Morphological and Molecular Identification of Ixodid Ticks that Infest Ruminants in Erbil province, Kurdistan Region-Iraq

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ABSTRACT

A cross-sectional survey was conducted in Erbil province from April to July 2021 to estimate the prevalence of major Ixodid ticks on ruminants and to identify tick species using morphological and molecular tools. A total of 687 animals (202 cattle, 287 sheep and 198 goats) examined, and 254 (36.9 %) were infested. About 381 ticks were collected from examined animals. The result identified two genera of six species of the hard ticks based on morphological, molecular investigation. The identified adult ticks morphologically were belonging to the two Ixodida genera, among which three species belonged to the genus Hyalomma (Hyalomma marginatum, Hyalomma anatolicum and H. excavatum), and three species belonged to the genus Rhipocephalus (Rhipocephalus sanguineu, Rhipocephalus turanicus, and Rhipocephalus B. annulatus). R. annulatus was the dominant tick species infesting cattle with 50% which was significantly higher at P<0.05 than the other isolated species. Whereas H. excavatum and R. sanguineus was the predominant tick species infesting sheep with 29.1% and 28.3 % respectively. While the infestation rate on goats was 16.7%, R. sanguineus was the most prevalent species infesting goats with 45.4% at (P<0.05). R. sanguineus were the predominant tick reported in Erbil governorate with 29.1% at (P<0.05)., while H. excavatum was reported in low percentage in Erbil at 10.2%. DNA samples from sixty ticks were chosen for molecular studies in order to detect tick species using a conventional PCR targeting the 16S rRNA gene. All sequences were subjected to a Basic Local Alignment Search Tool (BLAST) to determine their identities and assess their homologues and similarities to those in the Gen Bank. PCR and the sequencing have confirmed the morphological-based identification. Phylogenetic study revealed that the three Rhipocephalus genotypes revealed from the current study with accession number (MZ663757-MZ663759) were have a highly identical nucleotide sequence 99-100% with a strain of a Rhipocephalus turanicus, R. annulatus and R. sanguineus strain sequence (KY383068, MN594491 and MN594492) from China, and Iraq respectively. On the other hand phylogenetic analysis of Hyalomma genotypes from the present work with accession number (MZ663760-MZ663762) were closely related to a Hyalomma anatolicum, H. marginatum and Hyalomma excavatum (MK829042, MG418663, and KP210047) from Egypt, Turkey and India respectively.

Keywords: Molecular, Morphology, Ixodid tick, Ruminants, Erbil.

1. Introduction

Ticks are universal obligatory blood sucking ectoparasite in the class Arachnida, nonpermanent arthropods that infesting both animal and human. They are causing several diseases resulting in economic impact through morbidity or mortality, due to act as a vector of several pathogens including: haemoprototozoan, bacteria and viruses [1, 2]. Ticks are ectoparasites causing severe economic losses to their hosts through sucking blood and toxic secretions in their saliva leading to anemia, skin damage, reducing weight gain and milk production, inflammation and paralysis [3]. Besides, ticks are responsible for the transmission of various pathogens than any other arthropod vectors groups and they are considered among the most important vectors cause diseases in animals [4, 5].

Ticks have a worldwide distribution, with the highest species diversity in the tropical and subtropical regions [6]. There are approximately 900 tick species which nearly 10% lead to transmit the pathogens in worldwide; the majority of them belong to the two families including: Ixodidae and Argasidae [7, 8]. Hard ticks of tropical livestock belonging to the genera Rhipicephalus, Boophilus, Hyalomma, and Amblyomma and these are the most ecologically and veterinary significant [9]. The sub genus Rhipicephalus (Boophilus) and genus Hyalomma are predominating and have significant impacts on economy in many

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parts of the different countries \cite{10}. Tick-borne diseases remain a concern for global animal management, and it is a priority for many states in tropical and subtropical climates due to their economic and veterinary relevance \cite{10}.

