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Molecular Study of Ticks; *Hyalomma Anatolicum*, Isolated from Sheep in Al-Diwaniyah Province, Iraq

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Abstract:

Almost all livestock animals are susceptible to ticks considered vectors and reservoirs for many tick-borne pthogenes, causing severe and fatal zoonotic diseases affecting humans and animals' health, such as Crimean Congo hemorrhagic fever (CCHF) virus. Due to this importance, ticks must be characterized using different tools, such as molecular, which helps epidemiological and control programs related to ticks. The current study was conducted during September 2018 and January 2019; fifty ticks were collected from sheep raised in Al-Saniyah territory, Al-Diwaniyah Province, Iraq. Then, the ticks were classified according to morphology, and 20 ticks were identified using polymerase chain reaction (PCR) to confirm the morphological specifications using the cytochrome c oxidase subunit I (COX1) gene as a target. Finally, six purified PCR products were conducted to a partial gene sequencing (PGS) method to identify the tick species and compare them to the global isolates. The morphological identification showed that ticks appeared to be from the genus Hyalomma, which was PCR-confirmed. Furthermore, the PGS revealed that the local tick isolates belonged to Hyalomma anatolicum, and they were firmly nucleotide-similar to strains from Pakistan and China. The results of the phylogenetic analysis showed probable evolutionary links to the Pakistanian and Chinese isolates.

Keywords: COX1, DNA sequencing, Hyalomma anatolicum, tick.

Introduction:

Ticks are ectoparasites, which feed on humans, mammals, reptiles and amphibians by hematophagy. Ticks are mainly responsible for casualties incurred by sucking blood from animals such as sheep and goats. Many tick species are reservoirs for many domestic animal diseases such as babesiosis, anaplasmosis, rickettsiosis, borreliosis, and ehrlichiosis, well-known health conditions of tick-borne transmission (1,2).

Ticks are from the class Arachnida that comprises spiders and are very similar to mites. The 'soft ticks' (Argasidae or argasids) and the 'hard ticks" (Ixodidae or ixodids) occur as two separate classes of ticks according to morphological and biological characterization.

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Hyalomma ticks, such as those that vector numerous diseases in humans and livestock, are prevalent in North Africa, South Europe, Middle East, Central Asia, and China (3,4).

After the first publication, a phylogenetic tree has provided the idea that ticks and their host can bring fresh insights into the creation and study of the historical biogeography of ticks regarding the relative morphology and life cycles of the pests. Current strategies for the analysis of tick genomes have been extended to the creation of biology and species phenotypes by studying these creatures' phylogeny and reproduction. Ticks evolved during the pre-mid-Cretaceous era (both the Argasidae and Ixodidae were formed in the middle Cretaceous). Throughout the Paleozoic and Mesozoic periods (around 225 million years), Ixodids developed presumably from reptile parasites. Ticks lose their primary somatic segmentation during development, introducing tagma as body-division areas. They have a tagma named gnathosoma, which contains the parts of the mouth similar to other arthropods' heads. During the early larval period of development, only three legs emerge, and the fourth pair appears as it grows into an adult. By their presence or absence, the respiratory spiracles are crucial for the classification using the taxonomic methods, especially when linked to the tagma positions (5,6).

Materials and methods

Collection of ticks:

From Al-Saniyah County (Al-Diwaniyah Province, Iraq), 50 ticks were collected from sheep during September 2018 and January 2019. The ticks were removed from those sheep's skin using forceps and placed in specific containers in highly aseptic precautions. Later, the containers, including the ticks, were transferred to the Laboratory of the Unit of Zoonosis, University of Al-Qadisiyah, Veterinary Medicine College, Iraq, then the samples were submitted to genotypic and morphological classification (7).

Morphological identification:

The genus of *Hyalomma* was identified by distinguishing the characteristic morphological features of the genus mentioned by Walker (7). The distinctive female parts of the mouth, genital aperture, the dorsal festoons, and the ventral located Coxa I, Coxa II, and Coxa III were explored to complete the identification.

