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(RESEARCH ARTICLE)

Molecular phylogeny of tick species in naturally infested cattle

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Abstract

Background: Tick is the second largest vector of diseases, which infests many domestic and wild animals resulting in great economic losses due to morbidities and mortalities.

Aim: Clinical investigation of naturally infested cattle found in Al-Diwanyah city (Al-Qadisiyah, Iraq), morphological diagnosis of ticks' species, and molecular phylogeny of local isolates to identify its identity to global NCBI-GenBank isolates.

Materials and methods: Totally, 438 cattle of different ages and sexes were examined clinically during June-July (2024) to identify and collection of tick samples to be examined traditionally based on morphological characterization. Then, the tick samples were examined molecularly using the conventional PCR, and some of positive samples were sequenced, submitted in the NCBI-database and analysed phylogenetically using the MEGA-11 Software.

Results: A total of 72.37% of study cattle were infested clinically with ticks that existed high significantly in hind-limbs (97.48%) and ears (91.17%), but reduced in back (14.51%) when compared to other body parts including neck (79.2%), udder (71.92%), perineum (52.68%), and forelimbs (35.96%). Regarding age, the prevalence rate of tick was increased significantly in cattle aged 6-9 years (96.72% (59/61)] and cattle aged ≥ 10 years (96.3%) and decreased significantly in cattle aged ≤ 1 year (15.22%) in comparison with those aged 2-5 years (84.5%). Among the positive infested animals, ticks were detected significantly in cattle aged 2-5 years (68.77%) and decreased in those aged ≤ year (4.42%) and ≥ 10 years (8.2%) when compared to cattle aged 2-5 years (18.61%). Based on their morphology, microscopic characteristic suspected largely that all collected samples were hard ticks and belong to *Hyalomma anatolicum*. Targeting the *18S rRNA* gene, the PCR results confirmed that all samples are belonged to *H. anatolicum*. The sequence data of 11 local isolates were named as "Ghirban-Iraq1 to Ghirban-Iraq11", and submitted in the NCBI-GenBank database under the accession numbers "PV615532.1 to PV615542.1". Phylogenetic analysis of study isolates revealed its significant identity to the global NCBI-BLAST Chinese *H. anatolicum* XJ076 isolate (JX051052.1) at a similarity ranged 98.65-99.87% with presence of mutation / changes at 0.017-0.0011%.

Conclusion: Hard ticks, especially *H. anatolicum*, remain a high prevalent ectoparasite in Iraqi cattle causing severe economic losses due to morbidities and mortalities. The combination of traditional morphological characteristics and molecular phylogeny revealed a high efficacy in identification of tick species. Therefore, we recommended to collection the ticks from all field animals to investigate the tick species based on their morphology, and demonstrating their species and origins following the molecular phylogeny due to the definite role of this external parasite in transmission of various diseases to both animals and humans.

Keywords: Ixodid; Cattle Ectoparasites; *Hyalomma Anatolicum*; Polymeras Chain Reaction (PCR); *18s rRNA* Gene; Iraq

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1. Introduction

Tick is an ectoparasite of marked distribution, and considers the second largest vector of diseases in the world as infects many wild and domestic animals in several tropical and subtropical areas, resulting in great economic losses caused by morbidities and mortalities (Brites-Neto et al., 2015; Boulanger et al., 2019). Ticks scientific organization of three families are, Argasidae (soft), Ixodidae (hard) and Nuttalliellidae (Monotypic) which is a member of the Ixodida Order belonging to the Arthropoda phylum (Kelava et al., 2021). Hard ticks referred to so because of scutum's presence on their bodies are the most common and important bearer of pathogens as they took one, two or three hosts while its life cycle had four stages; egg, larva, nymph and adult (Apanaskevich, et al., 2014; Kahl, 2018; Okely, et al., 2021). Globally, spread of infectious diseases are more and more compromising the health of the global population as the number of affected people continues to increase, hence, better understanding of interactions, vectors and pathogens can help in development of prevention and control strategies (De la Fuente et al., 2017; Bouchard et al., 2019).