Tick species identification are essential for managing and treating the tick-borne diseases because tick-borne infections are generally transmitted by variant of tick species \cite{11}. Using phenotypic strategies for the determination and differentiation of tick’s species are worldwide gold standard, while significantly limited by the possible variation in ticks life cycle stages and also needs to enough training and experience \cite{12, 13}. However, different genetic markers based on sequence analysis of DNA; were employed to identify, classification and phylogenetic studies of arthropods \cite{14}. Nuclear rDNA (18S and 28S) and mitochondrial rDNA (12S and 16S) are of highly important in tick phylogenetic and genetic diversity studies \cite{8, 15}. Genes particularly, 16S rRNA have been used for molecular recognition and phylogenetic analysis of ticks and is considered well known for barcoding \cite{16, 17}. Molecular analytical tools have proven valuable and complementary for overcoming this ineffectiveness associated with morphological identification of ticks and have been used to identify and differentiate tick species \cite{18, 19}.

Several species of Ixodid ticks species were identified morphologically in Erbil Province; such species are \textit{H. marginatum}, \textit{H. anatolicum}, \textit{H. excavitum}, \textit{Boophilus microplus}, \textit{Boophilus annulatus}, \textit{Boophilus microplus}, \textit{Rhipecephalus turanicus} and \textit{R. sanguineus} \cite{20, 21}. As there are no studies on molecular features of hard ticks in in Erbil province, the current study was designed to investigate morphological and molecular characters of ruminants Ixodid ticks using PCR and sequencing.

2. Materials and Methods

This cross-sectional study was performed from April to July 2021 in Erbil province, Kurdistan Regional, Iraq. Totally 687 ruminants (cattle, sheep and goat) were thoroughly investigated for tick infestations. Ticks were carefully collected from different part of host body including: eye, ears, neck, udder and external genitalia, per femoral region, legs, inguinal and under tail areas. A total of 381 adult ticks were collected from selected animals varying in age and sex and stored in 70% ethanol at 4°C until morphological and molecular identification of tick’s species.

Prior to molecular and phylogenetic analysis the ticks were identified based on morphological classification and taxonomy using stereoscopic microscope (Hertel & Reuss Stereo microscope STE 6, Kassel, Germany, - Catawiki) according to the identification keys \cite{12, 22, 23}. After appropriate identification, from each species of Ixodid ticks (10) samples of, \textit{R. sanguineous}, \textit{R. turanicus}, \textit{Rh. (Boophilus) annulatus}, \textit{H. anatolicum}, \textit{H. marginatum} and \textit{H. excavatum} DNA was extracted using PrimePrepTM Genomic DNA Extraction kit (from Tissue) (GeNet Bio, Korea) following manufacture instructions.

In this study, universal primers of 16S rRNA gene were used to get fragment of size 460 bp, and this help to catch different species of hard tick spp., foreword 5'-CCG GTC TGA ACT CAG ATC AAG T-3' and reverse 5'-GCT CAA TGG TTT TTT AAA TTG CTG TGG T-3' \cite{24}. The PCR program was followed according to previous study by \cite{24}, the reactions were performed in a final volume of (25 μl) table (1). The cycling conditions were as shown in table (2). Eventually, for 1:40 minutes, 10μl of PCR products were visualized under UV on 1% agarose gel which run at 85 Volts.

Table 1: PCR components for tick’s species subject to conventional PCR assay.

| PCR assay   | Chemicals       | Concentration | Each reaction |
|-------------|-----------------|---------------|---------------|
| Conventional PCR | PCR Master Mix   | 2X            | 12.5 μl       |
|            | 100 μM F primer | 20 μM         | 1 μl          |
|            | 100 μM R primer | 20 μM         | 1 μl          |
|            | DNA Template    | 200 μM        | 2 μl          |
|            | dH2O            |               | 8.5           |
| Total      |                 |               | 25 μl         |

Table 2: The thermocycler program for 16S rRNA.

| Step | Function of each step | Temperature | Time    |
|------|-----------------------|-------------|---------|
| 1    | Preheating the lid    | 100 °C      | 5-6 min |
| 2    | Initial denaturation  | 95 °C       | 5 min   |
| 3    | Denaturation          | 95 °C       | 30 Sec  |
| 4    | Annealing             | 55 °C       | 30 Sec  |
| 5    | Extension             | 72 °C       | 30 Sec  |
| 6    | Cycling               | Repeat steps 3-5 | 35X     |
| 7    | Final extension       | 72 °C       | 5 min   |
| 8    | Storage until removal | 4 °C        | variable|

In this study a total of 6 positive PCR products were sent for purification and sequencing into the commercial company (Macrogen Inc. South Korea). Database searches and sequence similarity were performed using the BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST) and comparisons with previous sequences published in GenBank. MEGAX version \cite{25} was used to align sequences. Sequences were aligned together with 16S rRNA gene of tick species sequences, derived from GenBank.
Statistical Analysis

Statistical analysis data were interpreted using general linear model (GLM) procedures (SAS, 2002) in completely randomized design (CRD). Duncans New multiple range test (26) was used to separate the mean.

