتج الـ PCR عرضت لاختبار معرفة تسلسل الـ DNA لتسمية انواع القراد ومقارنتهم مع بعض انواع القراد المعروفة الاخرى في بلدان الجوار والعالم. حددت نتائج اختبار معرفة تسلسل الـ DNA القراد على انها *Hyalomma anatolicum*. عزلة واحدة كانت مشابهة بشكل كبير لعزلات هندية وايرانية, ووقعت العزلة الاخرى لوحدها وبنفسها في فرع واحد في الشجرة. تشير النتائج على ان الـ *H. anatolicum* نوع من القراد المنتشر والذي يؤثر على الابقار في محافظة النجف, العراق.

Introduction

The prevalence of cattle-infesting ticks is very high in Al-Najaf City, Iraq, especially during the beginning of summer (1). Tick-borne diseases of cattle such as theileriosis and babesiosis are important due to the losses they cause to animal health and industries (2). These diseases record high presence in the Arabian Gulf regions and place severe impact on their economies (3). Ticks can be transmitted and spread out by various ways including but not limited to moving of animals from an area to another searching for food and water. In addition, importation of

animals from tick-endemic countries is also a main method to invade new regions (4,5).

Using primary tools such as macroscopic and microscopic examination to identify tick (6) may give unconfirmed results with no information about the tick epidemiological history. On other hand, DNA-based methods such as DNA sequencing follow up the primary procedures and supply details about the tick genetic background. However; researchers that use molecular entomology should also utilize primary methods in their studies (7). So tracking the tick isolate isolated from an area needs to employ genetic tools such as DNA sequencing to recognize the isolate origin and how it has been transmitted to the studied area (8).

Cox1 is a well-known genetic barcode that could be utilized to molecularly identify tick species, and using this unique gene have improved epidemiological and evolutional studies that deal with ticks (9). Therefore; the Cox1-based recognition of ticks helps entomologists reaching the species level of ticks and even more to differentiate between closely related isolates (10). Moreover, knowing the species of ticks using this gene helps to recognize the breeds of animals that these ticks infest on, and if there is any alteration in the tick adaption to parasitize on different animal breeds (11). Sequencing a reliable piece of this gene also allows researchers to track down the origin of ticks and how fast they acclimate to the climatic conditions in an invaded area (12).

In Iraq, few-genetic studies that pay attention to ticks and the possibilities behind their presence in this country. For these reasons, the current study was initiated to identify the presence of *Hyalomma anatolicum* that infests cattle in Al Najaf, Iraq and to see what known tick isolates that they match with from neighbor and world countries.

Materials and methods

Sample collection

In Najaf Province, 50 hard ticks were collected from infested skin of cattle brought to a slaughterhouse. The period of the study was from October 1 to December 1, 2017. The sampling procedures followed aseptic criteria by using sterile forceps and sterile containers. Then the samples were transported to the laboratory of parasitology, college of veterinary medicine, University of Al-Qadisiyah, Diwaniyah, Iraq, where they were examined and photographed for the external features and stored in -20°C until the genomic DNA extraction was performed (13).

Morphological Identification

The ticks were morphologically identified using a microscope and according to a textbook (13). Briefly, ticks were detected relying on genital aperture, mouth parts. The presence of Coxa I, Coxa II, and Coxa III in the ventral part of the ticks was also used to perform the process. There is the dorsal surface that has the festoons. In males, characteristics of ventral surface of *Hyalomma anatolicum* are represented by the presence of Coxa II, Coxa III, Coxa IV, accessory shield, anus, adanal shields, anal groove, and sub-anal shields.

DNA extraction

Using gSYNCTM DNA Extraction Insect kit (Geneaid, USA), the tick-genomic DNA was extracted. To initiate the process, 50 mg of tissues from each tick and liquid N_2 were placed in a mortar to thoroughly grind the tick tissues. Then, the ground tissue was transferred into 1.5 ml tubes. After that, 200µl GST Buffer and 20µl of proteinase K were added to the tubes and vortexed thoroughly. Next, the tubes were incubated at 60ºC for 1 hour. Finally, the DNA extraction was done according to the kit manufacturer instructions. The purified DNA was eluted using a buffer from the kit and stored at -20°C. The DNA was checked for quality and quantity using a NanoDrop spectrophotometer.