For diagnosis, the primary methods usage, such as macroscopic and microscopic examination to define tick may provide unconfirmed results in case of the absence epidemiological history (Kemal et al., 2016). In a study of differentiation of some of *Hyalomma apanaskevich*, Horak (2006) revealed that the detection based on the morphological characteristics such size and color of the scutum of different tick stages is extremely complicated and requires great expertise. In addition, differentiating of tick species based on the morphology may lead to misclassification specifically when the method of collecting ticks is so damaging that they get physically hurt due to low expertise and engorging ticks with blood (Estrada-Pena et al., 2017< Nava et al., 2017). For this reason, DNA based methods like polymerase chain reaction (PCR) assays are precise diagnostic methods this offers a real valuable very sensitive and valid information in particular facing epidemiological research (Lv et al., 2014 a, b; Amira et al., 2021). In Iraq however studies have been made on characterization and distribution of ticks on various animal species (Al-gharban and Dhahir, 2015). Based on the works of other studies, these data are under expected since ticks and their incriminated diseases still exist in bigger numbers (Mohammad, 2015; Al-Fatlawi and Al-Fatlawi, 2019; Ali et al., 2021). Hence, this study aims to clinical investigation of naturally infested cattle found in Al-Diwanyah city (Al-Qadisiyah, Iraq), morphological diagnosis of ticks' species, and molecular phylogeny of local isolates to identify its identity to global NCBI-GenBank isolates.

2. Materials and methods

2.1. Samples

Totally, 438 cattle of different ages and sexes were examined clinically during June-July (2024) to identify and collection of tick samples in various areas in Al-Diwanyah city (Al-Qadisiyah, Iraq). The tick samples of each infested animal were sprayed by ethanol 70% and collected by rotating manner using of forceps to avoid damaging of their mouthparts, and then, kept into labeled plastic containers that transported cooled using ice-box. In laboratory, ticks of each sample were divided into two parts, one for morphology which saved cooled and the other for molecular assay which saved frozen.

2.2. Morphological Identification

Tick samples were identified microscopically in the Iraqi Natural History Research Center and Museum (University of Baghdad, Baghdad, Iraq) based on the reference classification keys of other researchers (Walker et al., 2003; Estrada-Pena et al, 2004).

2.3. Molecular analysis

Following the manufacturer instructions of the AddPrep Genomic DNA Extraction Kit (AddBio, Korea), DNAs were extracted from the ticks, and checked for its concentration and pusrity using the Nanodrop System (Thermo Fisher, USA). Targeting the *18S rRNA* gene, one set of primers [(F: 5′-GCAAAAGGAGCCGTTCGTTT-3′) and (R: 5′-GACAGACGAAGCCAAGGGAA-3′)] was designed based on sequence data of the NCBI global *Hyalomma anatolicum* isolate (JQ737105.1) and used to preparing the MasterMix (AddBio, Korea) tubes at a final volume of 20 µl. For PCR reaction, the thermocycler conditions were included 1 cycle (94°C, 7min) for initial denaturation, 35 cycles for denaturation (94°C, 45sec), annealing (58°C, 45sec) and extension (72°C, 1min), and 1 cycle for final extension (72°C, 10min). Electrophoresis using the agarose-gel stained with Ethidium Bromide was done at 80Am, 100volt for 1 hour. The PCR products were visualized using the UV-transilluminator and photographed using the digital camera. The product size of PCR positive samples to *H. anatolicum* were detected at 966bp.

2.4. Phylogenetic analysis

The DNAs of some positive *H. anatolicum* samples were selected and sent to the Macrogen Company (Korea). The received sequence data were submitted initially in the NCBI-GenBank database to get the specific access numbers for the local *H. anatolicum* study isolates, and then subjected to the multiple sequence alignment analysis and phylogenetic tree analysis in the MEGA-11 Software to identify its identity to the NCBI-BLAST *H. anatolicum* isolates (Al-Graibawi et al., 2021).

2.5. Statistical analysis

One-Way ANOVA in the GraphPad Prism (version 6.0.1) Software were applied to detect significant variation between values of study groups at P<0.05 (*) and P<0.01 (**), (Gharban et al., 2023, 2023).

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3. Results

A total of 72.37% (317 / 438) of study cattle were detected clinically to be infested with different species and numbers of ticks; whereas, 27.63% (121/438) were seen to be free of ticks (Figure 1).

The tick isolates were existed high significantly (p-value = 0.0015; 95%CI = 35.24 to 91.31) in hind-limbs [97.48% (309/317)] and followed by ears [91.17% (289/317)], but reduced significantly in back [14.51% (46/317)] when compared to other body parts including neck [79.2% (251/317)], udder [71.92% (228/317)], perineum [52.68% (167/317)], and forelimbs [35.96% (114/317)] (Figure 2). Regarding age of study cattle, the findings showed that the prevalence rate of tick was increased significantly (p-value = 0.0332; 95%CI = 11.04 to 135.3) in cattle aged 6-9 years [96.72% (59/61)] and cattle aged ≥ 10 years [96.3% (26/27)] and decreased significantly in cattle aged ≤ 1 year [15.22% (14/92)] in comparison with those aged 2-5 years [84.5% (218/258)] (Figure 3). Among the positive infested animals, ticks were detected significantly (p-value = 0.0192; 95%CI = 22.40 to 72.40) in cattle aged 2-5 years [68.77% (218/317)] and decreased in those aged ≤ year [4.42% (14/317] and ≥ 10 years [8.2% (26/317)] when compared to cattle aged 2-5 years [18.61% (59/317)] (Figure 4).

