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Histamine release factor encoding gene in *Hyalomma anatolicum* ticks is over-expressed during deltamethrin exposure

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Abstract

Ticks have evolved complex strategies to parasitize their hosts successfully. They are considered vectors for various pathogens that can infect humans and animals. Moreover, using acaricide has increased dramatically all over the world. Nevertheless, ticks have developed resistance to numerous acaricides. Thus, demonstrating the effect of deltamethrin on a hard tick (*Hyalomma anatolicum*) to estimate the level of Histamine Release Factor (HRF) using an RT-qPCR assay is important for future experimental studies, such as resistance tick identification. Also, conventional PCR was performed to confirm the species of ticks. To do this, three pairs of primers were designed in this study to demonstrate the expression level of HRF while identifying the optimal conditions. The RT-qPCR assay revealed successful amplification of the targeted HRF gene to quantify its mRNA expression. Ticks from each group were exposed to deltamethrin for two hours and then analysed for HRF mRNA expression. Also, genomic DNA was extracted for tick identification. The results revealed a significant upregulation in the exposed group with a fold change value of 4.3±0.5. At the same time, the level was lower in the control group at 1.69±0.4. *Hyalomma anatolicum* was found to be the predominant species in the studied region. This represents the first study in Iraq to target the effect of deltamethrin on the expression of HRF. Therefore, further studies are important to detect other tick genes that could contribute to their sensitivity or resistance to different types of acaricides and the following strategies for controlling ticks.

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Introduction

Hyalomma anatolicum has garnered significant attention due to its role as a vector for various pathogens, such as bacteria, protozoa, and viruses that can infect human and animal populations (1). This tick species is widely distributed across a diverse range of habitats in Eurasia, Africa, and the Middle East to parasitize a wide variety of hosts such as cattle, sheep, goats, camels, and various species of wild ungulates (2,3). Ticks can infest various hosts and survive despite harsh environmental conditions. This has spread ticks in many areas and transmitted several pathogenic agents to humans and animals (4–8). Ticks involved many arthropods that successfully developed complicated strategies to parasitize their hosts (9). One of these important aspects is their ability to modulate the host's immune responses, allowing ticks to evade the host and feed on blood without any interpretation (10). This activity occurs by secretion of a protein known as HRF from the salivary glands of a tick, which manipulates the host's immune response and suppresses the inflammatory responses resulting from the stimulation releasing of histamine from

the mast cells (11). Across various species of ticks, HRF is highly conserved, suggesting its essential role in the blood-feeding process. Moreover, the secretion of this protein is interestingly correlated with the capability of ticks to transmit different microorganisms to their hosts (12). In the field of tick biology, the evolutionary significance and understanding of the mechanism of the action of HRF have been the active areas of research (13). As the geographic ranges of ticks and the pathogens they transmit continue to expand, elucidating the role of their salivary protein may provide valuable insights into tick-host interactions and the development of novel strategies for tick control (14,15). Deltamethrin is an insecticide belonging to the synthetic pyrethroid family. It was first synthesized and registered by the United States Environmental Protection Agency in 1974 and marketed for use in agricultural and public health applications in 1977 (16). Deltamethrin is effective against ectoparasites that developed resistance to other insecticide classes (17,18). For this reason, deltamethrin was applied as a valuable tool throughout the management strategies, especially in regions where traditional insecticides are ineffective (19,20). However, in the last decade, the overuse of deltamethrin has related to the development of resistance among some ectoparasites, underscoring the need for a more active and sustainable approach to management strategies (21).

No available data describe the association of HRF in ticks with deltamethrin in Iraq; therefore, this study was aimed at identifying the effect of deltamethrin on the hard tick (*Hyalomma anatolicum*) for the first time in Iraq through quantitative molecular estimation of the level of the HRF gene using RT-PCR.

Materials and methods

Ethical approval

The Scientific Committee in the Department of Parasitology, College of Veterinary Medicine, University of Al-Qadisiyah provided the license for the current study issued 515 dated 28/11/2024.

Samples

One hundred fifty cattle were inspected clinically in June 2023) at different Basra province (Iraq) areas and collected from various body parts of each study animal, preserved in et andanol (70 %) into labeled plastic containers to prevent degradation of morphological features (22).

Morphological identification

The next step involves examining key morphological characteristics by microscopic inspection of body shape, arrangement and structure of mouthparts, presence and distribution of setae on the body, as well as unique patterns and features of dorsal and ventral surfaces. These features were observed using a stereomicroscope or high-magnification light microscope (Olympus, Japan), allowing for detailed examination and comparison to taxonomic keys and reference materials (23).

