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MOLECULAR DIAGNOSIS OF IXODIDAE FROM CANIDAE IN IRAQ

Hind Dyia Hadi[1], Afkar Muslim Hadi*[2] and Suhad Y. Jassim[3]
Iraq Natural History Research Center and Museum, University of Baghdad, Baghdad, Iraq
*Corresponding author email: afkar_hadi_iraq@yahoo.com

Abstract

A total of 294 specimens of hard ticks (141 male and 153 female) were collected from dog (*Canis familiaris*) 22 (13 / 9) specimens, from red fox (*Vulpes vulpes*) 97 (45/52) specimens, from Asiatic jackal (*Canis aureus*) 170 (80 / 90) specimens and from wolf (*Canis lupus*) 5 (3/2) specimens; that were caught from the middle, western and southern regions of Iraq. The canine infected with hard ticks in different places of the body that ear, dorsal and all the body. Ticks were removed from the animals using forceps and kept in small tube containing 70% Ethanol and transported to the laboratory in the Iraq Natural History Research Center and Museum for species identification. The current study revealed to three species of hard ticks morphologically identified as *Rhipicephalus turanicus*, *Rhipicephalus leporis* and *Rhipicephalus sp.*in all Canidae species. The results of the specific PCR assay allowing rapid and reliable identification of *Rhipicephalus* sp.by the fragment size amplified was 780bp in *18S rRNA* gene, in four species of Canidae family: dog, red fox, Asiatic jackal and wolf respectively. The sequencing data has reported 4 species of *Rhipicephalus,* Seq1 (MN999872), Seq2 (MN999873), Seq3 (MN999874) and Seq4 (MN999875), these species revealed close matching on the phylogenetic tree to an isolate of *Rhipicephalus turanicus* from Israel (KF958452), matches the isolation of *R. turanicus* (KF958440) of Israel, too, it matches the isolation of *R. turanicus* (KY996841) of China. And therefore, it matches the isolation of *R. turanicus* (JX987495) of Spanish strain. This study represents the first evidence of 18S rRNA gene in *Rhipicephalus* in Canidae in Iraq.

***Keywords*:** Canidae, hard ticks, Ixodidae, molecular, *Rhipicephalus*, Phylogenetic tree.

Introduction

Hard ticks (Ixodidae) are obligatory parasites of vertebrates including mammals, birds, reptiles and amphibians (Dantas-Torres *et al*., 2008, Woods & Sergil, 2001; Maldonado & Guad, 1977 and Morel, 1967). They are external parasites of medical and veterinary significance (Dantas-Torres *et al*., 2012), causing losses to the livestock industry and vector of disease for both animals and humans around the world (Estrada-Peña, 2015). The distribution and epidemiology of tick-borne diseases effect in many factors like: climate changes, biodiversity loss, animals and humans population movements, deforestation, change in land-use etc. (Dantas-Torres, 2015).

Taxonomist classified of the genus *Rhipicephalus* within *Boophilus* genus that because genetic and mating data suggested that *Boophilus* is nested within *Rhipicephalus*; since the *Boophilus* subgenus had been synonmize into *Rhipicephalus* (Horak, 2009).

The aim of the current study is to determine the biodiversity of ticks in Canidae Family in Iraq would be useful in understanding the epidemiology of ticks. And also, to determine the species by genetic analyzes and phylogenetic tree as a first time in Iraq.

Materials and Methods

Study area and tick samples

Through scientific trips and surveys conducted by the research team of the Iraqi Natural History Museum for the period 1977 to 2019, the hard ticks were collected from wild animals (Canidae) that were caught from the middle, western and southern regions of Iraq. The Canidae samples were 63 animals as follow: dog (*Canis familiaris*) 14, red fox (*Vulpes vulpes*) 19, Asiatic Jackal (*Canis aureus*) 27 and wolf (*Canis lupus*) 3. The canine infected with hard ticks in different places of the body that ear, dorsal and all the body. Ticks were removed from the animals using forceps and kept in small tube containing 70% Ethanol and transported to the laboratory in the Iraq Natural History Research Center and Museum for species identification. *Rhipicephalus* sp. was identified using illustrated keys (Strickland *et al*., 1965; Barker and Walker 2014).