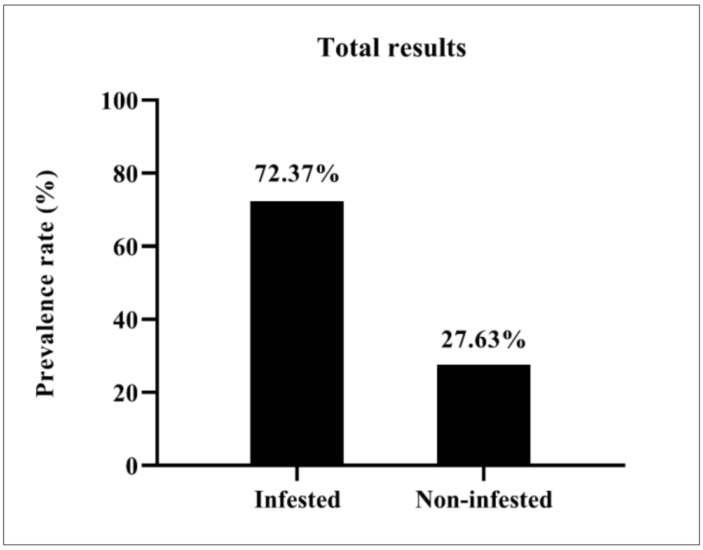

Figure 1 Total results of clinical examination to detect the ticks in totally 438 study cattle

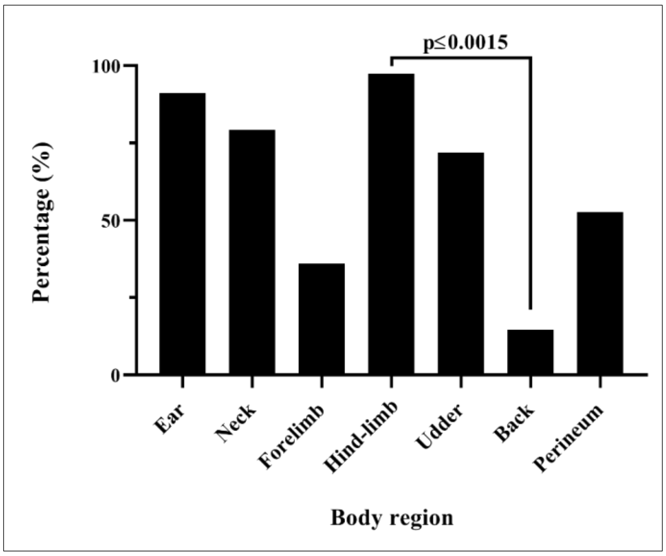

Figure 2 Distribution of ticks on the body regions of infested cattle (total number = 317)

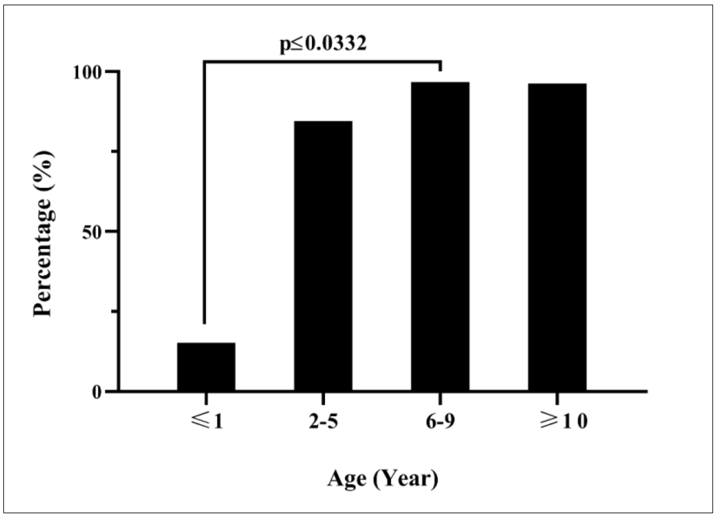

Figure 3 Association of tick infestation to age of study cattle (total number = 438)

