Assiut Veterinary Medical Journal **Assiut Vet. Med. J. Vol. 70 No. 183 October 2024, 480-492**

Assiut University web-site: www.aun.edu.eg

MICROSCOPIC AND MOLECULAR DIAGNOSIS OF TICKS INFESTING BUFFALOES IN BABYLON PROVINCE, IRAQ

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Received: 18 July 2024; **Accepted:** 18 September 2024

ABSTRACT

Ticks are detrimental ectoparasites that consume the blood of humans and animals. They serve as crucial carriers for various parasitic diseases in mammals. Existing data indicates that the study of ticks infesting buffaloes in Babylon City is incomplete, and their presence and identification have not been fully examined. This work aimed to use microscopic and molecular approaches to isolate and identify tick species that infest buffaloes. The results revealed that 213 out of 1000 animals (21.3%) were found infested by ticks based on the clinical inspection and microscopic examination in different areas, including Mahaweli, Al-Qasim, Al Saddah, Kifl, Al-Musayyab, Al-Wardia, and Awfi, from September 2023 to January 2024. The microscopic findings found that buffaloes were only infested by Hyalomma spp. The infection rate in females was higher than males in the Al Saddah region. A molecular study using PCR and DNA sequencing techniques identified two genera of hard ticks, Rhipicephalus and Hyalomma, in all infested buffaloes. The sequencing technique demonstrated three species of the genus Hyalomma, including *H. anatolicum, H. excavatum, H. scupense,* while *R. turanicus* was only identified during this study. Interestingly, *H. scupense* was identified for the first time in Babylon province based on DNA sequencing. Overall, this is the first molecular study to identify tick species infesting buffaloes in Babylon province in Iraq, which recommends more hygienic measures to overcome the tick species of Rhipicephalus and Hyalomma.

Keywords: Hard ticks, Buffaloes; PCR, DNA Sequencing, Phylogenetic analysis.

INTRODUCTION

Ticks have considerable medicinal and veterinary significance, since they are common hematophagous ectoparasites of birds, reptiles, and mammals (Akhtar *et al.*, 2011; Al-Lahaibi and Al-Taee *2019;* Sharifa *et al., 2020*). Moreover, they carry many bacterial, protozoal, spirochetal, rickettsial, and viral species, which infect humans and domestic animals and cause several diseases **(**Perveen *et al.,* 2021b**)**. For example, they host and transmit the deadly protozoa, *theileria annulata* **(**Nepveu-Traersy *et al.*, 2024). Ticks can be found worldwide and prefer humid and temperate climates; they typically attach to their host's legs, underarms, and abdomen **(**Nuttall 1905**)**. One of the most prevalent ticks of livestock in the tropics and subtropics is called *Hyalomma sp*. This parasite reduces milk supply, results in

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weight loss, increases mortality, physically harms the leather industry, and necessitates the use of acaricides **(**Kumar *et al.,* 2020**)**. Hard Ticks (Ixodidae), the most prominent tick family, involved 713 valid species **(**Makawi and Hadi 2023**)**. The life cycle of Ixodid ticks is influenced by many intrinsic and extrinsic factors: feeding behaviour, host relations, survival of the host, and the impact of weather and climate **(**Saadoon 2022**,** Kahl and Gray 2023**)**.

Water buffalo are of great value due to their high economic importance **(**Alsaedy 2007**)**. They are well adapted to swamps and areas subject to flooding. There are four general types of Asian water buffalo: wild and domestic **(**Zhang and Barker 2020**)**.