Morphological test were applied for all samples of hard ticks and genetic analyses and phylogenetic tree were conducted to four male samples randomly.

Sample 1 = Hard tick from dog (Fig. 1), sample 2 = Hard tick from red fox (Fig. 2), sample 3= Hard tick from Asiatic jackal (Fig. 3), and sample 4 = Hard tick from wolf (Fig. 4)

Polymerase chain reaction

A polymerase chain reaction (PCR) method targeting the *18S rRNA* gene of 4 specimens of ticks. The total volume for the PCR reaction solution was 40µl. Each mixture of the PCR included gDNA at 50ng/µl, each primer (18SF: 5' CATTAAATCAGTTATGGTTCC 3' and 18SR 5' CGCCGCAATACGAATGC 3' targeted the *18S rRNA* gene, (Lv *et al*., 2014) at 10 pM/µl, 25µl of ×2 PCR master mix (×2 buffer, 0.4mMdNTP and 4mM of $MgCl_2$ (abm, Canada). A volume of 10µl of nuclease free water (bioworld) was added to the mixture to have 40µl of the total volume. One cycle of 3-min-initial denaturation at 94°C, 35 cycles of (30sec-denaturation at 94°C, 50-sec-annealing at 45°C, and 30sec- -elongation at 72°C), and the 10-min-final elongation at 72°C. After the thermocycler-based reactions were done, 1.5% agarose gel, ethidium-bromide-stained (US-USA), was use in an electrophoresis process, and the gel was explored under a UV-screener (K&K).

Sequencing of the obtained PCR products

All positive PCR products were sent out to sequencing targeting the *18S rRNA*gene using the primers; 18SF 5' CATTAAATCAGTTATGGTTCC 3' and 18SR 5' CGCCGCAATACGAATGC 3. The purification of the PCR

products was performed using abm Cleanup Kit (abm, Canada) and depending on the kit procedures. The sequencing data were aligned, assembled, and corrected employing FinchTVsequence viewer 1.4 GenBank (http://www.ncbi.nlm.nih.gov/genbank/), and each sequence was recorded in the database.

Phylogenetic analysis

After that, a phylogenetic analysis was conducted to understand the evolution of the ticks. BLASTN in the NCBI website; http://www. ncbi.nlm.nih.gov/BLAST/, was utilized for searching and identifying sequence homologies of tick strains in the GenBank database deposited from around the world. ClustalW 1.8® program was used to infer the similarity between the current study strains and the similar or close-similar strains from around the world after the use of Mega X.

Results and Discussion

A total of 294 specimens of hard ticks (141 male and 153 female) were collected from dog 22 (13 / 9) specimens, from red fox 97 (45 / 52) specimens, from Asiatic jackal 170 (80 / 90) specimens and from wolf 5 (3 / 2) specimens (Table 1).

Table 1: Distribution of hard ticks in some species of Canidae in Iraq.

Host/ species	Species/ticks	Collection regions	Location/ Iraq	Male /female
Dog *Canis familiairs*	*R. turanicus*	Mansoriyat Al- Shat	Middle	2 / 2
		Baghdad	Middle	4 / 3
	Rhipicephalus sp.	AL – Haretha	South	2 / 1
		AL – Dewaniya	South	0 / 1
		AL- Swaira	Middle	5 / 2
Red Fox *Vulpes vulpes*	*R. leporis*	Nukhaib	West	1 / 0
		Samara	West	1 / 2
		AL- Kut	Middle	26 /22
		Said Dikheel	South	1 / 1
		AL- Najaf desert	South	2/ 1
	R. turanicus	AL- Swaira	Middle	4 / 2
		AL- Noamaniya	Middle	1 / 1
	Rhipicephalus sp.	AL- Hai	South	1 / 4
		AL- Tarmiya	Middle	2 / 5
		AL- Noamaniya	Middle	1 / 1
		Sammara	West	1 / 0
		Nukhaib	West	2 / 6
		Baghdad	Middle	1 / 4
		AL - Rutba	West	1 / 3
Asiatic Jackal *Canis aureus*	*R. turanicus*	Ain- Tammur	Middle	18 / 10
		Al – Khalis	Middle	32 / 12
		Baghdad	Middle	2 / 0
		Mansoriyat Al- Shat	Middle	2 / 3
		Summar	South	14 / 10
		Badra	Middle	1 / 0
		Al- Swaira	Middle	4 / 45
	R. leporis	Al- Swaira	Middle	1 / 3
		Haditha	West	1 / 1
	Rhipicephalus sp.	Al- Basrah (Fao)	South	2 / 4
		Baghdad	Middle	2 / 2
		Haditha	West	1 / 0
Wolf *Canis lupus*	*R. turanicus*	Baghdad	Middle	1/1
	Rhipicephalus sp.	Al- Anbar	West	2/1