Study experiment

After morphological identification of *H. anatolicum*, 80 tick samples were divided equally into two groups: the first is the control group, in which the ticks were exposed to distilled water, while the second is the treated group, in which the ticks were exposed to 1% deltamethrin for 1 hour. The tick samples of both groups were then kept frozen in liquid nitrogen for RNA extraction (24).

Molecular identification of the tested ticks

For molecular identification, five ticks with similar morphology from each group were processed for DNA extraction using a genomic DNA extraction kit (AddBio, South Korea) using the spin column method recommended by the manufacturer. Partial region within the 16S rRNA gene was amplified using primers designed by (25) as 5' CTG CTC AAT GAT TTT TTA AAT TGC TGT GG' 3 in forward and 5' CCG GTC TGA ACT CAG ATC AAG T' 3 in reverse primer using conventional PCR (BioRad, USA) with hot start Taq polymerase (AddBio, South Korea) in a total PCR volume 20 µl including 10 µl hot start Taq master mix, 1 µl of each primer (0.05 pmol/µl) and 2 µl template DNA, meanwhile the amplified products generated by thermocycler (BioRad, USA) including 35 cycles of 95 C for 20 seconds, 55 C for 15 seconds, and 71 C for 45 seconds. These products were electrophoresed by agarose gel (1.6 %) stained with 60 µl of ethidium bromide and imaged by a gel documentation machine (BioRad, USA). Finally, these amplicons were sequenced using the Sanger technique (Macrogen, South Korea).

Quantification of histamine release factor

This approach was carried out according to the comparative Ct approach (ΔΔCt), with the control group normalized to those of GAPDH mRNA in the presence of transcript levels following the procedure provided (26).

Primer design

To amplify the Histamine Release Factor gene, specific primers were designed in this study depending on the data provided at the National Centre for Biotechnology Information (NCBI) service with the relevant accession numbers of (KP184513) that corresponds to *Hyalomma anatolicum anatolicum* as seen in the (Figure 1) showing the site of the designed primers (three pairs) in the gene. The sequence of the designed primers and loci in the gene is demonstrated in (Table 1).

RNA extraction and cDNA synthesis

Frozen ticks were homogenized and then lysed chemically according to the manufacturer's recommendation

(AddBio, South Korea). Briefly, lysis of ticks was achieved by adding 400 µl of lysis buffer, 4 µl ß-mercapto-ethanol and 20 µl proteinase K solution (20 mg/ml) followed by incubation at 56 C for 10 minutes. The clear lysate was bound to the silica gel column and then washed twice using washing buffer and centrifugation at 13000 rpm for 1 min. These columns were treated with DNAse and then eluted with elution buffer. The concentration of the eluted RNA was measured by Quantus fluorometer using RNA dye as recommended by the manufacturer (Promega, USA). About 20 µl of the eluted RNA was converted into complementary DNA (cDNA) using reverse transcriptase enzyme from (AddBio, South Korea) in a total volume of 20 µl consisting of 10 µl of reverse transcriptase with 2 µl of dNTPs and 1 µl of random oligos. The thermal conditions were achieved in a conventional thermal cycler (BioRad, USA), including at 25 C for 10 min, followed by 50 C for 60 min, then 80 C for 5 min (27).

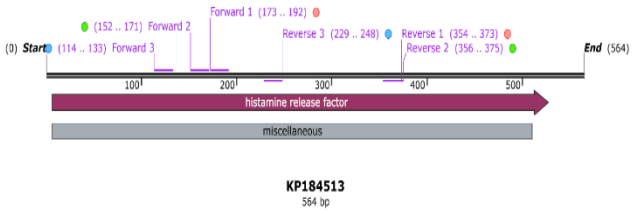

Figure 1: The site of the designed primers in the *HRF* gene of *H. anatolicum* tick.