Polymerase chain reaction (PCR)

The primary morphological detection of the collected ticks indicated that they belong to *Hyalomma spp*. Therefore, the PCR technique was performed on 16 tick samples using *Cox1* gene of this genus. The primers are F, AGGGTCCCCAGATATAGCATT and R, ACCGCCTGAAGGGTCAAAAA that amplify a 415 bp piece of the *Cox1* gene. These primers were provided by Bioneer Company, South Korea. The PCR master mix was prepared using AccuPower® PCR PreMix kit (Bioneer, South Korea). The PCR pre-mix contains Taq DNA polymerase, $MgCl_2$ 1.5mM, KCl 30mM, Tris-HCl 10mM, dNTPs 250µM, a dye, and a stabilizer. The kit directions were followed as 5µl DNA, 1.5µl from each of the primers, and 12µl PCR water were mixed. The thermocycler conditions were 1 cycle of initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 30 sec, annealing at 58 °C for 30 sec, and extension at 72 °C for 1min and ended by 1 cycle of final extension at 72 °C for 5 min. Agarose gel 1% stained by ethidium bromide was used to test the PCR products by electrophoresis and then explored using a UV imager.

DNA sequencing

To identify the species of the ticks collected, two of the generated PCR products were sent to sequencing at Macrogen Company, South Korea using AB DNA sequencing system. The alignment analysis was carried out using NCBI-Blast Alignment. The phylogenetic trees were drawn using Mega 6.0 software.

Results

Using the external characteristic features of genus *Hyalomma* mentioned in (13), the 50 ticks collected showed morphologically identification of *Hyalomma spp*. Those morphological features are illustrated in the Figure 1-6. These key features were genital aperture, mouth parts. The presence of Coxa I, Coxa II, and Coxa III in the ventral part of the ticks was also as an indicator. There was the dorsal surface that has the festoons. In males, characteristics of ventral surface of *Hyalomma anatolicum* were represented by the presence of Coxa II, Coxa III, Coxa IV, accessory shield, anus, adanal shields, anal groove, and sub-anal shields. The PCR results confirmed that the 16 samples belonged to genus *Hyalomma*, Figure 7. Interestingly, the sequencing results indicated that the 2 PCR products are *Hyalomma anatolicum*. The sequencing resulted isolates were deposited in the NCBI-Genbank database as *H. anatolicum* HA-IQ1, MG551987, and *H. anatolicum* HA-IQ2, MG551988. The phylogenetic analysis placed the MG551988 isolate in a domain together with 2 Indian isolates (KJ912622 and KP792577), 1 Iranian isolate (KP219872), and 1 Iraqi isolate (KM235704) (14). Amazingly, the current study isolate, MG551988, revealed 100% identity with the mentioned Indian, Iranian, and Iraqi isolates. However, it showed very close similarity to the Indian (KJ912622) and Iranian (KP219872) isolates, table 1 and Figure 8.

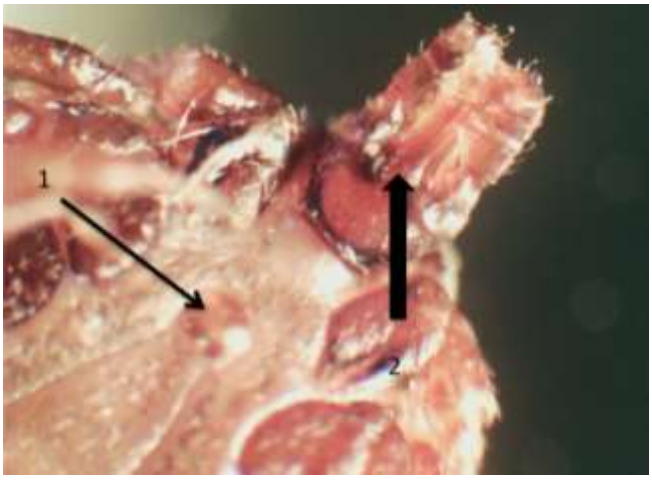

Figure 1: Ventral surface of *Hyalomma anatolicum*: 1. genital aperture 2. mouth parts. Olympus (2.5X).