Morphological study

The most important of morphological characteristics of the *Rhipicephalus* genus: Palps are short and the basis capituli usually hexagonal dorsally. Usually inornate. Eyes and festoons are present. The first coxa cleft deeply. Male has adanal shields and usually accessory shields. Spiracular

plates are comma –shaped. Caudal process present or absent in male (Strickland *et al*., 1965).

The current study revealed to three species of hard ticks morphologically identified as *Rhipicephalus turanicus* (Pomerantsef *et al*., 1940), *Rhipicephalus leporis* (Pomerantsef, 1946) and *Rhipicephalus sp.* in all Canidae species; this result is similar to Shamsuddin and Mohammad (1988), Mohammad (1996), Shubber *et al*. (2014), Shubber (2014) and Mohammad (2015) who revealed to the Asiatic jackal and the red fox were infected with *R. turanicus* and *R. leporis* with infested rate 100%. This result is not in accordance with some studies that recording hard ticks in dogs only like: Abdullah *et al*. (2016) revealed that 100% of dogs were infested by *R. sanguineus* in Egypt. And also, Dantas- Torres *et al*. (2018) who showed that the dogs in south- eastern Brazil infested with *R. sanguineus* only. In Pakistan, Cabezas-Cruz *et al*., (2019) reports that tick-borne pathogen co-infections are very common in *R. sanguineus* (s.l.) ticks that infested domestic dogs.

In general, genetic studies of the genus *Rhipicephalus* in Canidae are very few in the world, and the reason is indicated by Chitimia-Dobler *et al*. (2017) who revealed to the taxonomic status of these taxa will remain unresolved until new lines of evidence become available to complement the current results based on mitochondrial DNA.

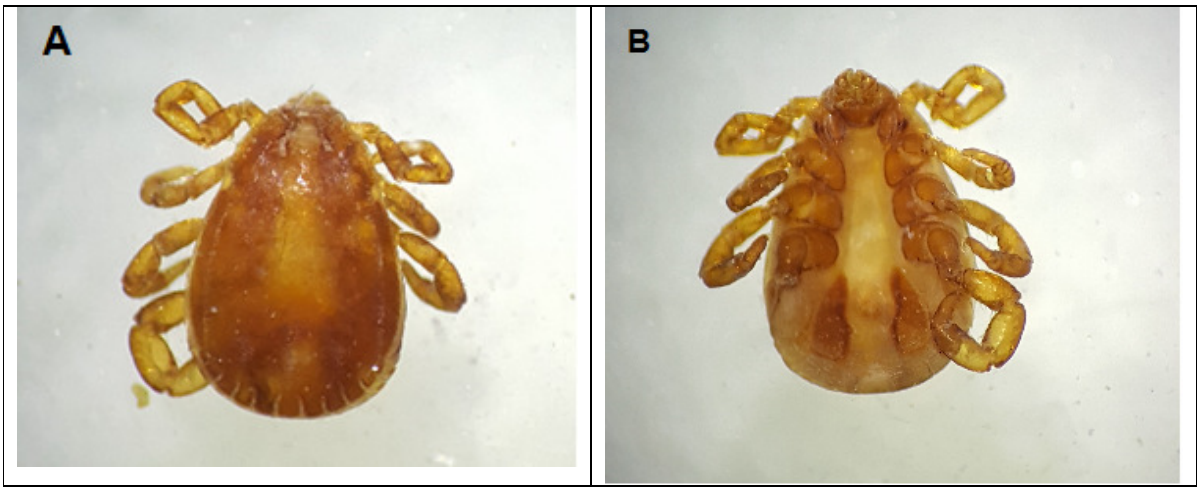

Fig. 1: Morphology of hard ticks from dog, A. Dorsal form. B. Ventral form.