Table 1: The characterization of the designed primers and their location in HRF

Primer name	Primer Sequence (5'----3')	Start	End	*Tm*	Access No.
HRF-forward 1	AGGGCACAGACGAGAATGTC	173	192	58	KP184513
HRF-Reverse 1	CCTTTTTGACTGCTGCGGTG	354	373	59	
HRF-forward 2	CCTCTGCTGAGGAGGTTGAC	152	171	58	KP184513
HRF-forward 2	CACCTTTTTGACTGCTGCGG	356	375	59	
HRF-forward 3	ACGCAAGCAGGGAGAGATTC	114	133	59	KP184513
HRF-reverse 3	TTGGTGAAGCAGGTTTCGGT	229	248	59	
GAPDH-F	AGGCTCAGCAGCACATTGAT	281	300	59	KU248453.1
GAPDH-R	ATGCCGAAGTTGTCGTGGAT	470	451	60	

RT-qPCR

The amplification was achieved using AddScript RT-qPCR Syber green master (AddBio, Korea) in a total volume of 20 µl including 10 µl of Syber green, two µl of each primer, and 2 µl of cDNA. The thermal conditions were conducted using a qPCR thermal cycler (BioRad, USA), including 95°C for 10 min as initial activation followed by 40 cycles of 95°C for 15 sec, 53.9°C for 30 sec, and 72°C for 30 sec. The amplification was evaluated by melting steps at the end of the amplification. The delta-delta Ct method was used to normalize transcript levels to those of GAPDH mRNA according to the 2^{-CT} method.

Statistical analysis

The t-test in the GraphPad Prism software (*version 8.0.2*) was applied to detect significant differences between the obtained results at P<0.05 (28).

Results

Molecular confirmation of the studied ticks

The conventional PCR showed highly specific products with 450 bp size (Figure 2A). Notably, the sequence results confirm that the studied ticks were *H. anatolicum* when aligned with the National Centre for Biotechnological Information (NCBI) (Figure 2B).

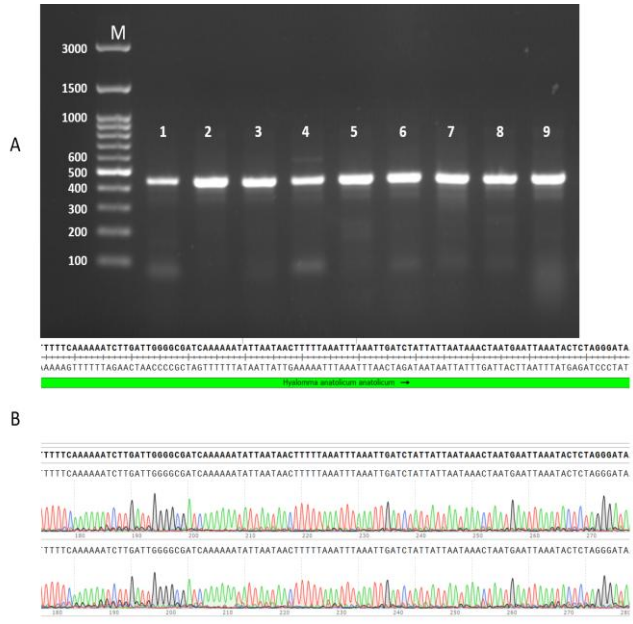

Figure 2: (A) Agarose gel electrophoresis image (1.5%) shows amplicons of the *H. anatolicum* targeting *16S rRNA* gene. M is a molecular marker (3000-100 bp) (GeneDirex, South Korea). (B) Chromatogram data from sequencing of *H. anatolicum.*

The optimisation results

The results of the gradient protocol from the tested three pairs of primers show specific amplification of all of them. In primer number 1, the optimal annealing temperature was 59.4°C, with the mean value of the curve threshold demonstrated as 29.3±0.15 (Table 2, Figure 3), while in primer number 2, the annealing temperature of 58.3°C resulted in a curve threshold 29.7±0.12 (Table 3, Figure 4). But primer number 3 shows better amplification at an annealing temperature of 53.9°C with a mean curve threshold value of 27.3±0.07 (Table 4, Figure 5). These results enable us to identify the optimal conditions. Thus, quantify the amount of HRF mRNA in exposed tick to acaricide.

Table 2: Gradient annealing temperatures for primer number 1

Annealing temperature	CT	1st run	2nd run	Mean	SE
60	29.23	29.52	29.54	29.4	0.1002
59.4	29.3	29.08	29.61	29.3	0.1537
58.3	28.98	29.15	29.5	29.2	0.1531
56.3	29.07	29.02	28.65	28.9	0.1325
53.9	28.33	27.98	27.86	28.1	0.141
52	27.85	27.92	27.28	27.7	0.2027
50.7	27.76	27.44	26.72	27.3	0.3075
50	27.48	27.32	27.2	27.3	0.0811