Currently, molecular tools and DNA markers have been widely used to identify tick species, such as ITS, 16S rDNA, 18S rDNA, and 28S rDNA **(**Perveen *et al.,* 2021a**)**. Some studies were carried out in Iraq to diagnose tick infestations in buffaloes, but most of them used the microscopic examinations only. For example, a study done in Waist province found the total prevalence of ticks genera *Rhipicephalus* 48%, *Hyalomma* 38%, and Boophilus 14% **(**Hassan and Al-Zubaidi 2014**)**. Another survey was conducted in the southern region of Iraq, namely in the marshes of Thi-Qar, Basra, and Misan provinces, between 2017 and 2018. The survey identified both *Hyalomma spp.* and *Rhipicephalus spp.* ticks in healthy and clinically unwell buffaloes (Khalaf *et al.,* 2018). In addition, a comprehensive project was conducted in 2018 to identify hard ticks in Iraq **(**Abdullah *et al.,* 2018**)**. A total of 3421 tick samples were gathered from all 18 provinces of Iraq, covering the entire country. The findings indicated that the ticks belonged to the Ixodidae family. The analysis of prevalence and density of ticks per host revealed that the genus Hyalomma was the most prevalent. In Duhok, the rate reached a peak of 88.6%, while the minimum rate of 46.94% was in Nineveh. The Hyalomma genus consists of eight species: *H. anatolicum, H. turanicum, H. scupense, H. dromedarii,* and *H. excavatum*. Among the Rhipicephalus species, the most common were *R. annulatus, R. sanguineous,* and *R. turanicus*. The maximum occurrence rate of these species was found in Al-Anbar at 48.9%, while the lowest incidence was seen in Duhok at 11.4%. The Dermacentor spp. genus is present in Basra with a prevalence of 1.72% **(**Abdullah *et al.,* 2018**).** The study in the central and southern regions of Iraq investigated the prevalence of ticks by the examination of 150 buffaloes; the microscopic findings recorded eight species of hard ticks with infestation rates as follows: *H. truncatum* (50.66%), *H. excavatum* (24%), *Hyalomma anatolicum* (16%), *Hyalomma marginatum* (8%), *H. impeltatum* (8%), *Hyalomma rufipes* (5.33%), *H. scupense* (4%), and *H. dromedary* (2.66%), respectively **(**Obaid *et al.,* 2023**).** Studies on ectoparasites infesting buffaloes are still little or incompletely identified in Iraq, particularly in Babylon city. Thus, this study aimed to identify the species of ticks that infest buffaloes in the areas of abundance of buffaloes in the Babylon province of Iraq, utilizing the molecular techniques besides the traditional microscopic analysis.

MATERIALS AND METHODS

Ethical Approval

The Department of Parasitology at Al-Qasim Green University provided an ethical permission letter (No: 1152, 7/18/2023) to authorize the conduct of this scientific experiment in the College of Veterinary Medicine. Prior to collecting samples on each visit, a data collection permit is obtained from all owners.

Ticks' collection

About 1000 buffaloes were examined in different areas of Babylon province, including: Mahaweli, Al-Qasim, Al Saddah, Kifl, Al-Musayyab, Al-Wardia, and Awfi from September. 2023 to January 2024. Various stages of ticks were found and collected in special containers according to morphological characteristics. The tick specimens were taxonomically classified at the species level using the taxonomic keys (Elhachimi *et al.,* 2023), which rely on many morphological characteristics such as shape, size, color, capitulum (mouth parts), scutum (dorsal shield), and festoons (posterior abdominal markings) **(**Asyikha *et al.,* 2022**)**.

Microscopic examination

The ticks were thoroughly examined in the laboratory using a dissecting microscope and a morphological key **(**Makawi and Hadi 2023**)**. They were then categorized into groups based on their species and kept in 70% ethanol for subsequent genetic diagnosis **(**Hoogsteaal and Kaiser 1958**)**.

Preparation and fixation of tick samples

The ectoparasites were boiled on potassium hydroxide, and then they were dehydrated by alcohol series. First, they were kept at 30%, then 50%, then 70% and 90% and on 100%. Then, they were kept on xylene to confirm whether they were dehydrated well. Then, using Distyrene Plasticizer Xylene (D.P.X), they were mounted on the slides and covered by coverslips, and these slides were observed upon 10X, 4X and photographs were taken **(**Cable 1950**).**