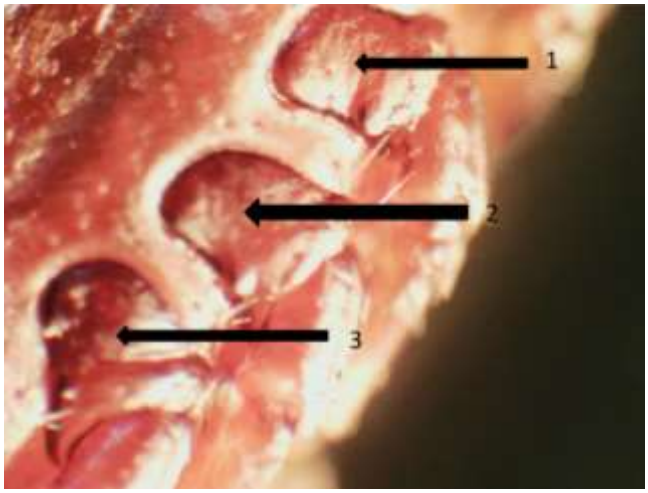

Figure 2: Ventral surface of *Hyalomma anatolicum*: 1. Coxa I, 2. Coxa II, 3. Coxa III. Olympus (2.5X).

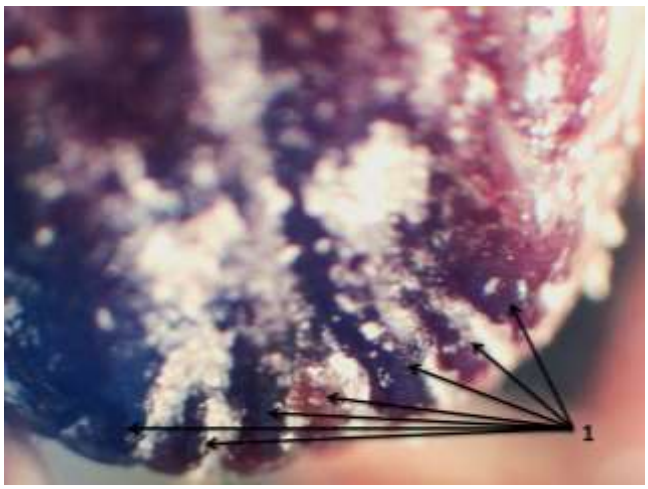

Figure 3: Dorsal surface of *Hyalomma anatolicum* 1. Festoons. Olympus (2.5X).

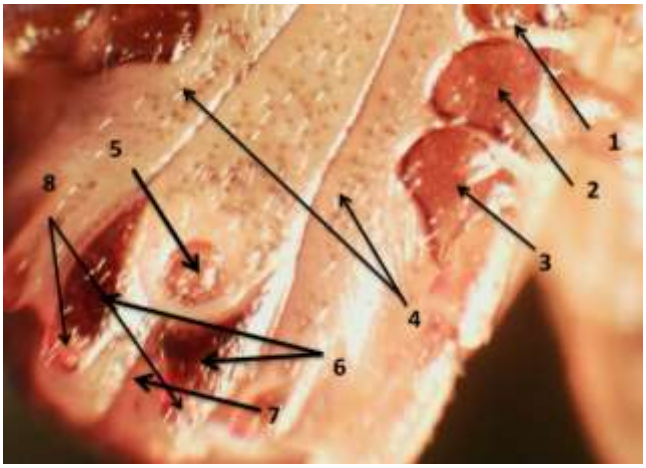

Figure 4: Ventral surface of *Hyalomma anatolicum* (Male): 1. Coxa II, 2. Coxa III, 3. Coxa IV 4. Accessory shielda, 5. Anus, 6. Adanal shields, 7. Anal groove, 8. Sub-anal shields. Olympus (2.5X).

