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Molecular Incidence of Theileriosis in Morphologically Identified Hard Ticks

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Abstract: The current study aims for molecular detection of theileriosis caused by *Theileria annulata* in hard ticks (*Hyalomma anatolicum*) using the conventional PCR. A total of 200 buffaloes existed in different areas in Wasit province (Iraq) were subjected to collection the tick samples during April to July (2023) to be examined morphologically and molecularly. Among study cattle examined grossly, 74% were found positives for presence of tick infestation. Regarding distribution of ticks on different bodily parts of study buffaloes, significant higher infestation of ticks was seen in udder (26.9%) and perineal region (23.1%); while, significant lowering was seen in forelimb (9.76%) and neck (9.76%); when compared to ear (15.48%) and hindlimb (18.57%). Concerning the month of samples collection, the findings showed a significant increase (P<0.0436) in prevalence of ticks in June (80%) and July (86%) and significant decrease in April (58%) when compared to May (72%). Based on their morphological characteristics, microscopic examination of collected ticks referred that all samples were hard ticks belongs to *Hyalomma* genus in particular *H. anatolicum* species. Targeting *18S rRNA* gene, molecular examination of 148 samples of ticks by the conventional PCR assay revealed that 10.14% were positively infected with *T. annulata*. Distribution of PCR results reported a significant elevation (P<0.0467) in positive *Theileria annulata* infections in July (20.93%) but significant reduction in April (3.45%) and May (2.78%) in comparison with June (10%). In conclusion, this study demonstrated the highly prevalence of ticks in buffaloes especially with increasing of environmental temperature in summer months. Hard ticks of *Hyalomma* genus in particular *H. anatolicum* remain the most incidence ticks among buffaloes population. Molecular detection of *Theileria annulata* in study ticks demonstrated their roles in transmission of infection to other healthy buffaloes. Moreover studies on ticks of infested buffaloes or other field animals using the molecular assays appear of great importance to estimate its role in transmission of other pathogens.
Keywords: *Theileria annulata*, *Hyalomma anatolicum*, Water buffalo, *Bubalus bubalis*, Tick-borne disease, Ectoparasite

Introduction

Ticks, ectoparasites of worldwide distribution in several tropical and subtropical countries, are the main vector for different viral, bacterial and parasitic infections resulting in variable illnesses in both human and animals (Boulanger et al., 2019; Muhammad et al., 2021). Theileriosis, caused by *Theileria* spp., is one of most prevalent infectious diseases of bovine and other domestic animals which need two types of hosts to complete its lifecycle (Abdela

and Bekele, 2016). Transmission and survival of *Theileria* might depend on several factors, including different host stages, the ability of sporozoites and merozoites of the mammalian host, and zygotes and kinetochores of the tick vector to sense and enter specific cells (Farhat, 2020; Gubbels et al., 2020). Although, transmission of *Theileria* occurs mainly through feeding of adult ticks on blood of a host, sporozoites infiltrate lymphocytes and differentiate into schizonts resulting in lymphocyte degeneration. In lymphocytes, schizonts differentiate into merozoites that infect erythrocytes (Tajeri et al., 2022). Repeated asexual divisions have been shown to occur in erythrocytes and lymphocytes of species such as *T. annulata*. Inside the infected erythrocytes, merozoites develop into piroplasms, a parasitic stage that infects ticks (Weir et al., 2011; Tirloni et al., 2015; Jalovecka et al., 2018). In ticks, sexual developmental phase of *Theileria* occurs in gut producing the zygote that invades the gut cells and remains there throughout the moulting cycle and grows into a single kinete. Kinete escapes from the gut cell, invades salivary glands, and remains in the salivary glands until transmitted to another mammalian host post moult nymph or adult feeds (Mans et al., 2015; Kiara et al., 2018; Akhtar et al., 2023). Tick feeding initiates rapid development of sporozoite that released during the final feeding period (Nene et al., 2016; Lakew et al., 2023).

In suspected animals, diagnosis of the parasite can be based on traditional or molecular methods. Traditional methods include detection of preliminary clinical signs, analysis of necropsy results, microscopic and serological examinations (Lempereur et al., 2017; Gebrekidan et al., 2020). In ticks, traditional diagnostic methods could be either not applicable or having several drawbacks and providing low-valuable data. These limitations can be overcome by molecular methods, especially polymerase chain reaction (PCR) that characterized by their highly sensitivity and specificity in identification, characterization, isolation and comparison of different strains (Bogema et al., 2015; Nangru et al., 2022). In Iraq, almost researchers have focused on diagnosis of *Theileria* in the hosts such as cattle (Ahmed et al., 2021), sheep (Abdullah et al., 2022), goats (Mahmoud et al., 2019) and camels; however, the number of carried out studies in ticks remains very limited and need moreover investigations (Al-Fatlawi and Al-Fatlawi, 2019). Hence, the current study aims for molecular detection of theileriosis caused by *Theileria annulata* in hard ticks existed on buffaloes using the conventional PCR.