Extraction of DNA from ticks

Each tick from every genus was subjected to a washing process using various concentrations of ethanol (10, 30, 50, and 70 percent) for one hour at each concentration, followed by two washes in PBS. The tick was pulverized using a portal homogenizer with 0.5 ml of PBS, then subjected to centrifugation and stored at a temperature of 18ºC until DNA extraction was performed. The tick's entire genome was collected using a specialized tissue kit known as a DNA extraction kit from Add Bio / Korea. The purity and quality of DNA samples were assessed using spectroscopy, while the samples were analyzed on a 1.5% Agarose gel using gel electrophoresis (Ismael and Omer 2020). This work utilized a single pair of primers to target a specific region of the 16S rRNA gene, which had an approximate size of 460 base pairs. The forward primer sequence was 5' CTG CTC AAT GAT TTT TTA AAT TGC TGT GG-3', while the reverse primer sequence was 5'- CCG GTC TGA ACT CAG ATC AAGT -3'. The primer pair successfully detected various species of hard ticks. The reference for the primer sequences is (Ismael and Omer 2020). The PCR reactions were conducted with a master mix kit provided by ADD Bio, South Korea. The PCR reactions were carried out using a final volume of 25µl. The reaction mixture consisted of 12.5 µl of GeNet Bio master mix, 1 µl each of forward and reverse primers, 2 µl of Template DNA at a concentration of 10 pmol/µL for both forward and reverse primers. The total volume was adjusted to 25 µl by adding 9.5 µl of nuclease-free water. The denaturing process occurred at a temperature of 95 ◦C for 5 minutes. This was followed by 39 cycles of denaturing for 30 seconds at 95 ◦C, annealing for 35 seconds at 55 ◦C, and extension for 35 seconds at 72 ◦C. Finally, there was a final extension at 72 ◦C for 5 minutes**.**

Sequencing of 16S rRNA gene fragment

Tick species were identified by DNA sequencing study. The set of twelve PCR products ~~were~~ was submitted to the Macrogen Company for tick species identification. A homology search was performed using the Basic Local Alignment Search Tool (BLAST) tool, which is accessible at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov), together with the Bio Edit program. The results were compared with data obtained from the Gene Bank, which is accessible online through the National Center for Biotechnology Information (NCBI) **(**Sanger *et al.,* 1977**)**.

Phylogenetic Tree:

Evolutionary analysis determined the maximum likelihood method of the identified *Hyalomma spp* and *Rhipicephalus* spp. sequences. The evolutionary history was deduced via the Maximum Likelihood technique and the Tamura-Nei model. The tree is accurately depicted, with branch lengths quantified in terms of substitutions per site (located below the branches). Evolutionary analyses were performed using the MEGA11 software **(**Mohamed *et al.*, 2022**)**.

Statistical analysis:

Statistical analysis was done using the Statistical Package for Social Science (SPSS) version 27 for Windows software and Microsoft Excel 2010 **(**Kwashabawa 2021).

RESULTS

Microscopic results

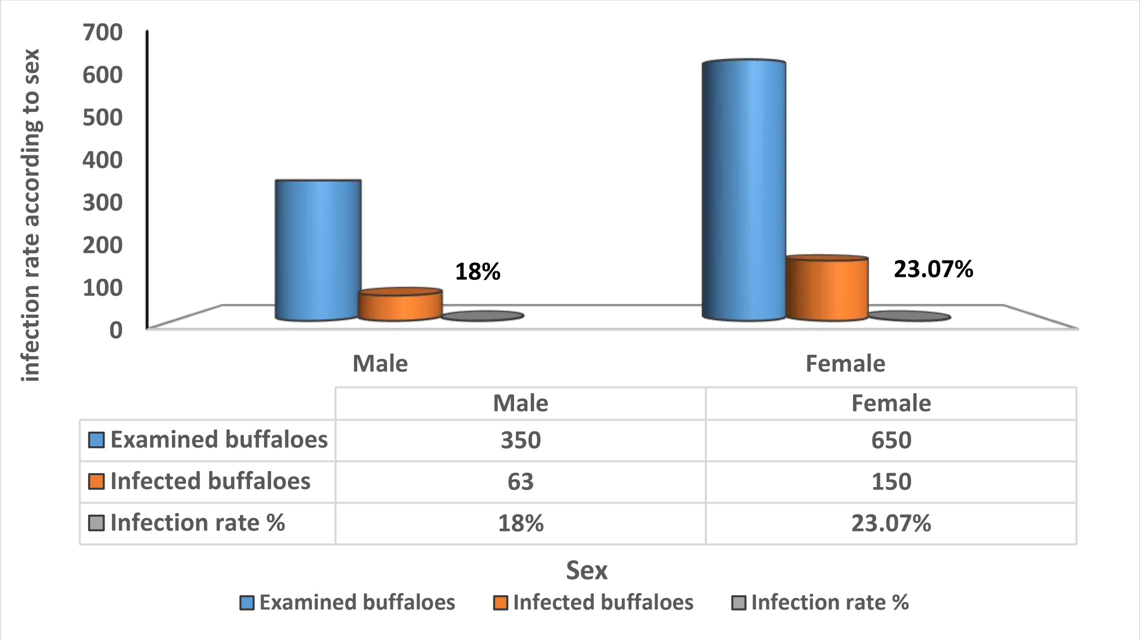
This study showed that 213 ticks were isolated from 1000 buffaloes from Babylon, Iraq, and depending on the microscopic identification of the tick, one genus, *Hyalomma* was prevalent on infested buffaloes (Figure 1). Some (engorged female ticks) remained for molecular identification, because it was difficult to identify them morphologically under the dissecting microscope (Figure 2).