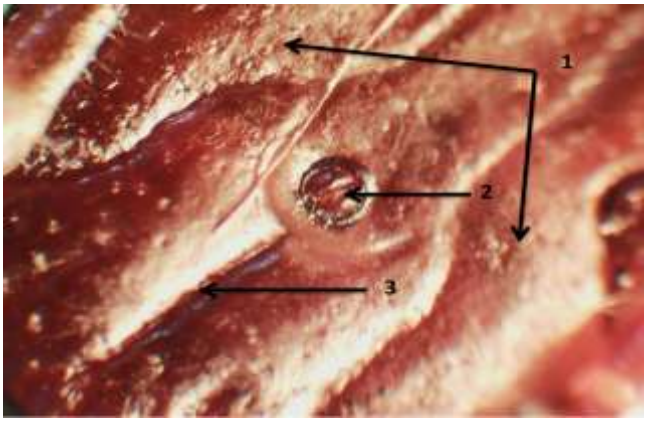

Figure 5: Ventral surface of *Hyalomma anatolicum* (Female): 1. Accessory shields 2. Anus 3. Anal groove. Olympus (2.5X).

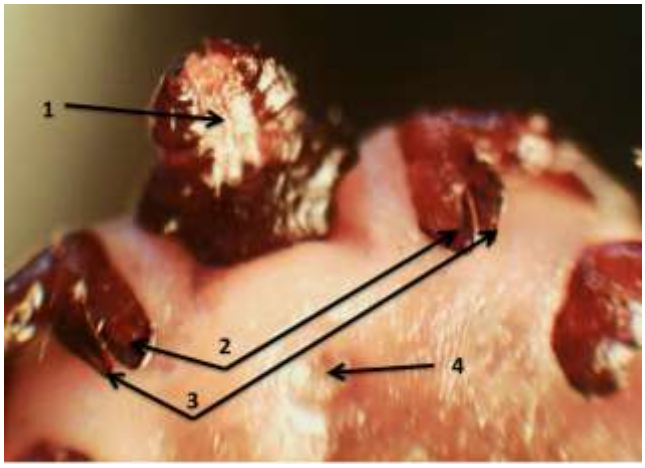

Figure 6: Ventral surface of *Hyalomma anatolicum*. 1. mouth parts 2. Internal spur 3. External spur 4. genital aperture. Olympus (2.5X).

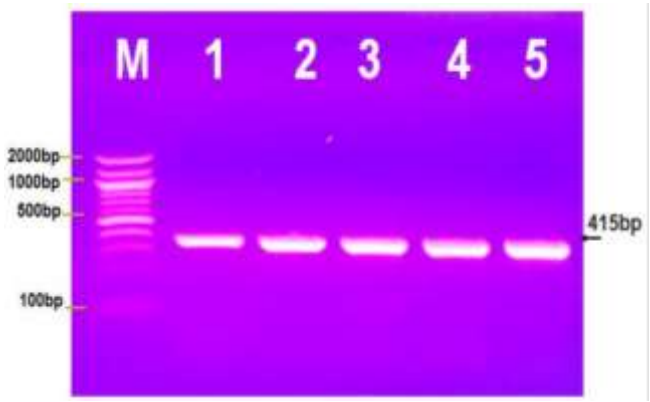

Figure 7: 1%-Agarose gel electrophoresis of PCR products for *Cox*1gene of *Hyalomma spp.* Lane (M), DNA ladder (2000-100bp), Lane (1-5) positive PCR amplification at 415 bp.

Table 1: Genetic Identity between the current study isolates of tick and some isolates of local and world countries

NCBI-BLAST Isolate	Accession number	Homology sequence identity	
		H.anatolicum HA-IQ1 isolate (MG551987)	*H.anatolicum* HA-IQ2 isolate (MG551988)
H.anatolicum Iraq	KM235704	99%	100%
H.anatolicum India	KJ912622	99%	100%
H.anatolicum Iran	KP219872	99%	100%
H.anatolicum India	KP792577	99%	100%
H.anatolicum China	KC203438	99%	99%
H.anatolicum China	JQ737067	99%	99%
H.anatolicum China	KF583577	99%	99%
H.anatolicum Iran	KT920180	99%	99%
H.anatolicum Pakistan	KU130581	99%	99%