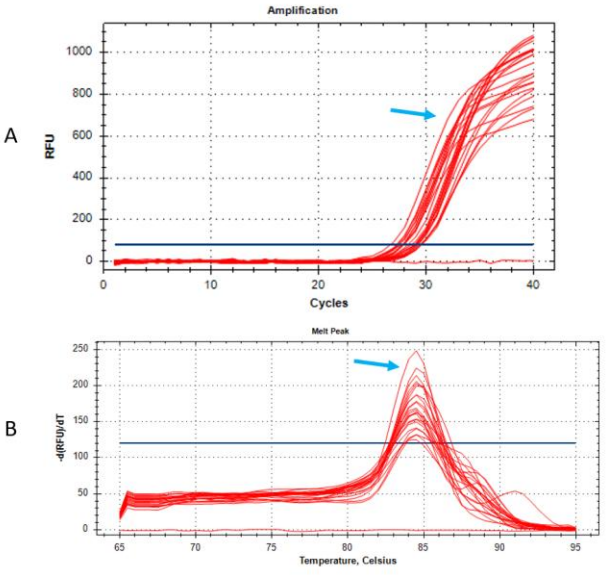

Figure 3: (A) Amplification curves of primer number 1 targeting HRF of *H. anatolicum* (B) melting curves of the amplified amplicons that show the specificity of the primer in the qPCR reaction (single peak referred to with blue arrow). This demonstrates the annealing temperature (59.4°C) as an optimal degree for the reaction with a mean value of curve threshold (29.3±0.15).

Table 3: Gradient annealing temperatures for primer number 2

Annealing temperature	CT	1st run	2nd run	Mean	SE
60	60	30.31	29.8	30.16	30.1
59.4	59.4	29.63	29.61	29.77	29.7
58.3	58.3	29.37	29.39	29.74	29.5
56.3	56.3	29.54	29.18	28.86	29.2
53.9	53.9	29.18	28.9	28.99	29
52	52	28.53	28.08	28.5	28.4
50.7	50.7	28.44	28.18	28.32	28.3
50	50	28.35	28.5	28.4	28.4

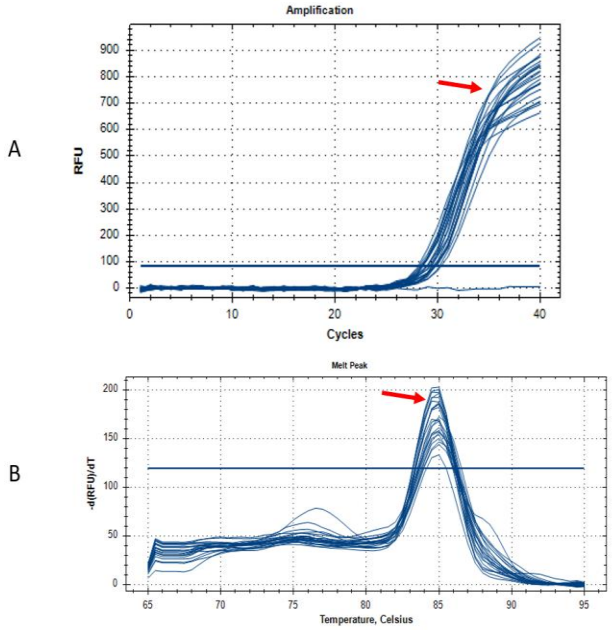

Figure 4: (A) Amplification curves of primer number 2 targeting HRF of *H. anatolicum* (B) melting curves of the amplified amplicons that show the specificity of the primer in the qPCR reaction (single peak). This demonstrates the annealing temperature (58.3°C) as an optimal degree for the reaction with a mean value of curve threshold (29.7±0.12).

Table 4: Gradient annealing temperatures for primer number 3

Annealing temperature	CT	1st run	2nd run	Mean	SE
60	29.67	29.07	29.32	29.4	0.174
59.4	28.95	29.13	29.19	29.1	0.07211
58.3	28.54	28.94	29.07	28.9	0.1595
56.3	27.89	28.16	28.28	28.1	0.1153
53.9	27.18	27.24	27.44	27.3	0.0786
52	27.02	26.47	27.14	26.9	0.2063
50.7	26.08	26.25	28.51	27	0.7832
50	26.28	26.25	26.3	26.3	0.01453