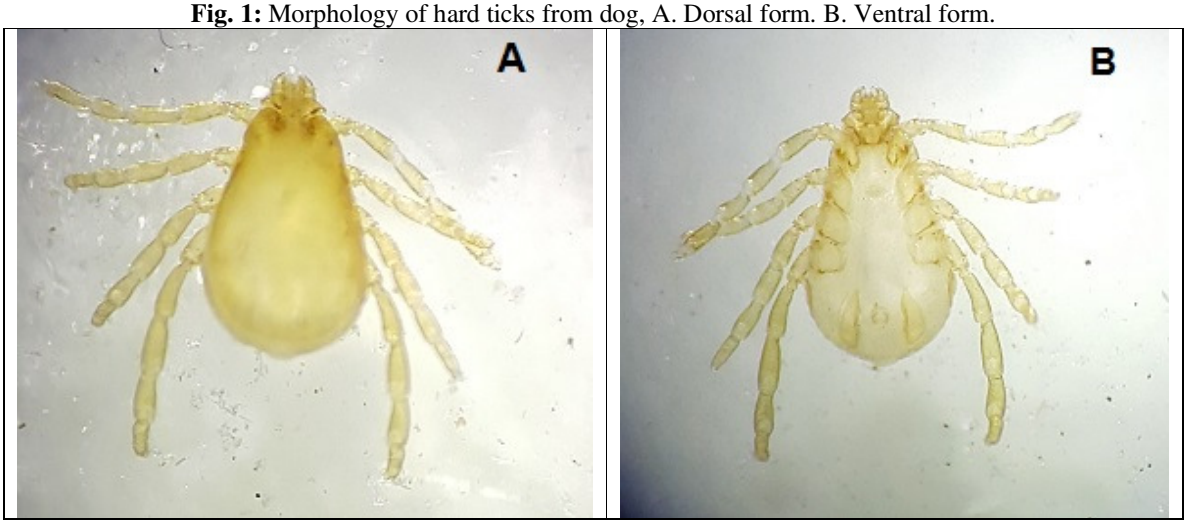

Fig. 2 : Morphology of hard ticks from fox, A. Dorsal form. B. Ventral form.

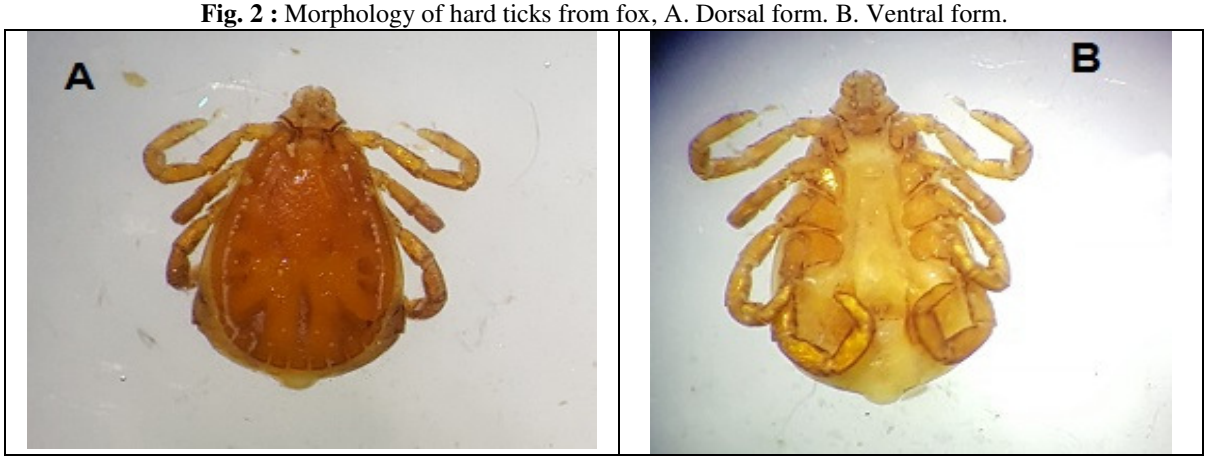

Fig. 3 : Morphology of hard ticks from jackal, A. Dorsal form. B. Ventral form.