Figure 1: *Hyalomma* Male (A: ventral and B: dorsal views)

Figure 2: *Hyalomma* Female (Dorsal Views).

Infection rates of ticks according to Sex

The results showed that the infection rate in females was higher than in males at 23.07% compared to males at 18% with a significant difference (P<0.05) (Figure 3).

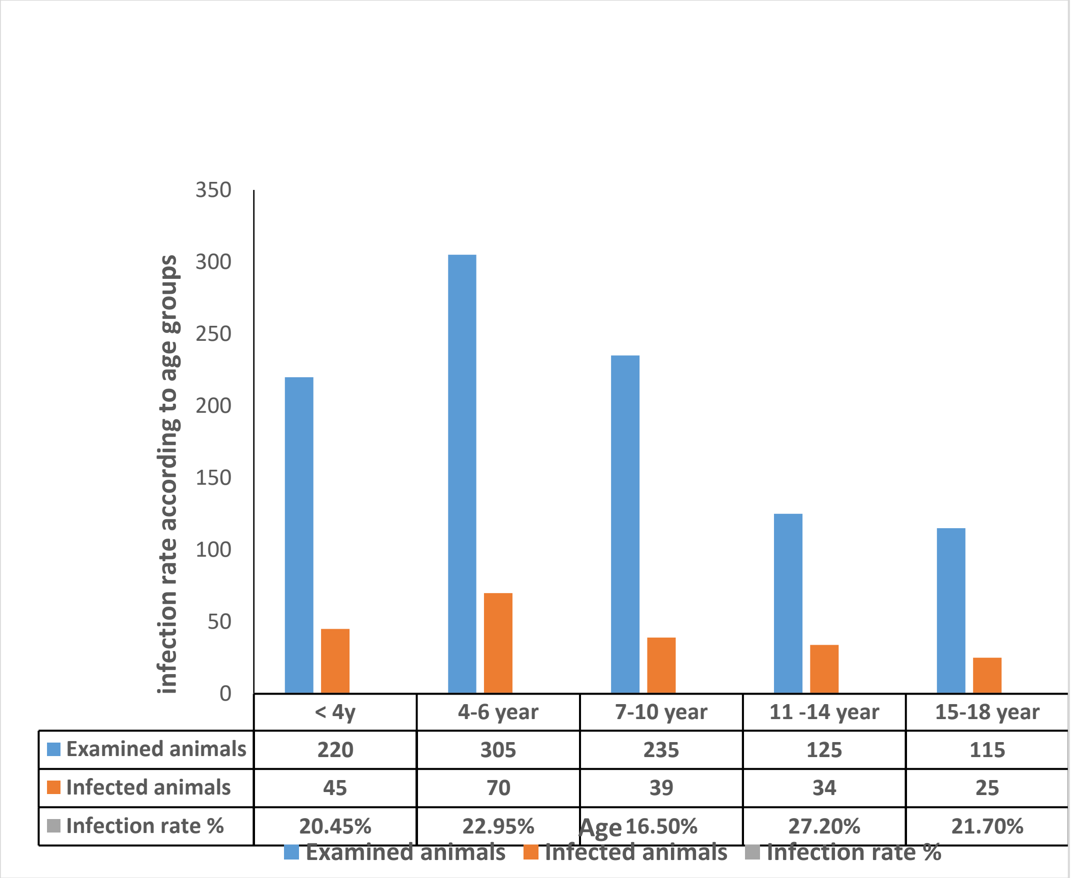


	Male	Female
■ Examined buffaloes	350	650
■ Infected buffaloes	63	150
■ Infection rate %	18%	23.07%

Figure 3: The distribution of hard ticks in buffaloes according to sex from different areas in Babylon province, Iraq

Infection rates of ticks according to age

The results demonstrated the distribution of infection rates of tick infestation in the examined buffaloes according to age groups as follows; in < 4y (20.45%), 4-6 years (22.95%), 7-10 years (16.5%), 11-14 years (27.2%), 15-18 years (21.7%), respectively, without any significant differences (P<0.39) (Figure 4).