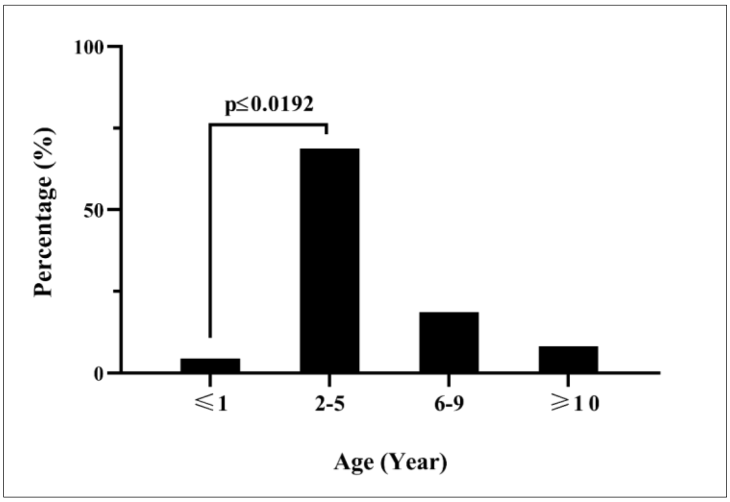

Figure 4 Association of tick infestation to age of positively infested cattle (total number = 317)

Based on their morphology, microscopic characteristic suspected largely that all collected samples were hard ticks and belong to *Hyalomma anatolicum*. Targeting the 18S rRNA gene, the PCR results confirmed that all samples are belonged to *H. anatolicum*. The sequence data of 11 local isolates were named as "Ghirban-Iraq1 to Ghirban-Iraq11", and submitted in the NCBI-GenBank database under the accession numbers "PV615532.1 to PV615542.1". Phylogenetic analysis of study isolates revealed its significant identity to the global NCBI-BLAST Chinese *H. anatolicum* XJ076 isolate (JX051052.1) at a similarity ranged 98.65-99.87% with presence of mutation / changes at 0.017-0.0011% (Figures 5-7, Table 1).