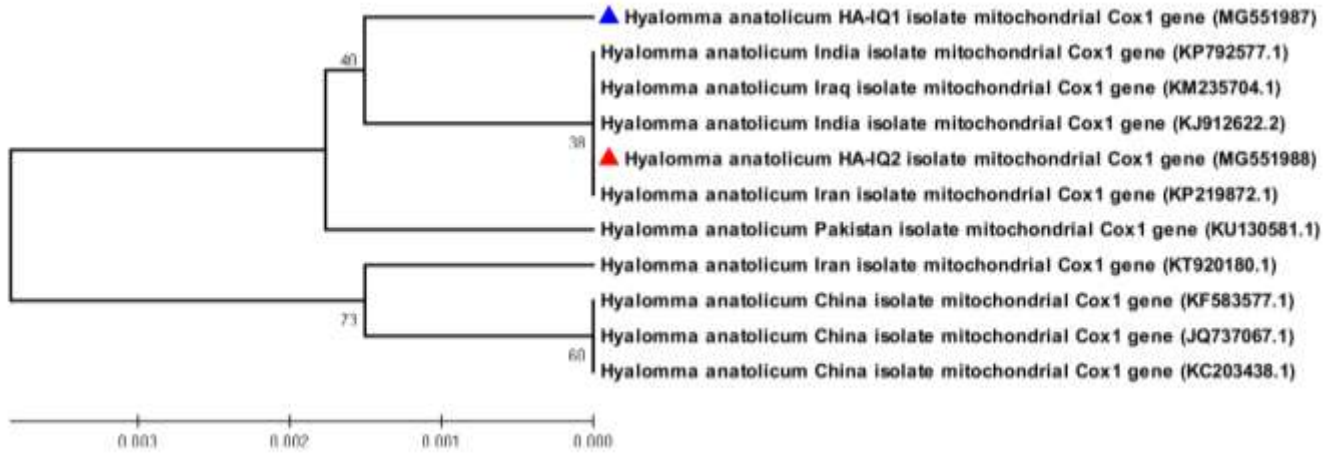

Figure 8: Phylogenetic tree of the present study isolates compared to some isolates of local and world countries. The analysis was based on the *Cox*1gene sequencing showing 2 isolates from the current study (colored triangles). The evolutionary distances were computed using the Maximum Composite Likelihood method.

Discussion

The results of the current study showed that the ticks collected were from genus *Hyalomma*, a primary detection that depended on the characteristics external differences. This genus is present in Asia, North Africa, and Europe (14,15). The genus has the ability to live in high temperature and humid areas (16). Iraq and more specifically the middle of Iraq, Al-Najaf, suffers very hot summers with temperatures could reach up to 120 °F (17).The current study results agree with (18) who found that genus *Hyalomma* was the most detected ticks in Iraq. The use of these external features to differentiate between tick genera is important to start the classification process of these Acari (19), however, more details of the species level are generated via studying the genetic components of these parasites (20). Because ticks transfer some diseases to cattle such as babesiosis and theileriosis (2), successful control of ticks should follow advanced scientific tools such as molecular entomology. In this study, utilizing unique genetic barcode sequences has enhanced confirming the available data from the primary tests.

Cox1 gene had the power of the required genetic barcode to confirm the identity of the genus discovered in this study. This is supported by (21) who proved that *Cox1* is a feasible and reliable genetic marker to detect the species of ticks. Using PCR and specific designed primers resulted in assuring that the ticks were from the genus *Hyalomma*. This agrees with (22) that found that using molecular tools such as PCR enhances the accurate detection of tick genera and species. Moreover, The name here (23) had assured the identification of life-cycle stages of ticks using *Cox1* based PCR assay. This allows future studies to use PCR and *Cox1*gene for different purposes with high confidence rate.

On the way to obtain more details about the current study isolates, *Cox1* was incorporated to recognize the species of these ticks. Interestingly, sequencing results of the *Cox1*-415bp piece gave interesting information about the species of the ticks and to which local and global isolates they align. The sequencing indicated that the species identified here was *H. anatolicum* and declared two distinct isolates belong to the same species. After generating the phylogenetic tree, one of the current study isolates (MG551988) showed high nucleotide similarity by 100% with Indian and Iranian isolates and very close placement to those isolates on the tree. This indicates that this isolate might belong geographically to the Indian region because importation and moving of animals and immigration of birds might play roles in transporting ticks from India to these countries (24,25). The second isolate (MG551987) of the present study is clustered alone by itself. This suggests that there are different amino acids in the putative peptides or proteins in this isolate (26). Moreover, this different composition of amino acids could have been induced by genetic material transferring from other species to the studied one (27). Together the results demonstrated here provide the first queue of genetic information regarding *Hyalomma anatolicum* in Al Najaf province, Iraq.

