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Molecular Diagnosis and Identification of Tick Strains from Bovine Origin Using Polymerase Chain Reaction in Mosul city

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ABSTRACT: Backgrounds and Objectives: Ticks are important ectoparasites of cattle and play a significant role in transmitting pathogens responsible for economically important diseases, including Theileria, Babesia, and Anaplasma. This study aimed to identify tick species collected from cattle in Mosul, Iraq, and investigate their genetic diversity using morphological and molecular approaches.

Methods Ticks were collected from cattle in Mosul and examined using morphological identification and molecular analysis. Mitochondrial 12S rRNA gene sequencing was used for molecular characterization, and phylogenetic analysis was performed to assess genetic relationships and variation among the identified tick strains.

Results: Molecular analysis revealed considerable genetic diversity among the examined ticks, with 10 genetically distinct tick strains reported as newly recorded in the study area. Phylogenetic analysis demonstrated genetic variation among the identified strains, suggesting differences in their genetic relationships and possible origins. These findings provide new insights into the genetic diversity of cattle ticks in Mosul, Iraq.

Conclusions:

The integration of morphological identification and 12S rRNA gene sequencing provided valuable information on the genetic diversity of cattle ticks in Mosul. The identification of 10 previously unrecorded strains highlights the importance of molecular surveillance for improving tick identification and understanding their distribution. Further studies are needed to investigate the potential associations between tick diversity, environmental and climatic factors, livestock movement, and the transmission of tick-borne pathogens, including Theileria, Babesia, and Anaplasma.

INTRODUCTION

Ticks are considered one of the most important blood-sucking arthropods within the order Ixodida and represent one of the most dangerous external parasites that infect both humans and animals, due to their vital role in transmitting many pathogens, including Babesia spp, Theileria spp., Anaplasma spp and Rickettsia spp. (Garcia et al.,2022). Ectoparasites can induce toxicosis and severe allergic irritation, they are known to pose a serious threat to native species. Additionally, ectoparasites are notorious for spreading a variety of bacterial and protozoan illnesses to both humans and animals (Jongejan

& Uilenberg, 2004, Hussain, et al., 2006, Qudoos, et al., 1995 & Dryden, et al., 1994). Ectoparasites, primarily ticks, are important when researchers discuss health issues with domestic animals. Approximately 80% of cattle worldwide are susceptible to tick infestation. (F. A. O, 1984).

Ticks of the genus Hyalomma carry the parasite from one cow to another (Gautam and Dhar, 2008). Through tick apprehension, damage to hides, blood loss and udders, toxin production, and decreased animal body weight, ticks can directly cause harm [Rafique, et al., 2015, Scholtz, et al., 1996 & Stachurski, 1996). Ticks have a complex life cycle consisting of four main stages: egg, larva, nymph, and adult. All of these stages—except the egg—require a blood meal to complete development (Okely et al., 2021). The life cycle often lasts from several months to more than two years, depending on the tick species, the host, and environmental conditions. Most tick species parasitize mammals, birds, and reptiles and exhibit a high degree of familial specialization, making them important ecological elements in veterinary and epidemiological systems. Hard tick species (Ixodidae), such as Hyalomma anatolicum, Rhipicephalus sanguineus, and Boophilus annulatus, are the most common in arid and semi-arid regions like Iraq, where conditions are favorable for their reproduction and survival (Aziz, 2022). Their danger lies in the fact that they are not just blood parasites but effective vectors of diseases with a wide economic and health impact on livestock and humans (Getange et al., 2025). Historically, tick species identification has relied on morphological characteristics such as mouthparts, scutum (back shield), and eyes. However, this approach faces significant challenges, particularly when dealing with immature or damaged specimens, limiting its accuracy and reliability (Galletti et al., 2024). With the development of modern biotechnologies, molecular diagnostic methods using polymerase chain reaction (PCR) have emerged as a precise and rapid means of species identification and monitoring associated parasites within ticks. Molecular diagnosis relies on amplifying conserved regions of DNA, such as the 16S rRNA gene, ITS2, or COI, which exhibit genetic differences between species, allowing for precise differentiation even in cases of high morphological similarity (Intirach et al., 2023). The aim of this study is to accurately diagnose and differentiate tick strains using polymerase chain reaction (PCR) techniques. By applying specific genetic markers, the research seeks to enhance the precision of tick identification, support epidemiological investigations, and contribute to the development of more effective tick control and management strategies.