Materials and methods

Samples

A total of 200 buffaloes existed in different areas in Wasit province (Iraq) were subjected for gross examination to detect and collect of tick samples during April to July (2023). Ticks were removed manually using the forceps and chloroform (CHEM-LAB, UK) to ovoid their damages due to mouthparts adhesion to skin of buffaloes, and collected into labeled plastic container. Ticks of each animal were considered as solitary sample during morphological examination of ticks and molecular investigation of *T. annulata*. Also, data concerned to distribution of ticks on different body parts in addition to month of samples collection were documented as risk factors. According to number of detected and collected ticks from each animal, the study buffaloes were graded into groups as following: A (1-5 ticks), B (6-10 ticks), C (11-15 ticks), D (16-20 ticks), E (21-25 ticks), F (26-30 ticks), G (31-35 ticks) and H (> 35 ticks).

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Morphological examination of ticks

Tick samples were identified morphologically based on the key features described by other researchers (Walker et al., 2003; Estrada-Pena et al., 2004).

Molecular detection of *T. annulata*

Following the manufacturer instructions of the gSYAN DNA Extraction Kit (Geneaid, Taiwan), DNAs were extracted from the ticks, and then evaluated for its purity and concentration using the Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). Targeting *18S rRNA* gene, one set of primers [F: (5´- GAC TCA ACA CGG GGA AAC TC-3´) and R: (5´- CAT TCC TCG TTC ACG ATT AAC A-3´)] was designed based on the GenBank database of NCBI isolate (MK737519.1), and the MasterMix tubes were prepared using the AccuPowerTM PCR PreMix Kit (Bioneer, Korea) at a final volume of 20 µl. For PCR reaction, the MasterMix tubes were transferred into the conventional PCR thermocycler system (BioRad/ USA) and subjected to the following conditions; 1 cycle initial denaturation (95°C / 7 min); 35 cycle denaturation (95°C / 30 sec), annealing (58°C / 30 sec) and extension (72°C / 30 sec); and 1 cycle final extension (72°C / 5 min). Electrophoresis of agarose-gel (1.5%) stained with Ethidium Bromide was carried out for PCR products at 80AM and 100 volt for 90 minutes, and the resultants were visualized using the UV transilluminator to detect the positive samples at a product size of approximately 400 bp.

Statistical analysis

The t-test and One-Way ANOVA in the GraphPad Prism Software were served for detection significant differences between study values at $P<0.05$ (Gharban et al., 2023).

Results

Among totally 200 buffaloes examined grossly, 148 (74%) were found positives for presence of tick infestation (Figure 1).

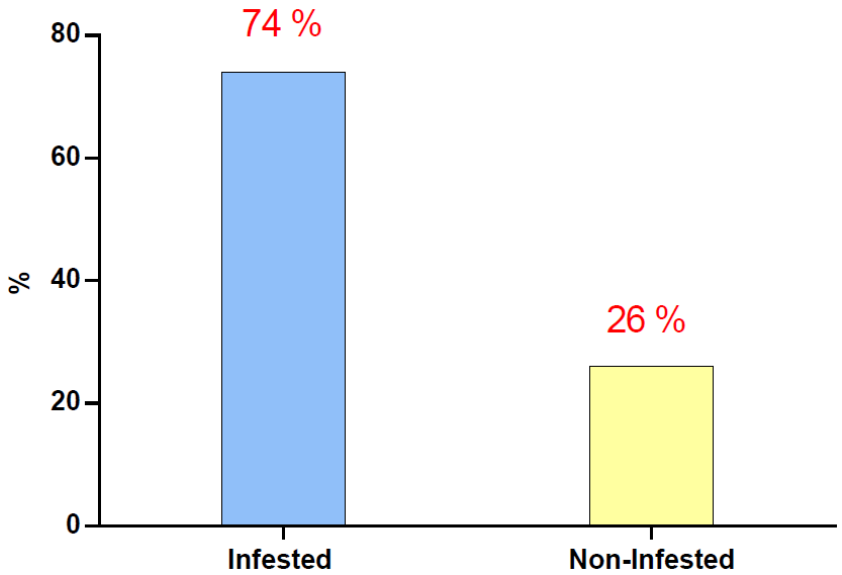

Figure (1): Total results for infested buffaloes with ticks (Total No: 200)

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According to number of collected ticks, significant increases (P<0.0387) in grade E (24.32%) and B (19.6%) while significant decreases were observed in grades A (9.46%), C (8.11%), F (9.46%), G (7.43%) and H (4.73%) when compared to D (16.89%), (Table 1). Regarding distribution of ticks on different bodily parts of study buffaloes, significant higher infestation of ticks was seen in udder (26.9%) and perineal region (23.1%); while, significant lowering was seen in forelimb (9.76%) and neck (9.76%); when compared to ear (15.48%) and hindlimb (18.57%), (Figure 2).