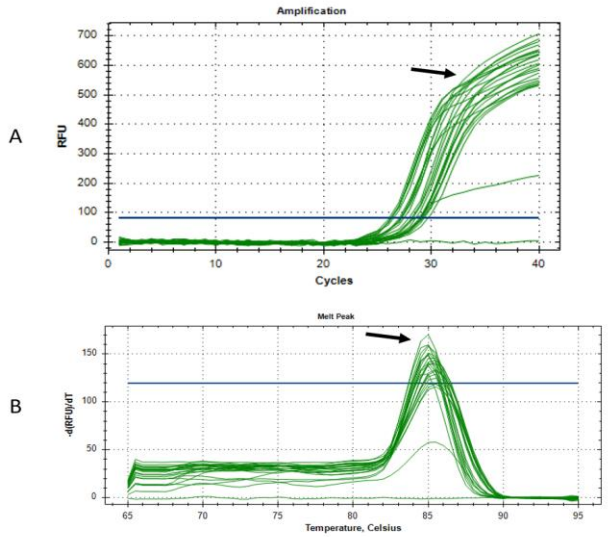

Figure 5: (A) Amplification curves of primer number 3 targeting HRF of *H. anatolicum* (B) melting curves of the amplified amplicons that show the specificity of the primer in the qPCR reaction (single peak). This demonstrates the annealing temperature (53.9°C) as an optimal degree for the reaction with a mean value of curve threshold (27.3±0.07).

Analysis of the RT-qPCR gene expression data in Histamine Release Factor gene

The RT-qPCR assay revealed successful amplification of the targeted gene (histamine release factor) to quantify its mRNA expression. This was conducted using primer number 3 because it revealed better results in the curve threshold value, less threshold value, with optimal melting curve. This enables the measurement of the level of the histamine release factor in the tested groups, as seen in (Figure 6). Ticks (n= 16) from each group were exposed to deltamethrin for two hours and then analyzed for histamine release factor mRNA expression. As shown in (Table 5); the results revealed a significant upregulation ($P < 0.01$) in the exposed group (1 ml of deltamethrin added into 99 ml of H_2O) with a fold change value of 4.3±0.5. At the same time, the level was lower in the control group as = 1.69±0.4 (Figure 7).

Discussion

Ticks are the major ectoparasites of livestock and wildlife, with significant economic and health risks globally. They act as vectors for various pathogens, including viruses, bacteria, and protozoa, causing animal morbidity (29–33). While standard tick eradication efforts have primarily focused on applying acaricides to cattle, the potential role in undermining these efforts is a growing concern (34,35). Natural organic compounds, such as plant-derived compounds, have emerged as a promising alternative for tick pest control and prevention (36).

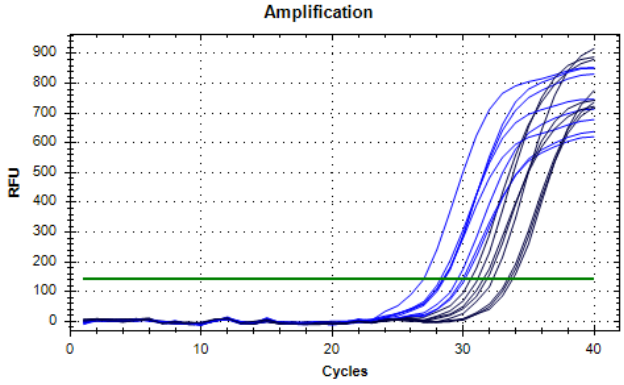

Figure 6: The amplification curves of the tested samples using primer number 3 to measure the level of histamine release factor expression.

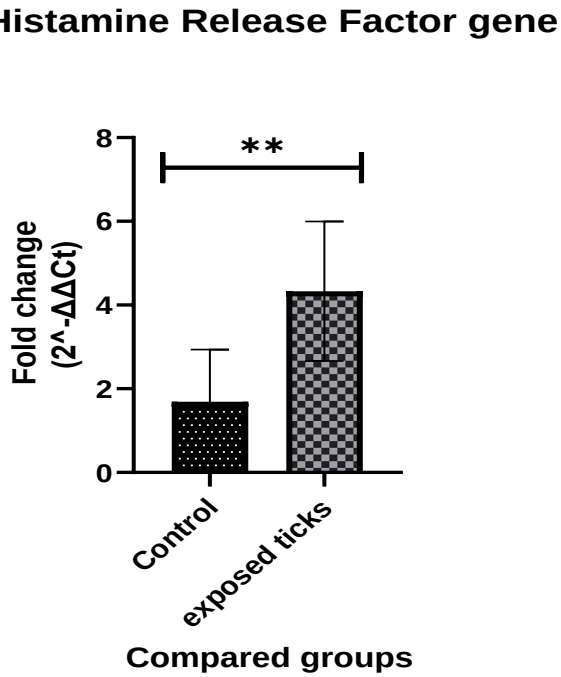

Figure 7: Fold change comparison between the compared groups expressed the HRF.