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Species/Abbrv                                                       * * * * * * * * * *   * * * * * * *   * *   * * * * * * * * * * * * * * * * * *
1. Hyalomma anatolicum Ghirban-Iraq1/Iraq (PV615532.1)              T G A G C C T G G A A C C C T T T G A G T T A C G G G T G C T T T T A T T A G A
2. Hyalomma anatolicum Ghirban-Iraq2/Iraq (PV615533.1)              T G A G C C T G G A A C C C T T T G A G T T A C G G G T G C T T T T A T T A G A
3. Hyalomma anatolicum Ghirban-Iraq3/Iraq (PV615534.1)              T G A G C C T G G A A C C C T T T G A G T T A C G G G T G C T T T T A T T A G A
4. Hyalomma anatolicum Ghirban-Iraq4/Iraq (PV615535.1)              T G A G C C T G G A A C C C T T T G A G T T A C G G G T G C T T T T A T T A G A
5. Hyalomma anatolicum Ghirban-Iraq5/Iraq (PV615536.1)              T G A G C C T G G A A C C C T T T G A G T T A C G G G T G C T T T T A T T A G A
6. Hyalomma anatolicum Ghirban-Iraq6/Iraq (PV615537.1)              T G A G C C T G G A A C C C T T T G A G T T A C G G G T G C T T T T A T T A G A
7. Hyalomma anatolicum Ghirban-Iraq7/Iraq (PV615538.1)              T G A G C C T G G A A C C C T T T G A G T T A C G G G T G C T T T T A T T A G A
8. Hyalomma anatolicum Ghirban-Iraq8/Iraq (PV615539.1)              T G A G C C T G G A A C C C T T T G A G T T A C G G G T G C T T T T A T T A G A
9. Hyalomma anatolicum Ghirban-Iraq9/Iraq (PV615540.1)              T G A G C C T G G A A C C C T T T G A G T T A C G G G T G C T T T T A T T A G A
10. Hyalomma anatolicum Ghirban-Iraq10/Iraq (PV615541.1)            T G A G C C T G G A A C C C T T T G A G T T A C G G G T G C T T T T A T T A G A
11. Hyalomma anatolicum Ghirban-Iraq11/Iraq (PV615542.1)            T G A G C C T G G A A C C C T T T G A G T T A C G G G T G C T T T T A T T A G A
12. Hyalomma anatolicum isolate XJ076/China (JX051052.1)            T G A G C C T G G A A C C C T T T G A G T T A C G G G T G C T T T T A T T A G A
13. Hyalomma anatolicum isolate HanatolTR1/Turkey (MW508350.1)      T G A G C C T G G A A C C C T T T G G G T T A C G G G T G C T T T T A T T A G A
14. Hyalomma anatolicum isolate ANM5/Iraq (MK719937.1)              T G A G C C T G G A A C C C T T T G G G T T A C G G G T G C T T T T A T T A G A
15. Hyalomma truncatum/Norway (DQ813264.1)                          T G A G C C T G G A A C C C T T T G G G T T A C G G G T G C T T T T A T T A G A
16. Rhipicephalus australis voucher QMS95164/Australia (KC769614.1) T G A G C C T G G A G C C C T T T G G G T A A C G G G T G C T T T T A T T A G A
17. Rhipicephalus turanicus isolate 50_T_He4/Palastin (KF958445.1)  T G A G C C T G G A G C C C T T T G G G T A A C G G G T G C T T T T A T T A G A
18. Rhipicephalus sanguineus isolate RSTAI/Brazil (JX987497.1)      T G A G C C T G G A G C C C T T T G G G T A A C G G G T G C T T T T A T T A G A
19. Rhipicephalus zambesiensis B304/Australia (AF018654.1)          T G A G C C T G G A G C C C T T T G G G T A A C G G G T G C T T T T A T T A G A
20. Rhipicephalus sanguineus isolate rs011/Uzbekistan (PP501922.1)  T G A G C C T G G A G C C C T T T G G G T A A C G G G T G C T T T T A T T A G A
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Species/Abbrv                                                       * * * * * * * * * * * * * * * * * * * * * * * * * * * *   * * * * * * * * * * *
1. Hyalomma anatolicum Ghirban-Iraq1/Iraq (PV615532.1)              G G A T A A C T T T G T G C T G A T C G C A T G G C C A T G A G C C G G C G A C
2. Hyalomma anatolicum Ghirban-Iraq2/Iraq (PV615533.1)              G G A T A A C T T T G T G C T G A T C G C A T G G C C A T G A G C C G G C G A C
3. Hyalomma anatolicum Ghirban-Iraq3/Iraq (PV615534.1)              G G A T A A C T T T G T G C T G A T C G C A T G G C C A T G A G C C G G C G A C
4. Hyalomma anatolicum Ghirban-Iraq4/Iraq (PV615535.1)              G G A T A A C T T T G T G C T G A T C G C A T G G C C A T G A G C C G G C G A C
5. Hyalomma anatolicum Ghirban-Iraq5/Iraq (PV615536.1)              G G A T A A C T T T G T G C T G A T C G C A T G G C C A T G A G C C G G C G A C
6. Hyalomma anatolicum Ghirban-Iraq6/Iraq (PV615537.1)              G G A T A A C T T T G T G C T G A T C G C A T G G C C A T G A G C C G G C G A C
7. Hyalomma anatolicum Ghirban-Iraq7/Iraq (PV615538.1)              G G A T A A C T T T G T G C T G A T C G C A T G G C C A T G A G C C G G C G A C
8. Hyalomma anatolicum Ghirban-Iraq8/Iraq (PV615539.1)              G G A T A A C T T T G T G C T G A T C G C A T G G C C A T G A G C C G G C G A C
9. Hyalomma anatolicum Ghirban-Iraq9/Iraq (PV615540.1)              G G A T A A C T T T G T G C T G A T C G C A T G G C C A T G A G C C G G C G A C
10. Hyalomma anatolicum Ghirban-Iraq10/Iraq (PV615541.1)            G G A T A A C T T T G T G C T G A T C G C A T G G C C A T G A G C C G G C G A C
11. Hyalomma anatolicum Ghirban-Iraq11/Iraq (PV615542.1)            G G A T A A C T T T G T G C T G A T C G C A T G G C C A T G A G C C G G C G A C
12. Hyalomma anatolicum isolate XJ076/China (JX051052.1)            G G A T A A C T T T G T G C T G A T C G C A T G G C C A T G A G C C G G C G A C
13. Hyalomma anatolicum isolate HanatolTR1/Turkey (MW508350.1)      G G A T A A C T T T G T G C T G A T C G C A T G G C C A T G A G C C G G C G A C
14. Hyalomma anatolicum isolate ANM5/Iraq (MK719937.1)              G G A T A A C T T T G T G C T G A T C G C A T G G C C A T G A G C C G G C G A C
15. Hyalomma truncatum/Norway (DQ813264.1)                          G G A T A A C T T T G T G C T G A T C G C A T G G C C A T G A G C C G G C G A C
16. Rhipicephalus australis voucher QMS95164/Australia (KC769614.1) G G A T A A C T T T G T G C T G A T C G C A T G G C C A C G A G C C G G C G A C
17. Rhipicephalus turanicus isolate 50_T_He4/Palastin (KF958445.1)  G G A T A A C T T T G T G C T G A T C G C A T G G C C A C G A G C C G G C G A C
18. Rhipicephalus sanguineus isolate RSTAI/Brazil (JX987497.1)      G G A T A A C T T T G T G C T G A T C G C A T G G C C A C G A G C C G G C G A C
19. Rhipicephalus zambesiensis B304/Australia (AF018654.1)          G G A T A A C T T T G T G C T G A T C G C A T G G C C A C G A G C C G G C G A C
20. Rhipicephalus sanguineus isolate rs011/Uzbekistan (PP501922.1)  G G A T A A C T T T G T G C T G A T C G C A T G G C C A C G A G C C G G C G A C
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Figure 5 Multiple sequence alignment of the local and NCBI-BLAST *H. anatolicum* isolates using of MEGA-11 software