Table (1): Grades for number of infested ticks on each study buffaloes

Grade	No. of tick infestation	Buffaloes	
		No.	%
A	1-5	14	9.46
B	6-10	29	19.6
C	11-15	12	8.11
D	16-20	25	16.89
E	21-25	36	24.32
F	26-30	14	9.46
G	31-35	11	7.43
H	> 35	7	4.73
Total		**148**	-
p-value		**0.0387**	

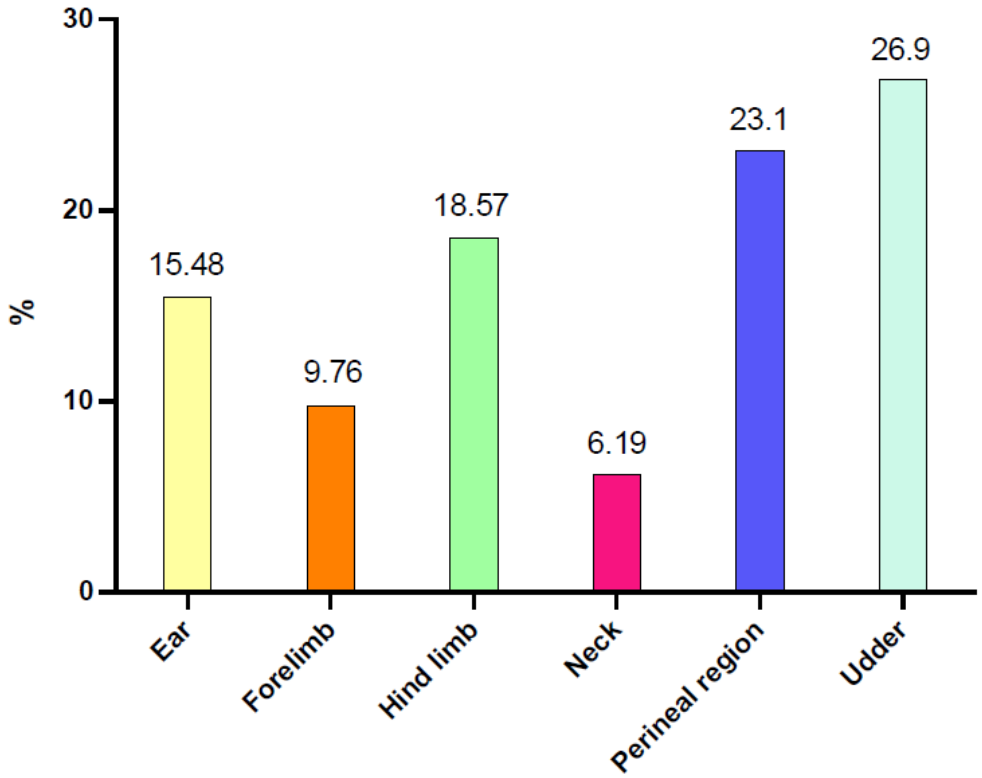

Figure (2): Distribution of ticks on each body part of study infested buffaloes

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Concerning the month of samples collection, the findings showed a significant increase (P<0.0436) in prevalence of ticks in June (80%) and July (86%) and significant decrease in April (58%) when compared to May (72%), (Table 2)

Table (2): Association between tick infestation and the month of samples collection

Month	Total No. of buffaloes	Positive infested buffaloes	
		No.	%
April	50	29	58
May	50	36	72
June	50	40	80
July	50	43	86
Total	200	148	-
p-value		0.0436	

Based on their morphological characteristics, microscopic examination of collected ticks referred that all samples were hard ticks belongs to *Hyalomma* genus in particular *H. anatolicum* species. The key features were based on the genital aperture and mouth parts as there were Coxa I, Coxa II, and Coxa III in the ventral part of the ticks, and the dorsal surface that has the festoons. In males, characteristics of ventral surface of *H. anatolicum* were represented by the presence of Coxa II, Coxa III, Coxa IV, accessory shield, anus, adanal plates, anal groove, and sub-anal shields (Figure 3).