Extraction of the mitochondrial DNA:

The gSYNCTM DNA Extraction Insect kit (Geneaid, USA) was recruited to extract the ticks' mitochondrial DNA. In brief, 50mg of each tick tissue was mixed with liquid N_2 using a mortar and smushed thoroughly. Then, the mixture was placed in 1.5ml tubes, and GST buffer at 200µl and proteinase K at 20µl were added, mixed thoroughly, and then 60ºC-incubated for one hour.

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In the end, the extraction steps implied from the kit protocol were conducted. The resulted DNA was NanoDrop-estimated for its purity and concentration.

Amplification and sequencing:

The PCR was performed using a primer set;

1- The forward: AGGGTCCCCAGATATAGCATT
2- The revers : ACCGCCTGAAGGGTCAAAAA
It is provided by (Bioneer, South Korea), for *Cox*1 gene (415) bp in twenty ticks.

The total volume of PCR was (20) µl; the PCR pre-mix formed from Taq DNA enzyme, KCl (30) mM, MgCl2 (1.5) mM, dye, dNTPs (250)µM, Tris-HCl (10) mM and a stabilizer. They were also adding 5 μ l DNA and primers (1.5) μ l and PCR water (12µl). The tubes of the PCR solutions were introduced into a thermocycler under one-cycled (95°C for 5min)-initial denaturation after that introduced in (30) cycles of for half minute at (95) °C (denaturation stage). Then submitted for half minute at (58) °C (Annealing stage), then submitted for 60 seconds at (72) °C (extension satge), followed by one-cycled for five minutes at (72) °C (final extension stage). Later, the final PCR products were run on a 1%-agarose gel pretreated with ethidium bromide. Finally, the gel was documented under a UV-based visualizer.

Macrogen Company (South Korea) sequenced six purified PCR products using the AB DNA sequencing system. The NCBI-Blast Alignment system was employed to perform the alignment analysis, and the Mega 6.0 software was utilized to build the phylogenetic tree.

The results:

Morphological characteristics of Hyalomma spp (50) ticks is tested after collected it as showed in Figure (1). The main characters were mouthparts and genital aperture and there are Coxa I, II, and III in the tick abdomen while the dorsal surface have festoons.

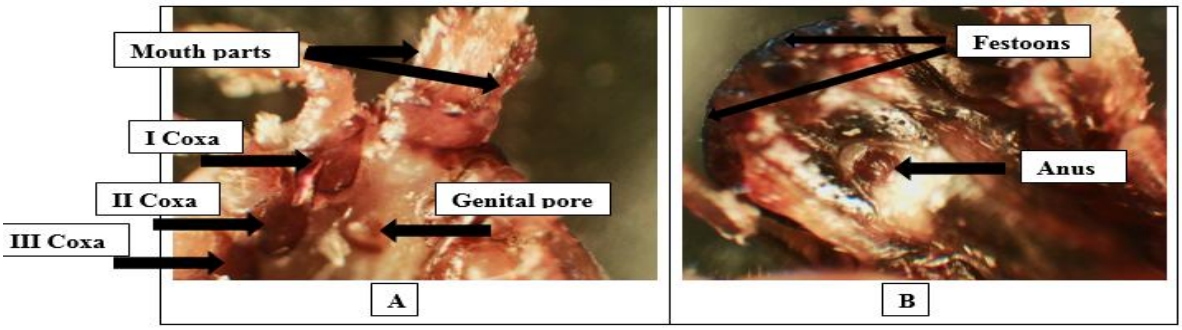

Figure (1) Microscopic examination of tick genus Hyalomma A.Ventral surface: mouthparts, genital pore and Coxa I, II, III B: Ventral surface: Festoons and anus: using Olympus microscope with (2.5X) power

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The results showed that the twelves samples back to Hyalomma genus as showed in Figure (2), the results of the sequencing illustrated that six samples were *Hyalomma anatolicum by* PCR. Isolates sequencing were recorded as *H. anatolicum* in the database of the NCBI-Genbank Under code MN460344.1,MN460345.1,MN460346.1,MN460349.1,MN460350.1,MN460355.1