Objectives of the study to:

1. Use morphological traits to identify tick species that were gathered from livestock in Mosul, Iraq.

2. Use mitochondrial 12S rRNA gene sequencing to describe the genetic diversity of cattle ticks.

3. Use phylogenetic analysis to determine the genetic links between the detected tick strains.

4. Examine genetic variation and locate newly discovered tick strains in the research region.

5. To aid future research on tick dispersal and their possible involvement in the spread of tick-borne infections such Theileria, Babesia, and Anaplasma, provide baseline genetic data.

METHODS

Samples collection

In August 2025, 10 ticks were extracted from the skin of ten sick cattle in the Kokjali region of the Ninewa Governorate in Iraq. A bottle containing 70% ethyl alcohol was used to preserve each tick that was collected from a single animal. After that, the samples were sent for partial identification to the Department of Life Sciences, College of Science, University of Mosul, Ninewa Governorate, Iraq. A 2.5x dissecting microscope was then used to analyze the samples morphologically.

DNA extraction from tick parasites:

The DNA extraction was performed using several analysis kits provided by Geneaid, as following: The strong wall of the tick parasite was broken down by crushing it with liquid nitrogen using a ceramic mortar and pestle. After that, a 1.5 ml Eppendorf tube is filled with the resulting parasite powder. Lysozyme at a concentration of 0.8 mg/200 ml was added in 200 microliters to the Eppendorf tube, and the mixture was vortexed. The tube was incubated for 30 minutes at 37 degrees Celsius, with the tube being inverted every three minutes. Additionally, 20 microliters of Proteinase K were added, and the mixture was vortexed. The combination was used for ten minutes at 60 degrees Celsius. A vortex mixer was used to combine 200 microliters of GB buffer solution, and the mixture was then incubated at 70 degrees Celsius. Absolute ethanol (200 microliters) was hand mixed. After that, move the mixture to a GD tube, place it inside a collection tube, and centrifuge

for 30 seconds at 16000 g. Throw away the supernatant. After that, 600 microliters of the washing solution were added, and the centrifuge was run at the same speed and duration. Throw away the supernatant. To get rid of any last remnants of the washing solution, centrifuge once more for three minutes. After transferring the GD column to a fresh 1.5 ml tube, 100 microliters of lysis solution were added. After three minutes, centrifuge for thirty seconds at 16,000 g. The DNA should be kept until needed.

Preparation of agarose gel and DNA electrophoresis procedure

A 1% agarose gel is made for DNA electrophoresis and detection. This concentration is achieved by dissolving 0.5 g of agarose powder in 50 ml of 1X TBE buffer and adding 3 microliters of RedSafe dye. This is accomplished by using a heat source and stirring constantly until it boils, at which point it is allowed to cool to between 50 and 60 degrees Celsius. The well-forming comb is then affixed to the gel's edges before the gel solution is poured into the relay device's tray. It must be poured carefully to prevent bubbles from forming; if they occur, a pipette is used to eliminate them. After that, the gel is allowed to solidify. The comb is then carefully lifted after the tray is set in the electrorelay tank with the proper volume of X1 TBE solution. Five microliters of the DNA sample and three microliters of the loading solution are combined to create the migration samples. After that, an electric current with a voltage of 5 V/cm is passed to activate the migration mechanism, which takes 1.5 to 2 hours. A gel documentation system is then used to scan the gel under ultraviolet light so that the PCR product and DNA bands can be seen. To achieve the necessary concentration for PCR reactions, which was (25) ng/µL for each study sample, the DNA concentration in each sample was diluted using TE buffer solution. In a 0.2 ml Eppendorf tube provided by Biolaps UK, the DNA sample and the particular primer for each gene were combined with the premix components to create the master reaction mixture for each PCR reaction. To make sure the reaction components were mixed, the mixture was centrifuged in a microfluidic device for three to five seconds after the reaction volume was stabilized to twenty microliters using purified water. The amplification reaction was then carried out using the particular software for each reaction by placing the reaction tubes in a thermocycler. (Mikić, et al., 2023). After that, the sample was added to a volumetric DNA ladder made by Biolabs in one of the wells of a 2% agarose gel that had already been prepared. Following a 60–70 minute electrophoresis of the materials, a gel documentation system was used to image the gel.