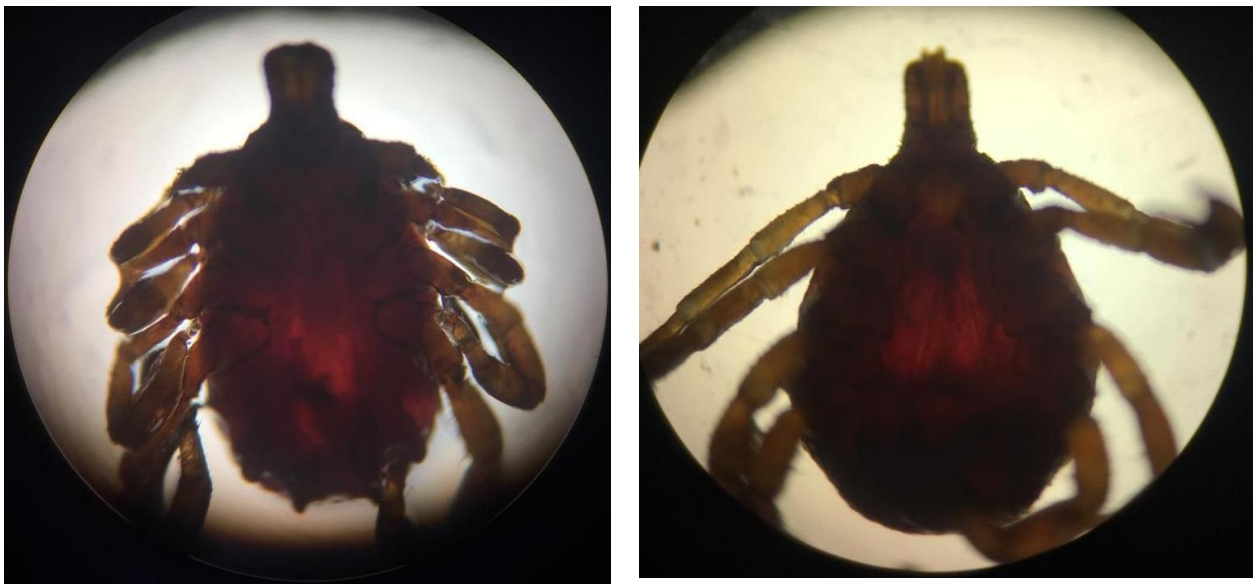

Figure (3): Microscopic examination for morphological appearances of ticks

Targeting *18S rRNA* gene, molecular examination of 148 samples of ticks by the conventional PCR assay revealed that 15 (10.14%) were positively infected with *Theileria annulata* (Table 3, Figure 4).

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Table (3): Total molecular results using the conventional PCR (Total No: 148)

Result	No.	%
Positive	15	10.14
Negative	133	89.86
Total	148	-

Figure (4): Agarose-gel electrophoresis of PCR products at 80AM and 100 volt for 90 minutes; in which, Lane M: Ladder marker (2000-100 bp), and Lanes (1-15) represent the positive PCR products for *Theileria annulata* at approximately 400 bp

Distribution of PCR results reported a significant elevation (P<0.0467) in positive *Theileria annulata* infections in July (20.93%) but significant reduction in April (3.45%) and May (2.78%) in comparison with June (10%), (Table 4).

Table (4): Association between positive PCR results and the month of samples collection

Month	Total No.	Positive	
		No.	%
April	29	1	3.45
May	36	1	2.78
June	40	4	10
July	43	9	20.93
Total	148	15	-
p-value		0.0467	

Discussion

The findings of current study that recorded the significant prevalence of hard ticks among study buffaloes were higher than observed previously by other local studies in bovine as recorded in Sulaimanyia (11.8%) by Kadir et al. (2012), and Baghdad (8.1%) by Hasson (2012) and 12.9% by Mallah and Rahif (2016); but 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 bovine 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).

Studies have shown that in healthy infected animals, ticks are also found on other parts of the body. According to Monfared et al. (2015), body regions and other researchers recorded the

most frequently found types of mites in the breast, mammary glands, scrotum, genitals and perineum (Ndhlova et al., 2009; Hasson, 2012; Ayana et al., 2021). Due to favorable temperatures and conditions in the warmest months of the year, the number of infections in these parts of the animal's body is high (Parola et al., 2008). Also, differences in the location of ticks on the body may be due to tick feeding triggered by odors coming from different parts of the body, especially the genitals, where there are more ticks that prefer the warm and moist hiding places with good and high blood supply. Also, ticks delicate the skin that is easy to penetrate and allow more nutrition is preferred (Tessema and Gashaw, 2010; Nejash, 2016; Makawi and Hadi, 2023).