3. Results

A total 381 adult ticks were collected from ruminants (cattle, sheep and goats) from different locations in Erbil province. Based on the morphological data, two genera of Ixodid tick (*Rhipicephalus* and *Hyalomma*) were identified; three species belong to the genus *Rhipicephalus* (*Rhipicephalus sanguineus*, *R. turanicus*, and *R. annulatus*) and three species belong to the genus *Hyalomma* (*Hyalomma anatolicum*, *H. marginatum* and *H. excavatum*), figure (1,2).

Figure 1: A and B: *Rhipicephalus sanguineous* dorsal and ventral view male; C and D) *Rhipicephalus turanicus* dorsal and ventral view male; E and F) *Rhipicephalus annulatus* dorsal and ventral view male.

Figure 2: A and B: *Hyalomma excavatum* dorsal and ventral view male; C and D) *Hyalomma anatolicum* dorsal and ventral view male; E and F) *Hyalomma marginatum* dorsal and ventral view male.
The data in table (3) shows that the infested rate of Ixodid ticks on cattle is 12%. *R. annulatus* the dominant tick species infesting cattle at a rate of 50% which was significantly higher at P<0.05 than the other isolated species. In sheep, the infestation rate with *H. excavatum* and *R. sanguineus* was the same, around 30%.

While the infestation rate on goats was 16.7% and *R. sanguineus* was the most common species infesting goats with a rate of 45.4%. In Erbil, among all ruminant animals, *R. sanguineus* was the most prevailed tick species with 29.1%, while *H. excavatum* was found in lowest rate at 10.2%.

### Table 3: The infested rate of ixodide tick species on ruminants.

| Tick species     | No. of ticks | %  | Host     | Cattle | %  | Sheep | %  | Goats | %  |
|------------------|--------------|----|----------|--------|----|-------|----|-------|----|
| *R. sanguineus*  | 111a         | 29.1 | 12bc     | 11.8   | 36a | 28.3  | 69a | 45.4  |
| *R. turnicus*    | 71b          | 18.6 | 16b      | 15.7   | 14bc| 11.0  | 47b | 30.9  |
| *R. annulatus*   | 65b          | 17.1 | 51a      | 50     | 9c  | 7.0   | 6c  | 3.9   |
| *H. marginatum*  | 51bc         | 13.4 | 6b       | 5.9    | 8c  | 6.3   | 12c | 7.9   |
| *H. anatolicum*  | 44c          | 11.6 | 14b      | 13.7   | 23b | 18.1  | 13c | 8.6   |
| *H. excavatum*   | 39c          | 10.2 | 3c       | 2.9    | 37a | 29.1  | 5c  | 3.3   |
| Total            | 381          | 16.7 | 102      | 12.0   | 127 | 16.6  | 152 | 16.7  |

The results of amplified PCR product using 16S rRNA gene amplification revealed that the DNA amplicon size for the reaction is 460 bp indicating positive samples for target gene figures (3).

The sequences were analyzed, verified and aligned using BioEdit sequence alignment editor. The sequence was submitted to GenBank (accession number from GenBank as following: MZ663757, MZ663758, MZ663759, MZ663760, MZ663761 and MZ663762) table (4). The “BLAST” tool on the National Center for Biotechnology Information (NCBI) website was used to identified and calculat the similarity of the sequence with homologous sequences in GenBank. Form this research, two genera of six species (each genera of three species) of Ixodid ticks were detected by molecular study and sequencing including *Rhipiciphalus* and *Hyalomma* were the identified genera that infect ruminants in Erbil province, Iraq table (4).