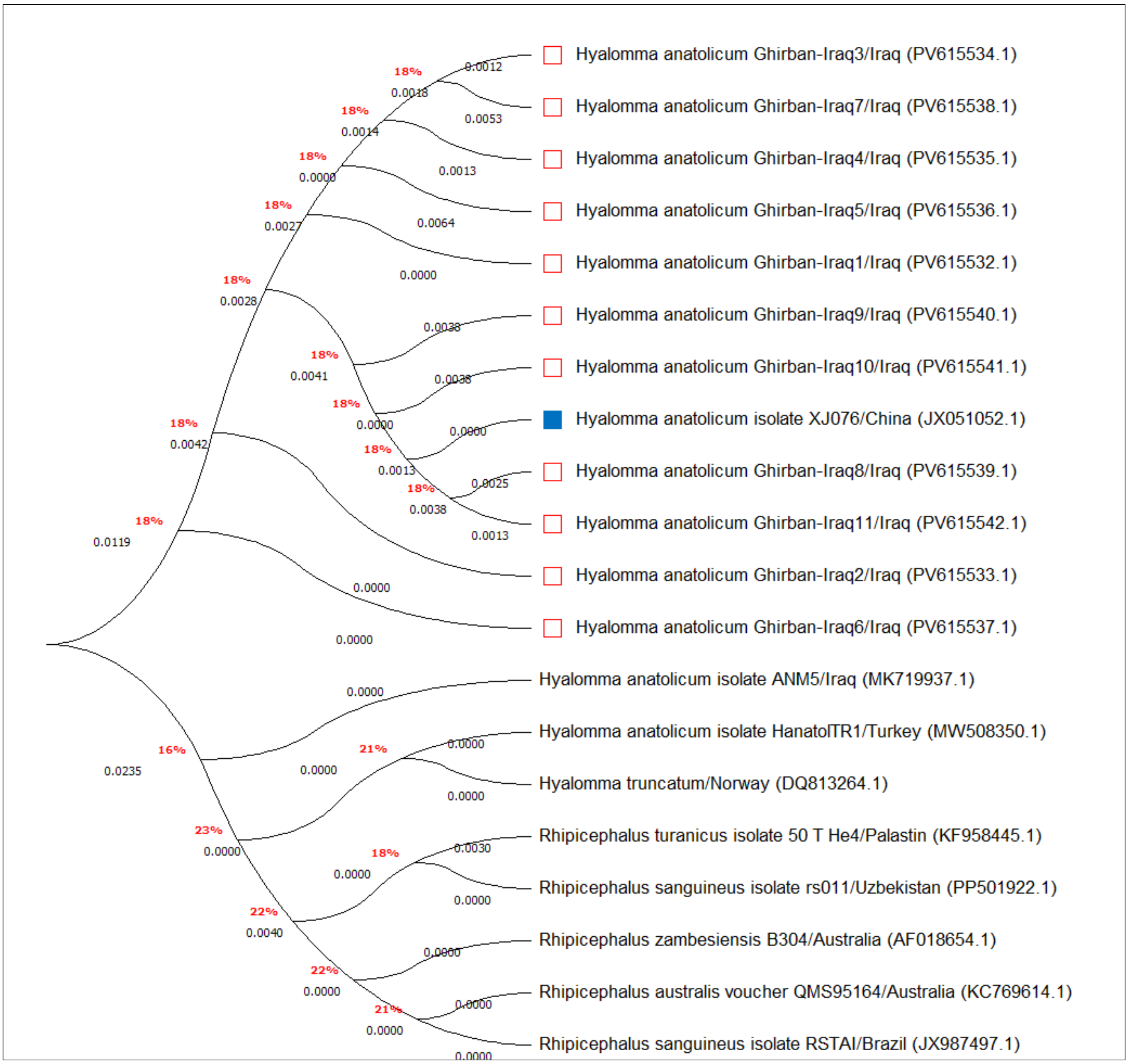

Figure 6 Phylogenetic tree analysis of the local and NCBI-BLAST *H. anatolicum* isolates/strains using of MEGA-11 software