Conclusion

Cox1 is a reliable gene to specifically identifying the species level via sequencing of a unique piece of this barcode gene. Using this genetic material, the present study

shows that one of the isolates is closely similar to an isolate from India and an isolate from Iran. The other isolate of this study is less similar to any of the compared isolates from local, neighbor, and global countries. This indicates that the process of tick evolution might have already started in the middle of Iraq, Al-Najaf, to generate a distinct local isolate of ticks.

Acknowledgement

The author would like to thank and appreciate University of Al-Qadisiyah, Diwaniya, Iraq.

References

1. Sands AF, Apanaskevich DA, Matthee S. 2017. Effects of tectonics and large scale climatic changes on the evolutionary history of Hyalomma ticks. Mol Phylogenet Evol. 114:153-165. doi:10.1016/j.ympev.2017.06.002.
2. Alsaad KM, AL-ObaidibQT, Esmaeel S A. Haematological and biochemical studyb on the effect of some common blood parasite in nagitve goat inMosul area. Iraqi J Vet Sci. 2009;23:101-106.
3. Al-Fahdi A, Alqamashoui B, Al-Hamidhi S, et al. Molecular surveillance of Theileria parasites of livestock in Oman. Ticks Tick Borne Dis. 2017;8(5):741-748. doi:10.1016/j.ttbdis.2017.05.008.
4. Scott JD. Studies abound on how far north Ixodes scapularis ticks are transported by birds. Ticks Tick Borne Dis. 2016;7(2):327-328. doi:10.1016/j.ttbdis.2015.12.001.
5. Hansford KM, Phipps LP, Cull B, Pietzsch ME, Medlock JM. Rhipicephalus sanguineus importation into the UK: surveillance, risk, public health awareness and One Health response. Vet Rec. 2017;180(5):119-119. doi:10.1136/vr.104061.
6. Kemal J, Tamerat N, Tuluka T. Infestation and Identification of Ixodid Tick in Cattle: The Case of Arbegona District, Southern Ethiopia. J Vet Med. 2016;2016:1-8. doi:10.1155/2016/9618291.
7. Muñoz-Leal S, Venzal JM, Nava S, et al. The geographic distribution of Argas (Persicargas) miniatus and Argas (Persicargas) persicus (Acari: Argasidae) in America, with morphological and molecular diagnoses from Brazil, Chile and Cuba. Ticks Tick Borne Dis. 2018;9(1):44-56. doi:10.1016/j.ttbdis.2017.10.009.
8. Kamani J, Apanaskevich DA, Gutiérrez R, Nachum-Biala Y, Baneth G, Harrus S. Morphological and molecular identification of Rhipicephalus (Boophilus) microplus in Nigeria, West Africa: a threat to livestock health. Exp Appl Acarol. 2017:1-14. doi:10.1007/s10493-017-0177-z.
9. Hornok S, Sándor AD, Beck R. Contributions to the phylogeny of Ixodes (Pholeoixodes) canisuga, I. (Ph.) kaiseri, I. (Ph.) hexagonus and a simple pictorial key for the identification of their females. Parasit Vectors. 2017;10(1):545. doi:10.1186/s13071-017-2424-x.
10. Csordas BG, Garcia MV, Cunha RC, Giachetto PF, Blecha IMZ, Andreotti R. New insights from molecular characterization of the tick Rhipicephalus (Boophilus) microplus in Brazil. Rev Bras Parasitol Veterinária. 2016;25(3):317-326. doi:10.1590/S1984-29612016053.
11. Shyma KP, Gupta JP, Singh V. Breeding strategies for tick resistance in tropical cattle: a sustainable approach for tick control. J Parasit Dis. 2015;39(1):1-6. doi:10.1007/s12639-013-0294-5.