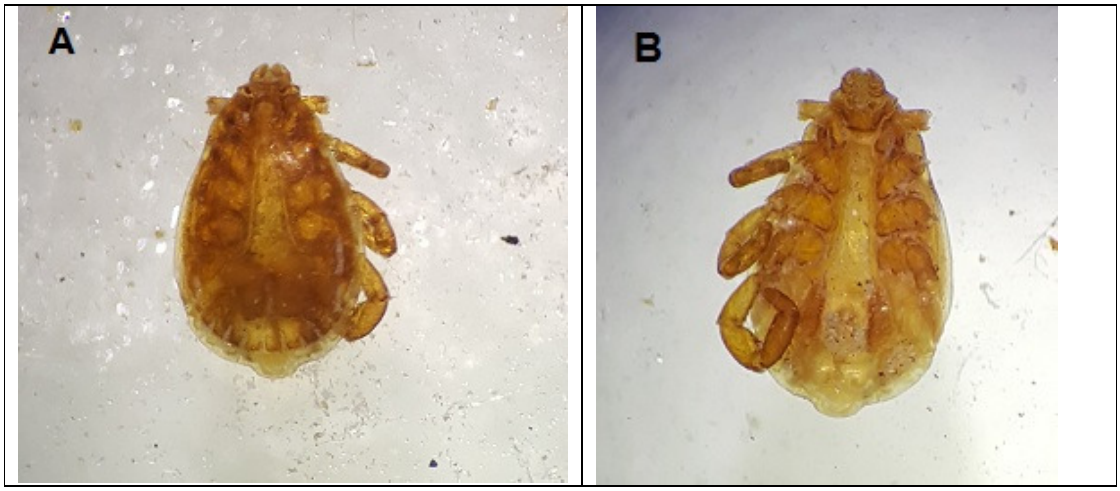

Fig. 4 : Morphology of hard ticks from wolf, A. Dorsal form. B. Ventral form.

Molecular study results

The current study reported the development of sensitive and specific PCR assay allowing rapid and reliable identification of *Rhipicephalus* sp.by the fragment size amplified was 780bp in *18S rRNA* gene, in four species of Canidae family: dog, red fox, Asiatic jackal and wolf respectively, (Fig. 5).