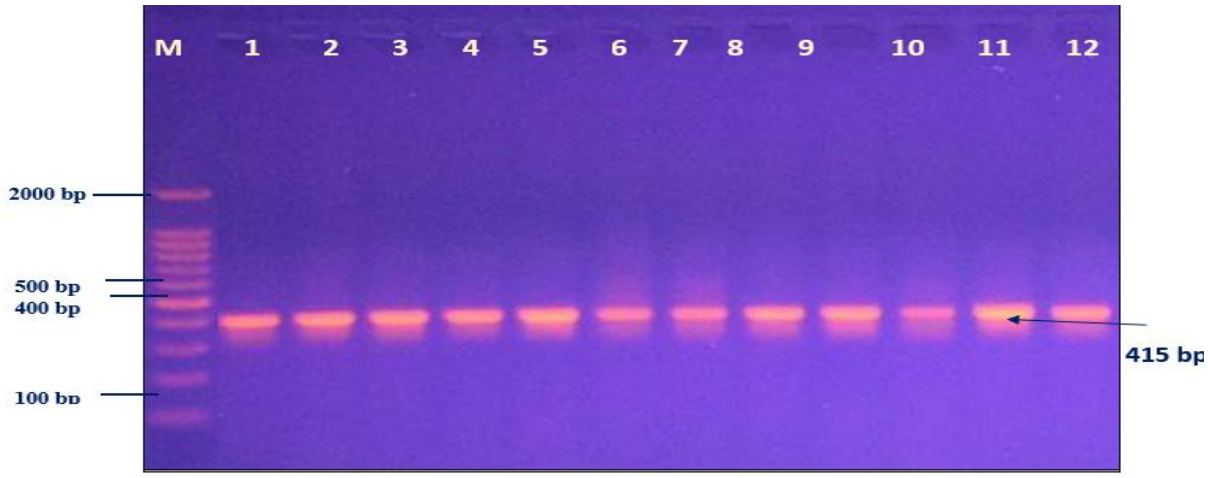

Figure (2): the electrophoresis showed band of Cox1 gene in samples of Hyalomma spp. Wherever, the Lane (M) (2000-100bp) represent DNA ladder while the Lane represent the positive PCR amplification (1-12)

The PGS revealed that the local tick isolates belonged to *Hyalomma anatolicum*, and they were firmly nucleotide-similar to strains from Pakistan and China (Table 1 and figure 3).

Table (1): The current study tick (*Hyalomma. anatolicum*) isolates and their nucleotide-based similar global isolates.

Iraqi current isolates	Aligned global isolates		
Accession No.	Accession No.	Country of origin/Identity	
MN460344	MK462197	Pakistan	99.46%
MN460345	JQ737067	China	99%
MN460346	MN728997	Pakistan	99%
MN460349	MK462199	Pakistan	99.47%
MN460350	JQ737067	China	98.83%
MN460355	KU130580.1	Pakistan	99%