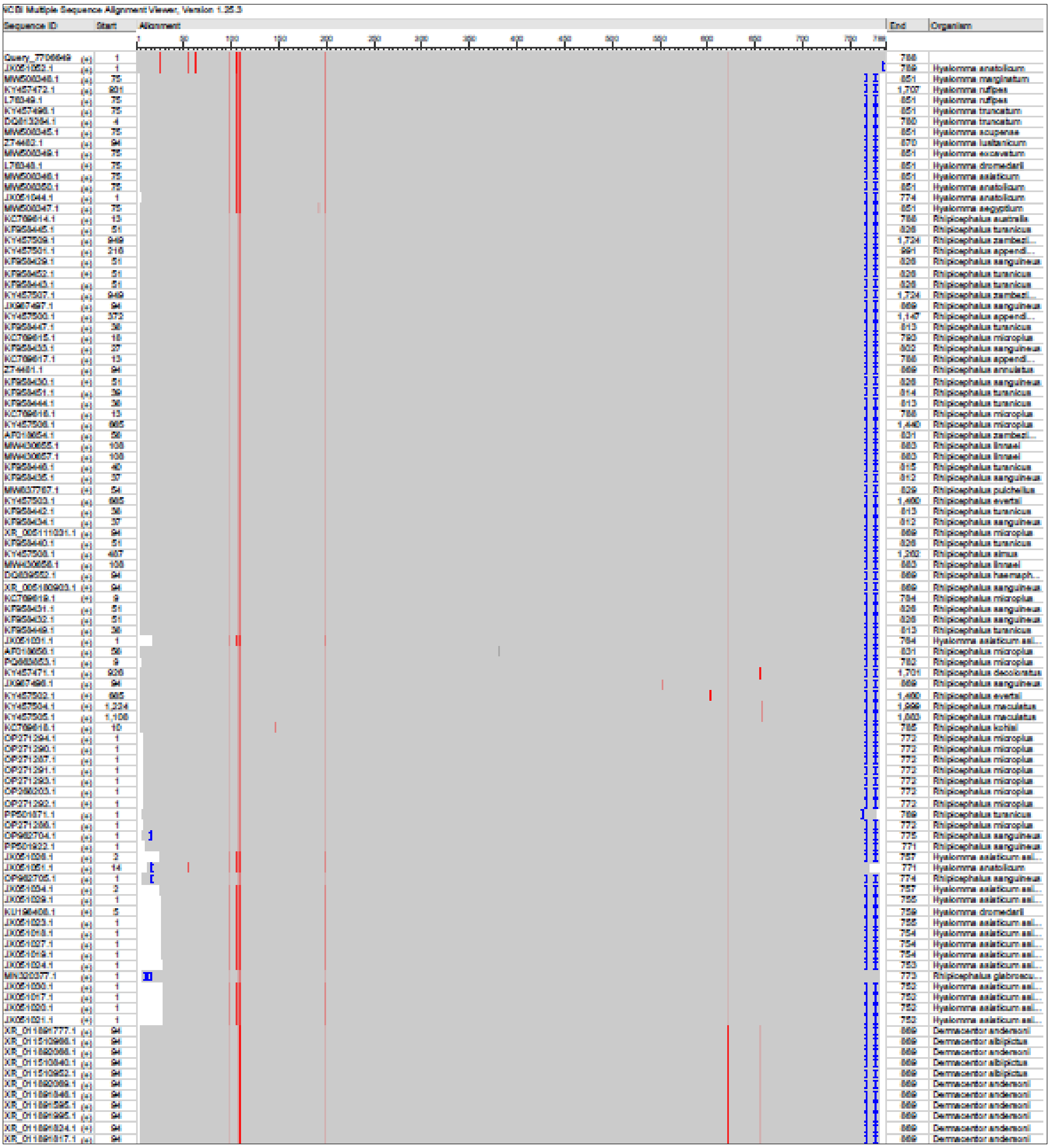

Figure 7 Multiple sequence alignment of the local and NCBI-BLAST *H. anatolicum* isolates/strains using NCBI MSA Viewer

Table 1 Homology Sequence identity (%) for local and NCBI-BLAST *H. anatolicum* isolates

Local isolate		**NCBI isolate**				
Name	Access No.	Species	Isolate	Country	Access No.	%
Ghirban-Iraq1	PV615532.1	*H. anatolicum*	XJ076	China	JX051052.1	99.87
Ghirban-Iraq2	PV615533.1	*H. anatolicum*	XJ076	China	JX051052.1	99
Ghirban-Iraq3	PV615534.1	*H. anatolicum*	XJ076	China	JX051052.1	98.97
Ghirban-Iraq4	PV615535.1	*H. anatolicum*	XJ076	China	JX051052.1	99.22
Ghirban-Iraq5	PV615536.1	*H. anatolicum*	XJ076	China	JX051052.1	99.62
Ghirban-Iraq6	PV615537.1	*H. anatolicum*	XJ076	China	JX051052.1	99.75
Ghirban-Iraq7	PV615538.1	*H. anatolicum*	XJ076	China	JX051052.1	98.98
Ghirban-Iraq8	PV615539.1	*H. anatolicum*	XJ076	China	JX051052.1	99.10
Ghirban-Iraq9	PV615540.1	*H. anatolicum*	XJ076	China	JX051052.1	98.65
Ghirban-Iraq10	PV615541.1	*H. anatolicum*	XJ076	China	JX051052.1	99.13
Ghirban-Iraq11	PV615542.1	*H. anatolicum*	XJ076	China	JX051052.1	99.10