As mentioned by Al-Fatlawi et al. (2018), the key features were based on the genital aperture and mouth parts. Prevalence of *Hyalomma* genus in particular *H. anatolicum* in this study was similar with that reported by other (Al-Abedi and Al-Amery, 2021) and global studies as in Turkey (Aktas et al., 2004), India (Haque et al., 2011), Pakistan (Atif, 2012), Iran (Razmi and Ramoon, 2012) and United Arab Emirates (Perveen et al., 2021). The dominance and spread of *Hyalomma* is due to the cold resistance of this species and its ability to survive in environments with low humidity and harsh climate (Kettle, 1995). This percentage is lower than the 94.2% recorded by Tarash (1982) for *Hyalomma* spp. in Al-Dahab Al-Abyad village, Baghdad, but higher than the 46% recorded by Al-Mawla (2001) for *Hyalomma* spp. in Mosul among others above the level of Abdul Hussain and Awad (2005) in Basra province.

In this study, application of conventional PCR assay demonstrates the presence of *T. annulata* in ticks. Various methods have been used to investigate the level of *Theileria* infection in the salivary glands of ticks worldwide (Abdigoudarzi, 2013; Tajeri et al., 2016). A shortcoming of histological and histochemical methods commonly used to detect infections is that they cannot accurately identify the type of parasite infecting a tick (Mallesh et al., 2017 a, b). Methylverdepironin (MGP) is one of the simplest and most sensitive histopathological methods and has traditionally been used to quantify tick-borne diseases (Lempereur et al., 2017). However, this can only be performed on freshly collected ticks and cannot distinguish between closely related *Theileria* species such as *T. lestoquardi* and *T. equi* that can infect the same ticks but not bovine (Kirvar et al., 2000). This can overcome by PCR targeting specific genes in *T. annulata*. One of the main advantages of the PCR test over traditional diagnostic methods is that it can distinguish between *T. annuluta* and other genera of *Theileria* and *Babesia* in bovine blood and *T. annuluta* from *T. lestoquardi* and *T. equi* in ticks. This work therefore proceeds in a way that addresses questions raised by other workers regarding the accurate assessment of *Hyalomma* parasites (Saleh et al., 2015; Kumar et al., 2022)

The sensitivity, specificity, and degree of cross-reactivity of a PCR assay will certainly depend on factors such as primer sequence, amplification characteristics, DNA extraction method, sample, and DNA storage. Similar criteria have been proposed for *Plasmodium* species. However, if we could test and develop a simple and inexpensive DNA extraction method for the detection of striped mites in the blood of animals and ticks, it would be useful for large-scale epidemiological studies (Costa et al., 2021; Yadav et al., 2021). Understanding the prevalence of *Theileria* infections in adult ticks is essential to the development of appropriate *Theileria* and tick control programs. In this study, *T. annulata* was detected in 10.14% of the studied samples. A previous study conducted in Sudan showed that *H.*

anatolicum had the highest prevalence of *Theileria* infections, ranging from 38% to 86% (Walker and McKellar, 1983). The prevalence of *H. anatolicum* and *T. annulata* is estimated to be 96% among ticks fed on infected calves (Bakheit, 1998) and 80% among ticks collected on farms (El Imam, 1999). This study suggests that the high rate of tick infestation is due to the agricultural system used, consisting of small livestock pens, which create a good microbial habitat for *H. anatolicum* ticks.

Ali et al. (2013) found *T. annulata* to be present only in *H. anatolicum* and *H. dromedari*, but not in *H. anatolicum*. These results suggest that *H. anatolicum* may play an important role in the spread of *T. annulata* in Iran. The findings are similar to other studies conducted in Ethiopia and Sudan. This is because *H. anatolicum* is the main causative agent of *T. annulata*, the main causative agent of tropical disease infections. Therefore, tropical diseases must be monitored within the range of *H. anatolicum* (Mossaad et al., 2021; Kaba, 2022).

Conclusion

This study demonstrated the highly prevalence of ticks in buffaloes especially with increasing of environmental temperature in summer months. Hard ticks of *Hyalomma* genus in particular *H. anatolicum* remain the most incidence ticks among buffaloes population. Molecular detection of *Theileria annulata* in study ticks demonstrated their roles in transmission of infection to other healthy buffaloes. Moreover studies on ticks of infested buffaloes or other field animals using the molecular assays appear of great importance to estimate its role in transmission of other pathogens.

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