12. Grech-Angelini S, Stachurski F, Lancelot R, et al. Ticks (Acari: Ixodidae) infesting cattle and some other domestic and wild hosts on the French Mediterranean island of Corsica. Parasit Vectors. 2016;9(1):582. doi:10.1186/s13071-016-1876-8.
13. Walker a.., Bouattour A, Camicas J., et al. Ticks of Domestic Animals in Africa: A Guide to Identification of Species.; 2003. http://www.researchgate. net/publication /259641898_ Ticks_of_domestic_animals_in_Africa_a_guide_to_identification_of_ species/file/5046352d0429878d7f.pdf.
14. Shubber HWK. Taxonomic, anatomic, and molecular study of ixodid ticks parasitizing some mammals and birds in the middle and south of Iraq. Ph.D. thesis, College of Education, Al-Qadisiya University, Iraq. 2014.
15. Estrada-Peña A, Pfäffle M, Baneth G, Kleinerman G, Petney TN. Ixodoidea of the Western Palaearctic: A review of available literature for identification of species. Ticks Tick Borne Dis. 2017;8(4):512-525. doi:10.1016/j.ttbdis.2017.02.013.
16. Ernieenor FCL, Ernna G, Mariana A. Phenotypic and genotypic identification of hard ticks of the genus Haemaphysalis (Acari: Ixodidae) in Peninsular Malaysia. Exp Appl Acarol. 2017;71(4):387-400. doi:10.1007/s10493-017-0120-3.
17. Patel G, Shanker D, Jaiswal AK, Sudan V, Verma SK. Prevalence and seasonal variation in ixodid ticks on cattle of Mathura district, Uttar Pradesh. J Parasit Dis. 2013;37(2):173-176. doi:10.1007/s12639-012-0154-8.
18. Day TK, Grimshaw D. An observational study on the spectrum of heat-related illness, with a proposal on classification. J R Army Med Corps. 2005;151(1):11-18. doi:10.1136/JRAMC-151-01-03.
19. Shanan SMH, Abbas SF, Mohammad MK. Ixodid Ticks Diversity and Seasonal Dynamic on Cattle in North, Middle and South of Iraq. Syst Appl Acarol. 2017;22(10):1651. doi:10.11158/saa.22.10.7.
20. Rivera-Páez FA, Labruna MB, Martins TF, et al. Contributions to the knowledge of hard ticks (Acari: Ixodidae) in Colombia. Ticks Tick Borne Dis. 2018;9(1):57-66. doi:10.1016/j.ttbdis.2017.10.008.
21. Alsarraf M, Mierzejewska EJ, Mohallal EME, Behnke JM, Bajer A. Genetic and phylogenetic analysis of the ticks from the Sinai Massif, Egypt, and their possible role in the transmission of Babesia behnkei. Exp Appl Acarol. 2017;72(4):415-427. doi:10.1007/s10493-017-0164-4.
22. Lv J, Wu S, Zhang Y, et al. Assessment of four DNA fragments (COI, 16S rDNA, ITS2, 12S rDNA) for species identification of the Ixodida (Acari: Ixodida). Parasit Vectors. 2014;7(1):93. doi:10.1186/1756-3305-7-93.
23. Amendt J, Krettek R, Zehner R. Forensic entomology. Naturwissenschaften. 2004;91(2):51-65. doi:10.1007/s00114-003-0493-5.
24. Faraliana E, Lah C, Yaakop S, Ahamad M, George E, Nor SM. Precise identification of different stages of a tick, Ixodes granulatus Supino, 1897 (Acari: Ixodidae). 2016. doi:10.1016/j.apjtb.2016.05.003.
25. Spinage CA. Cattle Plague : A History. Springer US; 2003. https://books.google.iq/books?id=uk3MBgAAQBAJ&dq=cattle+imported+to+iraq+from+india. Accessed November 27, 2017.
26. Kuljić-Kapulica N. Emerging diseases. Crimean-Congo hemorrhagic fever. Med Pregl. 2017;57(9-10):453-456. http://www.ncbi.nlm.nih.gov/pubmed/15675618.
27. Liu G-H, Chen F, Chen Y-Z, et al. Complete mitochondrial genome sequence data provides genetic evidence that the brown dog tick Rhipicephalus sanguineus (Acari: Ixodidae) represents a species complex. Int J Biol Sci. 2013;9(4):361-369. doi:10.7150/ijbs.6081.