4. Discussion

The findings of this study were in agreement with other local studies such as Suleiman (2018) who recorded 36.77% infested cattle with hard ticks; but higher than those recorded in Sulaimanyia (11.8%) by Kadir et al. (2012); and in Baghdad (8.1%) by Hasson (2012) and 12.9% by Mallah and Rahif (2016); while were lowered than reported in Basrah (42.5%) by AL-Mayah and Abdul-Karim (2020). In comparison with other global studies, the overall prevalence of hard ticks in cattle was 85% in Pakistan (Ali et al., 2013), 67.5% in Iran (Ghashghaei et al., 2017), 40.26% in Ethiopia (Yalew et al., 2017) and 41.93% in India (Debbarma et al., 2018).

Based on their morphology, microscopic characteristic demonstrated that all collected samples were hard ticks and belong to *Hyalomma anatolicum*. Our results were similar with that reported by other researchers (Intirach et al., 2023; Luz et al., 2023).

Targeting the *18S rRNA* gene, the PCR results confirmed that all samples belonged to genus of *Hyalomma anatolicum*.

These results were in agreement with other locally studies carried out in Al-Najaf (Al-Fatlawi et al., 2018) and Babylon (Saadoon and Abid, 2021) provinces. Globally, the findings of this study were similar with that detected in Turkey (Aktas et al., 2006), Tunisia (M'ghirbi et al., 2008), China (Yu et al., 2018), Pakistan (Zeb et al., 2020), Egypt (Amira et al., 2021) and Saudi Arabia (Omer et al., 2021).

Many researchers in Iraq and neighboring countries have studied the epidemiology of persistent ticks and reported that dairy cattle grazing on pastures can become infected with ticks during grazing (Al-gharban and Dhahir, 2015; Desta et al., 2016; Kasaija et al., 2021). In Iraq, six species have been recorded including *Rhipicephalus annulatus*, *R. sanguineus*, *H. anatolicum*, *H. impeltatum*, *H. marginatum* and *H. excavatum* (Omar et al., 2007; Mohammed, 2015; Al-Abidi and Al-Ameri, 2021). An extensive study of some cattle herds in Basra province showed that *H. excavatum* can infect cows, calves and donkeys for the first time in Iraq. *Hyalomma asiaticum* was first recorded in animals in the south Iraq (Awad and Abdul-Hussein, 2006). In northern Iraq, Ismail and Omar (2021) recorded six species in Duhok city which belongs to two major tick genera, three species belonging to *Hyalomma* and three species of minor pollinators belonging to the genus *Rhipiciphalus*. A new species of the subgenus *Hyalomma* (*H. asiatium asiaticum*) was reported for the first time in the Duhok region. In addition, a study was conducted to determine the prevalence of ticks of the family Ixodidae among horses and some domestic animals in Erbil province (Aziz and AL-Barwary, 2020). Another survey was conducted between September and February 2020 to determine the prevalence of the epidemic in the northern region of Basra province using 250 animal samples of different ages and sexes from the same region (Faraj et al., 2021). In other countries, *H. anatolicum* is well adapted to the dry climates of the Mediterranean and North Africa and other desert climates on both continents (Walker, 2014). In Saudi Arabia, a study on several dairy farms in the Al-Ahsa in the eastern region found *H. excavatum* (18.33%), *H. dromedarii* (17.63%), *H. anatolicum* (14.29%) and *R. turanicus* (14.04%), *H. impeltatum* (11.28%), *R. Praetexttatus* (8.56%); *H. turanicum* (6.20%), *Haemaphysalis sulcata* (3.57%), *R. kohlsi* (2.33%), *H. rufipes* (2.09%), *H. schulzei* (1.03%), *H. variegatum* (0.47%) and *A. gemma* (0.18%), (Abdally et al., 2020).

5. Conclusion

Hard ticks, especially *H. anatolicum*, remain a high prevalent ectoparasite in Iraqi cattle causing severe economic losses due to morbidities and mortalities. The combination of traditional morphological characteristics and molecular phylogeny revealed a high efficacy in identification of tick species. Therefore, we recommended to collection the ticks from all field animals to investigate the tick species based on their morphology, and demonstrating their species and origins following the molecular phylogeny due to the definite role of this external parasite in transmission of various diseases to both animals and humans.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

Statement of ethical approval

This study was licensed by the Scientific Committee in the College of Veterinary Medicine (University of Wasit) and performed under the approval No: CVM-UW-4/4/2024-158.

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