Molecular diagnosis of the ticks parasite based on the 16S rRNA region:

The 12S rRNA region was found to be present. The master mix was supplemented with one microliter (10 µL) of each gene-specific primer and four microliters (100 nanograms) of amplified template DNA.

Primer	Target gene	Primer Sequence(5'-3')	Product size (bp)	
Tick	12 SrRNA	CCGGTCTGAACTCAGATCAAGT	460	Mangold and Bargues 1998
		GCTCAATGATTTTTTAAATTGCTGT		

The reaction tubes were then inserted into the thermo cycler to carry out the polymerization reaction using the specific reaction program, as shown down:

No.	Stage	Temperature	Time	Cycle number
1	Initial denaturation	95	6 min.	1
2	denaturation	95	1.30 min.	35
3	Annealing	58	1.30	

			min.	
4	Extension	72	2 min.	
5	Final extension	72	5 min.	1

DNA Sequencing

The basic method for discovering and detecting genetic variants and for molecular diagnosis based on highly conserved areas is DNA sequencing. The sequences of amplified fragments in which genetic variants are to be ascertained are usually identified using the PCR reaction result. The foundation for registering isolates in the GenBank database has recently been DNA sequencing results. Additionally, the targeted fragment is separated from the gel and purified if the PCR reaction product has many bands. On the other hand, the reaction product can be used directly for sequencing if it is unique to a single band (Cheng et al., 2012). Using DNA sequencing to ascertain the amplified fragments' nucleotide sequence:

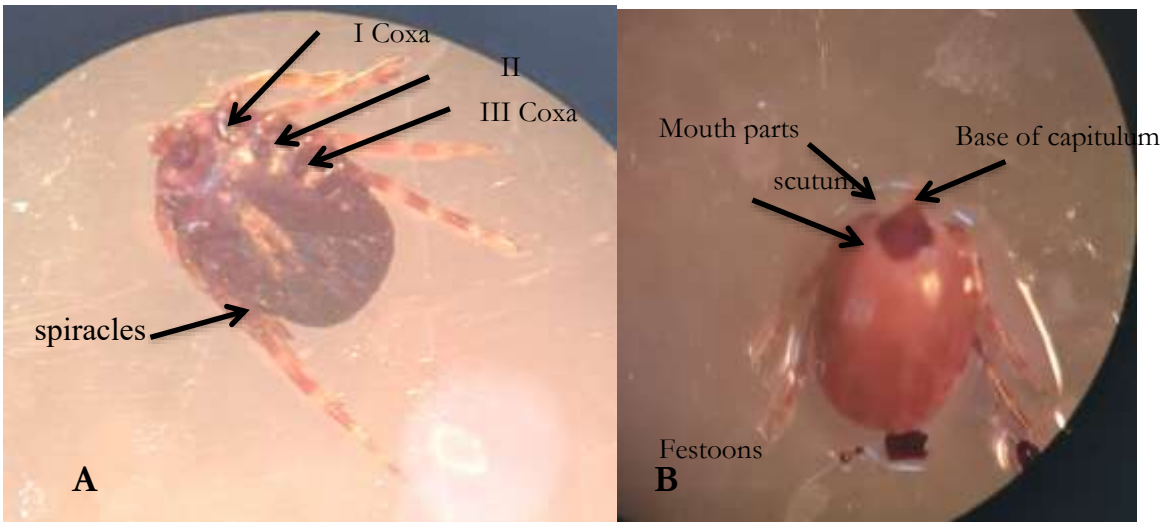
The nitrogenous base sequences of the investigated tick samples were identified. The primers of the final package were supplied with the PCR reaction products of the previously described samples, and the Somagene Center in the United States read the gene sequences.

BLAST software was used to assess the results of matching the gene sequences with those listed in the National Center for Biotechnology Information (NCBI).