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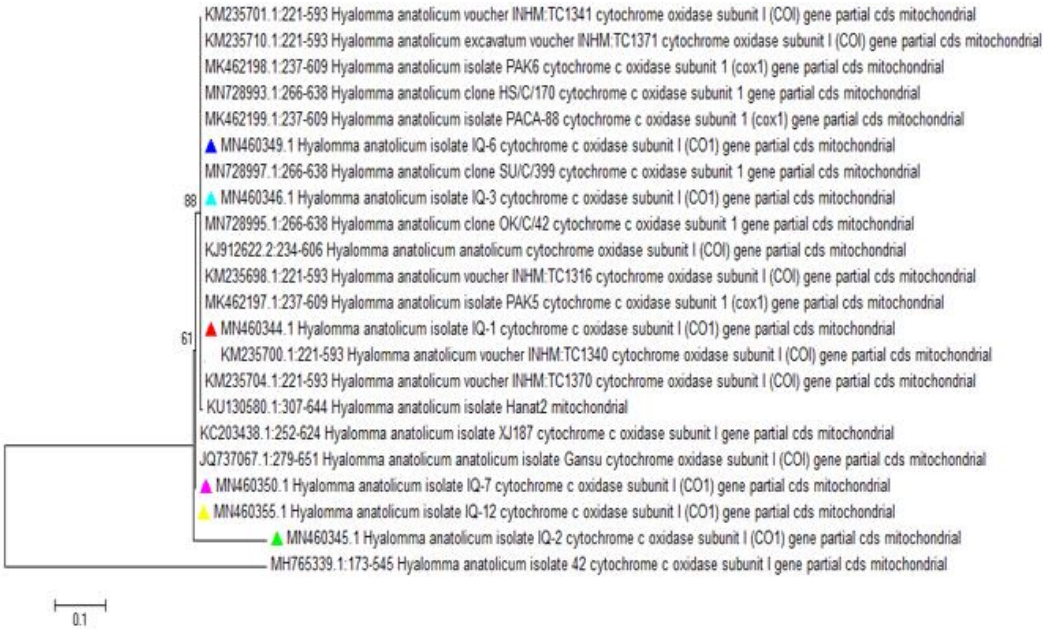

Figure 3: Molecular Phylogenetic analysis by Maximum Likelihood method, The Cox1gene-based phylogenetic tree of the ticks; H. anatolicum isolated from sheep (Al-Diwaniyah Province, Iraq. The tick isolates from the current study (coloured triangles) were firmly nucleotide-similar to Pakistan and China strains.

Discussion:

For many decades, experts have been dealing with ticks and tick-borne pathogens in urban areas, and ongoing work has been undertaken to learn about the prevalence of ticks and zoonoses in urban areas. The persistence conditions of tick species in urban habitats are linked to possibly tick-proliferating industrial regions, such as urban parks, private gardens, municipal parks and riparian forests. The efficient reproduction of ticks relies on the optimum temperature and humidity of their environments and sufficient tick hosts' availability within an urban environment. New experiments have shown that tick species persisting in wild natural environments around towns and cities in industrial regions and ecosystem conditions in urban areas are compatible with the circumstances in which the tick species reside in ecosystems and ideally fitted to ticks (8,10).

The present results revealed that the dominant tick species located in Iraq's tested area is *H. anatolicum*. Ticks transfer causative agents of diseases through their saliva, which is the key route by which these pathogens are transmitted to animals and humans. Ticks play a significant role in the transmission of blood-borne pathogens compared to this role by the hematophagous insects. During feeding, the ticks may have some negative consequences on the host, such as painful activity due to the biting of ticks, which establish a reaction cycle that affects the skin and other subcutaneous tissues. The cells and tissues may be crushed and lacerated, commonly

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correlated with pruritis, erythema, edema, excoria, papules, lichenification, ulceration, and the occurrence of secondary bacterial infections (11).

The phylogenetic analysis uncovered that the tick's local isolates are closely related to isolates from Pakistan and China. Evolutionary factors, such as adaptation to the local hosts and environment, can significantly change the nucleotide sequences of the mitochondrial *Cox*1 gene. *Cox*1 nucleotide alterations can be employed to explore insects' genetic evolution and provide insightful information in this regard (12,13). Similar isolates of ticks to those from Pakistan may be due to India's importation, a neighbour of Pakistan (3,14-19). The similarity with the Chinese isolates can be explained by factors such as transmitting ticks to new countries through aircraft, ships, and cargos (19).

Conclusion:

Cox1 is a specific gene for identifying the species level of Hyaloma anatolicum by sequencing specific sequence gene and using the molecular technique. The present study shows that the isolates (3,6) are closely similar to the Pakistanian isolates, while (1,2,7,12) are closely similar to the Chinese isolates. That meaning that the tick evolution was occur and started in Iraq, Al-Diwaniyah, to forming a distinct local isolate.

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