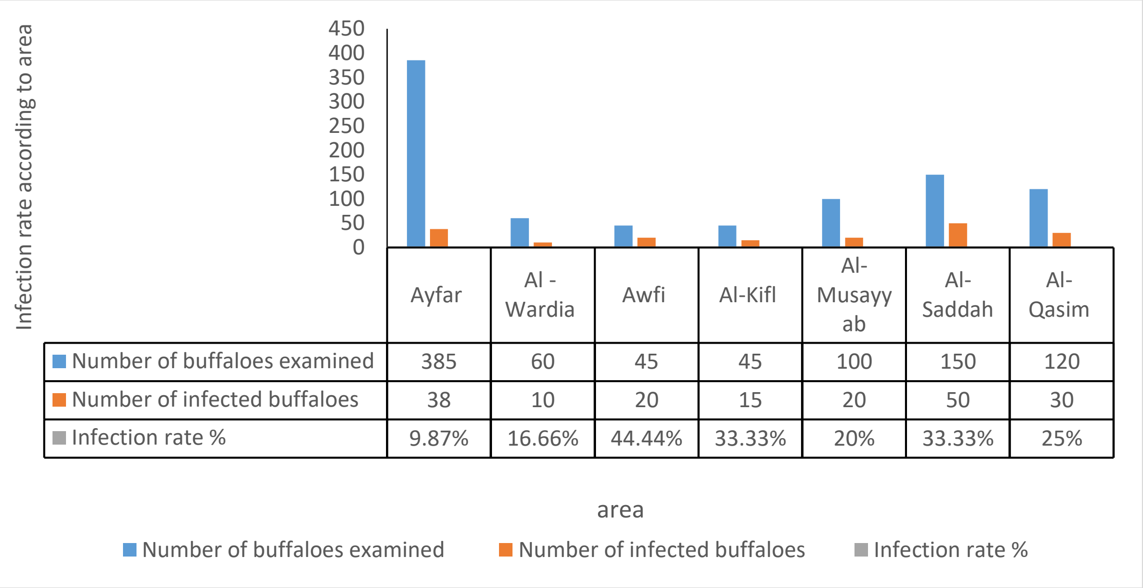


	< 4y	4-6 year	7-10 year	11 -14 year	15-18 year
■ Examined animals	220	305	235	125	115
■ Infected animals	45	70	39	34	25
■ Infection rate %	20.45%	22.95%	16.50%	27.20%	21.70%

Figure 4: The distribution of hard tick in Buffaloes according to age groups from different areas in Babylon province, Iraq.

Infection rates of ticks according to area

The findings revealed the distribution of infection rates of tick infestation in the examined Buffaloes according to the present study region, where they found that in Ayfar (9.87%), Al-Wardia (16.66%), Awfi (44.44%), Al-Kifl (33.33%), Al-Musayyab (20%), Al-Saddah (33.33%) and Al-Qasim (25%) (Figure 5).



	Ayfar	Al - Wardia	Awfi	Al-Kifl	Al-Musayyab	Al-Saddah	Al-Qasim
🟦 Number of buffaloes examined	385	60	45	45	100	150	120
🟧 Number of infected buffaloes	38	10	20	15	20	50	30
⬜ Infection rate %	9.87%	16.66%	44.44%	33.33%	20%	33.33%	25%

Figure 5: The distribution of hard tick in Buffaloes according to the region from different areas in Babylon province, Iraq.

Molecular study:

Conventional PCR

For confirmation, DNA extraction was performed for 50 ticks, including microscopically positive samples. The results showed the amplicons of ticks targeting a partial region within the 16SrRNA gene in isolated ticks' samples (Figure 6).