RESULTS:

Microscopic examination of Ticks:

Numbers of ticks were collected from cattle slaughtered at the Al-Sadoon abattoir, and these ticks were diagnosed by using a dissecting microscope, and the species of tick was Hyalomma anatolicum, which is characterized by the trunk being narrow at the peak and rounded. At the bottom, the mouthparts are long, the base of the capitulum is round in shape, and the cauda of the first pair of legs is branched into a thin external spur and a wide internal one. The festoons are present , and the spiracles are comma-shaped. and the two sub-plates that protrude (Walker et al., 2003).



The external appearance of tick

DNA Extraction

As explained in the techniques section, a commercial isolation kit from Geneaid was used to extract DNA from the ten tick parasite samples. As seen in Figure (1) below, 1% agarose gel electrophoresis was used to verify the integrity and purity of the recovered DNA from the seven (G1-G14) parasite samples.

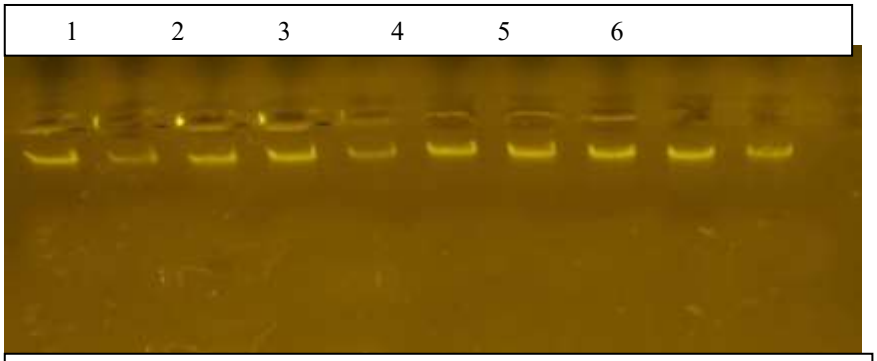


Figure (1): DNA extraction of ten samples of the *Hyalomma anatolicum* parasite and the 1% agarose gel.

12 SrRNA gene region amplification (PCR Reaction):

PCR equipment provided the 12 SrRNA gene PCR reaction findings for the ten Hyalomma anatolicum parasite samples (G1–G10). The approximate size of the target gene area was then ascertained by comparing the molecular size of the amplified DNA produced by the polymerase chain reaction (PCR) to a multi-size standard DNA ladder. On the 2% agarose gel, the gel electrophoresis results revealed a single, distinct band for the PCR product, demonstrating the reaction's effectiveness and specificity in the absence of contamination or non-specific products. As seen in Figure (2) below, this band's position was found to be within the range of 560 base pairs when compared to the standard size ladder. This is compatible with what has been documented or is available in the database.

In Duhok 2021, research was conducted on ticks of the same genus, but different in type. A completely different gene was used, which is 16S RRNA. It was noted that there was a percentage of identity for the gene between the current study and the study that was referred to, and the sequence was 460 bp (smael, S., & Omer, L. T., 2021). In another study in India in 2011, the same DNA primer used in the previous study in Duhok, northern Iraq, was used, and the target DNA sequence in the study was found to be 449 base pairs. This is almost identical to the sequence in the current study, except that it differs in the use of the 12S rDNA primer (Kumar, et al., 2011). In another study, two different genes were used, namely 16S rDNA and CO1 genes, and two different types of ticks were used, namely H. dromedarii and H. excavatum. It was observed that the sequence of nitrogenous bases was 440 and 850 respectively, which matched the sequence used in this study for the first gene (16S rDNA) and differed from the second gene used (CO1) (Abdullah., et al., 2018).

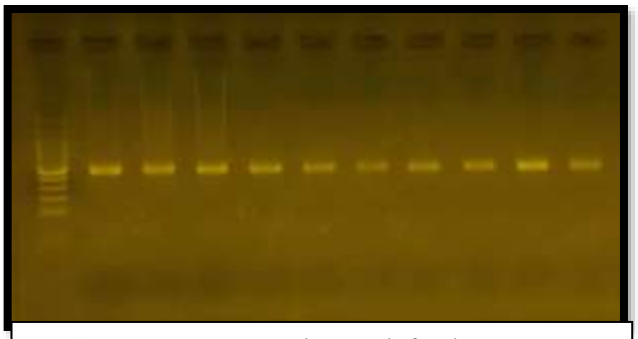


Figure (2): PCR reaction result for the 12srRNA region used in ticks *(Hyalomma anatolicum*) parasite diagnosis, with a 460 bp reaction result plated with 2%

Determining the Nucleotide Sequence of the 16 SrRNA Region.