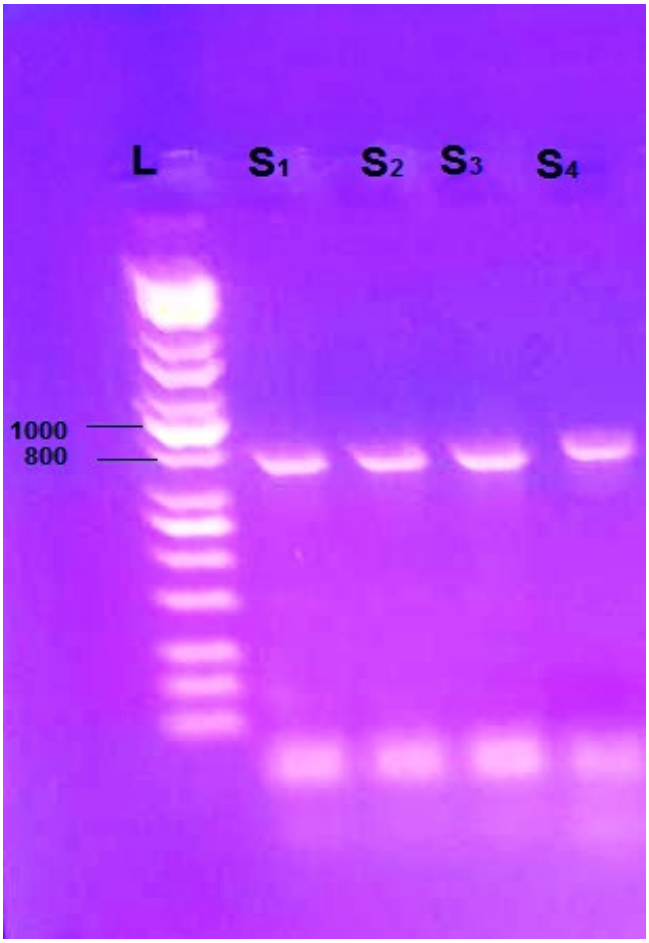

Fig. 5 : PCR product (780 bp) of fragment of *18S rRNA* gene of *Rhipicephalus* sp.in four species of Canidae.
Abbreviations: L = ladder, S =Sample.

GeneRular 1500 bp DNA ladder (lane 1) positive for *Rhipicephalus* banding pattern from dog, (lane 2) positive for *Rhipicephalus* banding pattern from fox , (lane 3) positive for *Rhipicephalus* banding pattern from Jackal, (lane 4) positive for *Rhipicephalus* banding pattern from wolf.

Phylogenetic analysis

The sequencing data has reported 4 species of *Rhipicephalus,* Seq1 (MN999872), Seq2 (MN999873), Seq3 (MN999874) and Seq4 (MN999875), these species revealed close matching on the phylogenetic tree to an isolate of *Rhipicephalus turanicus* 7_T_D1 18S ribosomal RNA gene, partial sequence from Israel (KF958452). On the other hand, it matches the isolation of *R. turanicus* 2_T_D1 18S ribosomal RNA gene, partial sequence (KF958440) of Israel, too. Then, it matches the isolation of *R. turanicus* mitochondrion, complete genome (KY996841) of China. And therefore, it matches the isolation of *R. turanicus* voucher RTE5 18S ribosomal RNA gene, partial sequence (JX987495) of Spanish strain (Diagram 1). Analysis of the genetic tree of the hard tick in the Canidae showed different results and matched with isolates far from Iraq, and the reason may be the lack of genetic recordings in the NCBI bank of the hard tick isolated from the Canidae in the areas adjacent to the study area.

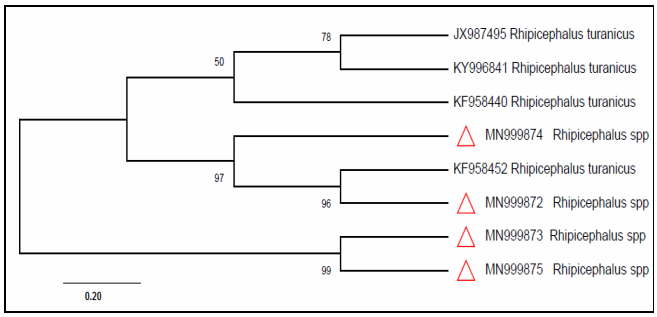

Diagram 1. Phylogentic tree analysis relied on 18SrRNA gene specific region sequencing. The sequencing has shown four species of *Rhipicephuls* spp. Seq1 Δ (MN999872), Seq2 Δ (MN999873), Seq3 Δ (MN999874) and Seq4 Δ (MN999875). These four species revealed close matching on the phylogenetic tree to an isolate from Israel (KF958452). The compression was performed using NCBI – based nucleotides website.

The results of the current study are encouraging to be a starting point for similar studies in Iraq and neighboring countries, especially in wild animals.

Conclusion

The current study sequenced the all positive PCR products targeting the *18S rRNA* gene. The sequencing data were aligned, assembled, and corrected employing Finch TV sequence viewer 1.4 GenBank (http://www.ncbi.nlm.nih.gov/genbank/), and each sequence was recorded in the database of hard ticks from Canidae Family for the first time in Iraq. Phylogenetic analyses revealed that *Rhypiciephalus* species close matching on the phylogenetic tree to an isolate of *Rhipicephalus turanicus* from Israel (KF958452). On the other hand, it matches the isolation of *R. turanicus* (KF958440) of Israel, too. Then, it matches the isolation of *R. turanicus* (KY996841) of China. And therefore, it matches the isolation of *R. turanicus* (JX987495) of Spanish strain.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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