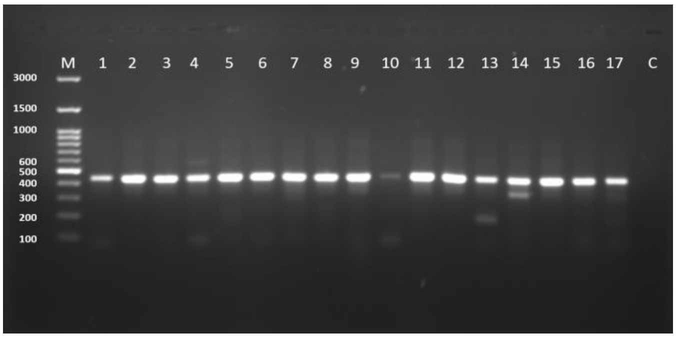


Figure 6: Gel electrophoresis image (1.5 % agarose) shows the amplicons of ticks targeting partial region within 16SrRNA gene (size= 450 bp) (1-17). C is a negative control in which similar PCR reaction components were used, except free nuclease water was added instead of genomic DNA. M is a Molecular marker from Gene Direx, Korea.

Sequence analysis of ticks:

The twelve PCR products were submitted to GenBank and assigned the following accession numbers: PP204225, PP204226, PP204227, PP204229, PP204230, PP204231, PP204232, PP204233, PP204234, PP204235, PP204236, and MN594494.1 (Table 1). The sequence's similarity to homologous sequences stored in GenBank was determined using the "BLAST" program available on the National Center for Biotechnology Information (NCBI) website. The molecular examination and sequencing of this research discovered four species belonging to two genera of hard ticks, Hyalomma and *Rhipicephalus*. Three species falling under the genus Hyalomma *(H. anatolicum, H.excavatum, H. scupense)* and one species under the genus *Rhipicephalus (R.turanicus)* (Table 2) infest buffaloes in Babylon city. Importantly, one of these species *(H. suspense)* with the accession number (PP204236) has been identified for the first time by the current study in Babylon province, Iraq (Table 1)**.**

Table1: Distribution of species of hard ticks *Hyalomma* species and the GenBank accession numbers of obtained sequences.

Sequence number	Obtained Accession number	Identical to	GenBank Accession number	Country	Identity (%)
1	PP204225	*H. anatolicum*	MT509435	China	100
2	PP204226	*H. anatolicum*	MK495916	Pakistan	100
3	PP204227	*H. anatolicum anatolicum*	LC651060	India	100
4	PP204229	*H. anatolicum excavatum*	KP210042	India	100
5	PP204230	*H. scupense*	OR807418	Pakistan	100
6	PP204231	*H. anatolicum*	MH447255	China	100
7	PP204232	*H. anatolicum excavatum*	KP210042	India	100
8	PP204233	*H. anatolicum*	OR486016	Kazakhstan	100
9	PP204234	*H. anatolicum excavatum*	KP210042	India	100
10	PP204235	*H. anatolicum anatolicum*	MK829042	Egypt	100
11	PP204236	*H. scupense*	ON679629	Pakistan	100

Table 2: Distribution of hard ticks *Rhipicephalus spp* and the GenBank accession numbers of obtained sequences.

Sequence number	Obtained Accession number	Identical to	GenBank Accession number	Country	Identity (%)
1	PP204228	*R. turanicus*	MG651940	China	99.73
2	PP204228	*R. turanicus*	OR486014	Kazakhstan	99.73
3	PP204228	*R. turanicus*	MG651935	China	99.45

Phylogenetic tree analysis

The results of the phylogenetic tree based on 16S rRNA gene partial sequence also determined the genetic identity between the locally isolated *Hyalomma species* from infested buffaloes compared to other NCBI-BLAST isolates, showing that the current isolates were PP204225, PP204226, PP204227, PP204229, PP204230, PP204231, PP204232, PP204233, PP204234, PP204235, PP204236 and MN594494.1) to isolates of *(*MT509435, MK495916, LC651060, KP210042, OR807418, MH447255, KP210042, OR486016, KP210042, MK829042, ON679629) as appeared in the Figure (7).

Figure 7: A phylogenetic tree analysis based on 16S rRNA gene partial sequence explains the genetic identity of the local isolates of *Hyalomma spp* in Buffaloes and NCBI-BLAST isolates.

The UPGMA method was used to infer the evolutionary history of *Rhipicephalus turanicus* in the phylogenetic tree analysis. The phylogenetic tree analysis of the 16S rRNA gene partial sequence revealed a genetic similarity between the *Rhipicephalus turanicus* isolated from Buffaloes and other isolates obtained from the NCBI-BLAST database, showing that the current isolates were similar to isolates of MG651940, R486014, and MG651935) Figure 8.