DNA Sequencing

The basic method for discovering and detecting genetic variants and for molecular diagnosis based on highly conserved areas is DNA sequencing. The sequences of amplified fragments in which genetic variants are to be ascertained are usually identified using the PCR reaction result. The foundation for registering isolates in the GenBank database has recently been

DNA sequencing results.Additionally, the targeted fragment is separated from the gel and purified if the PCR reaction product has many bands. On the other hand, the reaction product can be used directly for sequencing if it is unique to a single band (Cheng et al., 2012). Using DNA sequencing to ascertain the amplified fragments' nucleotide sequence:

The nitrogenous base sequences of the investigated tick samples were identified. The primers of the final package were supplied with the PCR reaction products of the previously described samples, and the Somagene Center in the United States read the gene sequences. The gene sequences were compared to the gene sequences listed in the National Center for Biotechnology Information (NCBI), and BLAST software was used to assess the results.

1- HBA1 = G1 - SUB15688523 HBA1 PX44794

2- HBA2=G2 - SUB15688523 HBA2 PX447948

3- HBA3 =G3 - SUB15688523 HBA3 PX447949

4- HBA4= G4 - SUB15688523 HBA4 PX447950

5- HBA5= G5 - SUB15688523 HBA5 PX447951

6- HBA6= G6 - SUB15688523 HBA6 PX447952

7- HBA7= G7 - SUB15688523 HBA7 PX447953

9- HBA8= G8 - SUB15688523 HBA8 PX447954

10- HBA9= G9 - SUB15688523 HBA9 PX447955

11. HBA10= G10 - SUB15688523 HBA10 PX447956

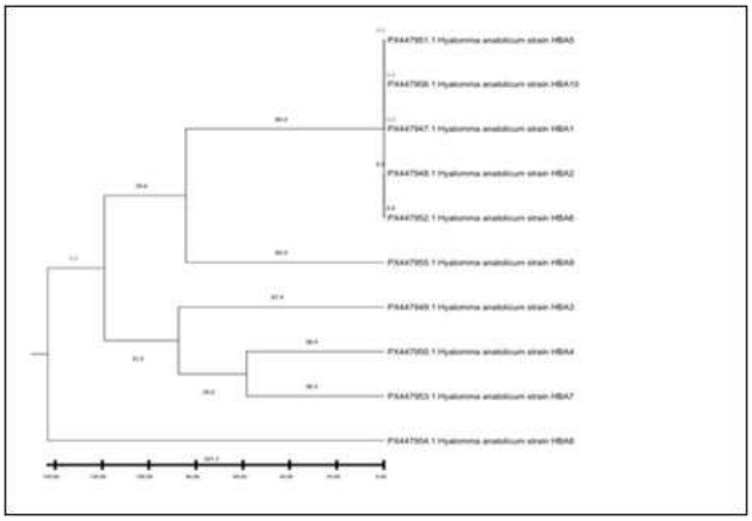
By sending sequencing samples for each of the ten samples of ticks (Hyalomma anatolicum) for sequencing and then subsequently reading the percentage of similarity of each sample with the GenBank sequence in NCBI, the results were as follows:

The second, third and six samples (G2, G3 and G6) were 100% identical to the GenBank sequence, with no differences in the sequence. While the rest of the samples (G1,G4,G5,G7,G8,G9 and G10) out of ten had a similarity percentage of 99% with GenBank. Through the analysis of the similarities above, it was observed that there was a match in three samples out of ten, while there was a slight difference in other samples. This is due to the nature of the animal from which the samples were taken, or it is due to the differences in the sequence of parasites in the organism's body.