### Table 4: Genus and species of Ixodid ticks and the GenBank accession numbers.

| Ticks genus   | Accession No. | Species       | Similarity % | Reference accession No. | Country |
|---------------|---------------|---------------|--------------|-------------------------|---------|
| *Rhipiciphalus* | MZ663757     | *R. sanguineus* | 100          | MN594492                | Iraq    |
|               | MZ663758     | *R. turnicus*  | 99           | KY583068                | China   |
|               | MZ663759     | *R. annulatus* | 100          | MN594491                | Iraq    |
| *Hyalomma*    | MZ663760     | *H. marginatum* | 100          | MG418663                | Turkey  |
|               | MZ663761     | *H. anatolicum* | 99           | MK829042                | Egypt   |
|               | MZ663762     | *H. excavatum* | 99           | KP210047                | India   |

The Phylogenetic tree of six putative tick species by molecular methods confirmed the existence of species that reported by morphological identification in Erbil in table (4). This shows three major clades representing the *H. marginatum* (MZ663760) which clustered with *H. marginatum* in turkey with accession number (MG418663), *H. anatolicum anatolicum* (MZ663761)
were closely identical to *H. anatolicum* from Egypt (MK829042) while *Hyalomma excavatum* (MZ663762) clustered with *Hyalomma excavatum* Isolate Z7 in India with accession number (KP210047). *R. sanguineus* (MZ663757) and *R. annulatus* (MZ663759) clustered with sequences previously detected from Duhok, Iraq (MN594492), (MN594491) respectively. While *R. turnicus* (MZ663758) sequences were located close to sequences detected in China (KY583068), and using *Amblyomma transversale* used as out group as in figure (4).

**Figure 4**: phylogenic tree of tick species. Black diamond labeled sequences derived from the current study; others indicated sequences from references.

### 4. Discussions

The microscopical and molecular examination of Ixodid ticks in ruminant animals (cattle, sheep and goats) identified six species of Ixodid tick belong to two genera of hard ticks, (*Rhipicephalus* and *Hyalomma*). Three species of *Rhipicephalus* (*R. sanguineus*, *R. turinus* and *R. anulatus*), and also three species of *Hyalomma* (*H. anatolicum*, *H. excavatum* and *H. marginatum*) were identified in this study. The distribution of *Rhipicephalus* spp. appeared to be the predominant tick species in the ruminants. This finding were supported with previous several studies in Kurdistan region, Iraqi by [27, 21, 17] and in Iran by [28]. *Rhipicephalus* spp. appeared to be more predominant species in cattle, sheep and goats. These data are in line with the previously reported results of [29] who found that this species are more prevalent species among small ruminants in Duhok governorate. The ticks *Rhipicephalus sanguineus*, *Rhipicephalus turanicus* and *Hyalomma anatolicum* were found in the center and south of Iraq as well as in the north by [30]. In addition, [30] found some species of Ixodid ticks such as *Rhipicephalus appendiculatus*, *H. marginatum* and *H. anatolicum*. In contrast a study in south of Iraq was disagreeing with these results and reported that *Hyalomma* spp. were more predominant in south of Iraq [31]. The reason of these differences in tick infestations could be related to differences in animal rearing circumstances between ecosystems investigated in prior studies, as well as climate change variables in recent years. These finding are inline with what have been suggested in previous studies conducted by [32, 33]. Phylogenetic analysis and tree allow genetic relationships between closely related species in this country to be resolved with other countries, and it has become a helpful tool in a variety of biological study disciplines [34]. The current study’s phylogenetic tree was built using 16S rRNA sequences, with deletion, transition, and transversion of some nucleotide of sequenced samples which impacted on number of nucleotide, the 16S rRNA sequence of samples (MZ663757-MZ663762), almost was identical 99-100% to the sequences of dereference within the GenBank sequences reference with accession number (MG418663, MK829042, KP210047, KY583068, MN594492 and MN594491) was similar to Turkey, Egypt, India, China and Iraq respectively.

### 5. Conclusion

Overall, the findings suggest that *Rhipocephalus* and *Hyalomma* are the predominant hard ticks’ genera in Erbil based on morphological and molecular studies. This study has deposited 6 ticks sequences information for the 16S rDNA gene in GenBank. Phylogenetically, the identified genotypes were highly identical to that from China, Iraq, Egypt, Turkey and India. Based on the current data, actions need to be taken to control the distribution of the identified tick species to mitigate their economic impacts on the livestock within the region.

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