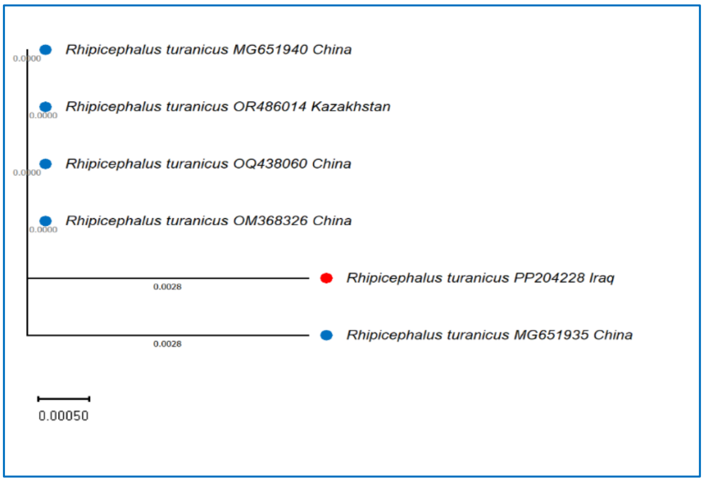


Figure 8: Phylogenetic tree analysis based on 16S rRNA gene partial sequence explains genetic identity between the locally isolated *Rhipicephalus turanicus* in buffaloes NCBI-BLAST isolates.

DISCUSSION

The microscopic examination of hard ticks revealed that one genus (*Hyalomma*) which was isolated from infested Buffaloes. The current study recorded an infestation rate with *Hyalomma* spp (21.3%) from a total number of 1000 buffaloes. The prevalence and proliferation of the Hyalomma species may be attributed to its high tolerance for adverse environmental circumstances, such as low humidity and harsh climates (Kettle 1995).

The percentages mentioned are lower than the ones documented by (Tarash 1982), specifically 94.2% for Hyalomma sp in the Al-Dehab Al-Abiad Village in Basrah, and lower than the rates reported by (Elhachimi *et al.,* 2021). Their percentages are lower compared to Abdul Hussein's (2006) findings, where he documented 73.6% of the genus Hyalomma in Basra province (Telmadarraiy *et al.,* 2004). The province of Babylon has a moderate level of humidity and high temperatures, which provides favorable conditions for the establishment of this genus. The disparities between the findings of the current investigation and prior research could potentially be attributed to variances in geographical locations, climatic circumstances, analytical methodologies, selection criteria for sampled animals, and the specific breed of animals under examination. The spatial arrangement of ticks on hosts in natural environments is determined by multiple factors, including the tick's morphology (specifically the length of its hypostome), the host's morphology (particularly the amount of its fur coat), and the immune responses of the host (Abdul Hussain 2006). The authors discussed the characteristics of the host (such as susceptibility, area, age, and sex) and the management strategies, which included the use of acaricides.

The data indicated a greater infection rate of buffaloes with hard ticks in females compared to males. The results obtained from this investigation exhibited a slight resemblance to the findings of the research conducted by (Estrada-Peña 2023) in Basrah, Iraq. This result did not agree with the results of a study conducted in Pakistan, where the infection rate in buffaloes was in males (87.71%) and in females (83.53%) (Miranpuri 1988). In addition, in the current study, significant differences (P≤0.05) were recorded in the rate of infection between age groups, with the highest rate observed in (11 -14 years) of buffaloes. These results were consistent with a study in Pakistan from October 2012 to September 2013 **(**Abubakar *et al.,* 2018**).** While infection rates according to the area of infection rates of tick infestation in the examined Buffaloes, according to the present study region, low in Ayfar (9.87%), and high in Awfi (44.44%).

The current study utilized molecular approaches, specifically the analysis of the ribonucleic acid 16S rRNA gene, to identify tick species. This gene sequencing method serves as an effective marker for identifying hard tick species, addressing challenges associated with morphological tick identification (Abouelhassan *et al.,* 2019; Hajeel and Abd Alfatlawi 2019). Occasionally, relying just on the morphological identification of ticks is inadequate for species detection. This investigation corroborated the findings of a study conducted by (Hajeel and Abd Alfatlawi 2019). The current study revealed that buffaloes in Babylon province are infested with four hard tick species belonging to two genera: *H. anatolicum, H. excavatum, H. scupense,* and *R. turanicus.* Thus, the utilization of 16S rRNA serves as a reliable indicator for identifying the specific hard tick species in this particular investigation. Prior research has documented the presence of identical species of hard ticks in Pakistan (Fukunaga *et al.,* 2000). The construction of the phylogenetic tree analysis for Hyalomma species was based on 16S rRNA sequences, which showed a complete similarity of 100% with isolates from various Asian countries. Similarly, the phylogenetic tree analysis for *Rhipicephalus turanicus* demonstrated a similarity of 99.73% with isolates from Kazakhstan and China. Phylogenetic analysis and trees have shown to be a significant tool in various sectors of biological research by enabling the resolution of genetic links among closely related species **(**Ali *et al.,* 2021**).**