Some of these differences in the alignment between the seven samples of the E. granulosus (G1-G7) parasite are due to the presence of specific mutations in the sequence. These mutations may be substitution, insertion, or deletion mutations, as observed in some alignments of the genetic sequences of the 12 SrRNA gene, or they may be due to different readings or specific sequences that the sequencing machine was unable to read due to nucleotide impurity. Therefore, these variations or differences between seven samples of ticks (Hyalomma anatolicum) and the GenBank database at the NCBI using a phylogenetic tree method, which will be discussed down.

In the discussion of this study about molecular analysis of mitochondrial 12S rRNA gene sequences were showed that samples G2, G3, and G6 were 100% identical to the reference sequences in GenBank, while the remaining samples (G1, G4, G5, G7, G8, G9, and G10) showed 99% similarity. This pattern of genetic similarity suggests that some samples exhibit genetically conservative lineages, while others show slight variations. This aligns with recent research indicating that the 12S rRNA gene is a strong indicator for tick classification and evolutionary analysis, exhibiting small, natural genetic variations within the same species) Beati & Keirans, 2001(. The complete match in some samples reflects the presence of genetically stable alleles within the same species or strain, making it suitable for use in international taxonomic studies. Recent studies have indicated that using mitochondrial regions such as 12S rRNA with COX1 improves the accuracy of distinguishing between genetically related tick strains, particularly when studying the diversity of insect communities in different environments. (Lv et al.,2014). The slight differences can also be explained by the influence of local environmental and climatic factors in Mosul, such as climate changes (temperature, humidity), pasture and livestock breeding patterns, and the environmental interaction between different hosts. These factors have been shown to affect the genetic distribution of ticks and contribute to the emergence of genetic differences even within the same species (Sánchez-Seco et al .,2024).

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DISCUSSION

The tested Hyalomma anatolicum isolates were showed a significant degree of genetic similarity, according to the dendrogram created using MEGA11 software based on genetic distance analysis. There was little intraspecific genetic variation among the examined samples, and all sequences clustered into a single main clade, confirming their taxonomic identity. The genetic tree or cluster sequencing (Dendrogram) revealed a very high degree of similarity, reaching 100 out of 100 (0.000), for samples from the fifth, tenth, sixth, ninth, and eighth generations. However, there was a slight genetic divergence of 0.002–0.006 between the first, second, third, and fourth generations. This may be due to point mutations or very minor differences in the nucleotide sequence. The only generation among the ten samples that showed significant divergence was the seventh generation, which may represent a sub-lineage of the original lineage.

Strong genetic homogeneity among the majority of isolates was suggested by the clustering pattern, which showed the creation of multiple closely related sub-clusters with very short branch lengths and nearly zero genetic distances. This high degree of resemblance could be the result of limited selective pressure on the examined genetic marker, ongoing gene flow between populations, or a shared ancestral origin. One isolate, however, formed a somewhat divergent branch within the main clade and showed a comparatively higher genetic distance than the other samples. Despite its limitations, this variation could be a sign of early-stage genetic differentiation linked to host-related selective pressures, environmental adaptability, or geographic isolation. However, the observed divergence does not support the existence of different species or subspecies and stays within the range of intraspecific variation.

Overall, the dendrogram's minimal genetic divergence points to a genetically stable Hyalomma anatolicum population structure. From the standpoint of genetic engineering and molecular biology, this uniformity offers a solid basis for subsequent applications, such as the creation of molecular markers, primer design, population genetics research, and studies pertaining to vector competence and acaricide resistance.

CONCLUSIONS

By integrating morphological identification with mitochondrial 12S rRNA gene sequencing, the current study showed significant genetic diversity among tick strains collected from cattle in Mosul, Iraq. Ten strains that were reported as newly discovered in the research region were identified by phylogenetic analysis, which also showed genetic variation among the strains under examination. These results demonstrate the value of molecular methods for precise cattle tick identification and genetic characterization. Additionally, the findings offer baseline data for upcoming studies on genetic diversity, tick dispersion, and the possible epidemiological implications of tick-borne infections like Theileria, Babesia, and Anaplasma. It is advised that more research be done to examine the connections between tick genetic diversity, environmental variables, livestock mobility, and pathogen prevalence in the area.

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CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

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