CONCLUSIONS

It can be concluded that the infection of hard ticks has an important effect on buffaloes living in Iraq, particularly in Babylon province. The overall infection rate of hard ticks in buffaloes was (21.3%), showing two genera: *Hayloma* and *Rhipicephalus*. The results of molecular techniques identified and confirmed the infection of buffaloes by three species of the genus *Hayloma* which are *(H. anatolicum, H. excavatum, and H. scupense)*, while one species under the genus *Rhipicephalus turanicus* was found. Importantly, it is believed that the current study has identified and recorded some *Hyalomma* species and *Rhipicephalus turanicus* for the first time in Babylon province/Iraq depending on the PCR and DNA sequencing techniques, which highlighted the importance of the presence and distribution of hard ticks infesting buffaloes in this area.

Highlights

1. Two genera of hard ticks (*Hayloma* and *Rhipicephalus*) were isolated in buffaloes living in Babylon province.
2. The infestation rate of hard ticks in females was higher than in males.
3. The tick infestation was observed in all ages of examined buffaloes.
4. This is the first study in this area that used the DNA sequencing technique for identification of species belonging to those genera, where it recorded three species belonging to the genus *Hayloma* (*H. anatolicum, H. excavatum, H. scupense*), and one species belonging to *Rhipicephalus (R. turanicus)*

ACKNOWLEDGEMENT

The authors are thankful to the College of Veterinary Medicine at Al-Qasim Green University for the facilities provided to complete this work.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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التشخيص المجهري والجزيئي للقراد المصيب للجاموس في محافظة بابل، العراق

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يعتبر القراد من الطفيليات الخارجية الضارة التي تتغذى على دم الانسان والحيوان. حيث تعد من النواقل الرئيسة لمختلف الامراض الطفيلية في اللبائن. تشير البيانات الحالية إلى أن وجود وتحديد اجناس وانواع القراد الذي يصيب الجاموس في مدينة بابل لم يتم دراسته بشكل كامل. لذلك فقد أجريت هذه الدراسة لعزل وتشخيص أنواع القراد التي تصيب الجاموس باستخدام التقنيات المجهرية والجزيئية. كشفت النتائج الحالية عن إصابة ٢١٣ حيواناً من أصل ١٠٠٠ بالقراد بناء على الفحص السريري والفحص المجهري في مناطق مختلفة منها المحاويل، القاسم، السدة، الكفل، المسيب، الوردية، وعوفي للفترة من ايلول ٢٠٢٣ الى كانون الثاني ٢٠٢٤. اظهرت نتائج الفحص المجهري ان الجاموس مصاب بجنس *Hayloma* فقط في هذه الدراسة. كما بينت النتائج أن معدل الإصابة بين الإناث كان أعلى منه بين الذكور في منطقة السدة. فيما يخص الفحص الجزيئي باستخدام تفاعل البوليميراز المتسلسل وتقنية تسلسل الحمض النووي ، اشارت النتائج الى ان الجاموس مصاب بجنسين من القراد الصلب هما *Hayloma* و*Rhipicephalus* ، حيث تم تحديد ثلاث انواع تابعة الى جنس *Hayloma* ونوع واحد تابع لجنس *Rhipicephalus H.anatolicum, H. excavatum, H. scupense, and R. turanicus* ومن المثير للاهتمام أنه تم التعرف على *H. scupense* لأول مرة في محافظة بابل باسخدام تقنية تسلسل الحمض النووي. بشكل عام ووفقاً للتقنيات الجزيئية المستخدمة والبيانات الحالية، تعتبر هذه الدراسة الجزيئية هي الأولى في التعرف على اجناس وأنواع القراد التي تصيب الجاموس في محافظة بابل، العراق.