Table 5: Statistical analysis of the expressed gene in the exposed and control ticks

	Control	Exposed ticks
Number of values	16	16
Minimum	0.2577	2.302
Maximum	3.253	6.559
Range	2.995	4.256
Mean	1.691	4.331
Std. Deviation	1.246	1.669
Std. Error of Mean	0.4404	0.5900

In vitro and *in vivo* assays have identified several molecules with repellent activities on ticks, including synthetic compounds like N, N-diethyl-meta-toluamide, IR3535, picaridin or KBR 3023, and para-menthanediol, as well as plant-derived molecules (37). One synthetic compound, deltamethrin, has shown significant efficacy in controlling ticks (38). Simulated interactions between ticks, climate variation, and habitat heterogeneity have demonstrated the potential impact of these factors on the efficacy of standard eradication protocols (39).

The application of acaricides to cattle every two weeks for nine or 12 consecutive months, or the removal of cattle for 12 consecutive months, has been shown to suppress the number of host-seeking larvae in the system to near zero (40). An integrated approach incorporating synthetic and natural compounds may be warranted to address this challenge. Exposure of ticks to 1% deltamethrin has been associated with considerable histological damage (41). This concentration has been studied for its effects on tick resistance and efficacy against species such as *Rhipicephalus microplus*. Frequent exposure or under-dosing of deltamethrin may increase the likelihood of resistance in this tick species (21).

The last decade has seen an increased functional discovery of tick salivary proteins and their critical interactions with the host and pathogens (42). This information has also offered a new approach to developing effective vaccines against ticks and the pathogens they transmit by simultaneously targeting the pathogen and the tick (43). Identifying tick proteins potentially involved in pathogen transmission is important in developing effective tick vaccines (43,44). Understanding the complex interactions between HRF, tick biology, and the host immune response could provide valuable insights into developing more effective tick control strategies and preventing tick-borne diseases (45). Probing the saliva of ticks, particularly *Ixodes ricinus*, has revealed a diverse array of bioactive molecules that play crucial roles in their parasitic lifestyle and ability to evade host defenses (46).

One of tick saliva's most extensively studied components is a 15-kDa salivary gland protein known as *Salp15*. It has been shown to suppress hosts' immune response through its pleiotropic action on several key defense pathways (47). The *Salp15* and its homolog from *I. ricinus*, *Iric1*, have also been demonstrated to bind to outer surface protein C, enabling the spirochetes to evade antibody-mediated killing in the human host (48). The host immune response to tick infestation is a complex and multifaceted process involving local and systemic reactions (14). Strong correlations in the expression of pro-inflammatory cytokines, such as IL1α and IL1β, and their antagonist IL1RN, have been observed in biopsies from tick-resistant bovine phenotypes.

Additionally, the up-regulation of IL12 and IL23 in blood leukocytes from tick-infested calves of all phenotypes suggests possible systemic recruitment of memory T cells, which may play a role in developing tick resistance (49). Interestingly, multiple tick bites have been shown to reduce the likelihood of contracting some diseases in humans, indicating that repeated exposure to tick saliva can induce a protective immune response (50,51). This observation underscores the potential for exploiting the host immune response to ticks to develop more effective tick control strategies and prevent tick-borne diseases (49).

Conclusion

This represents the first study in Iraq that targeted the effect of acaricide (deltamethrin) on the expression of the HRF gene. Our findings confirmed the high expression of the HRF gene after exposure to deltamethrin, suggesting that blocking tHRF might offer a viable strategy to complement ongoing efforts to develop vaccines to block tick feeding and transmission of tick-borne pathogens. Therefore, further studies are very important to detect other tick genes that could contribute to their sensitivity or resistance to different types of acaricides and then follow successful strategies to control ticks. Moreover, research is needed to evaluate the efficacy of deltamethrin and plant-derived compounds in controlling ticks while also considering the impact of climate variation and habitat heterogeneity on the effectiveness of tick eradication efforts.

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Conflict of interests

The authors declared that there was no conflict of interest.

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زيادة التعبير الجيني لعامل الهستامين في قراد الهايلوما المتعرض للدلتامثرين

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الخلاصة

لقد طور القراد الكثير من الاستراتيجيات التي تمكنه من على الكائن الذي يتطفل عليه فضلا عن مقاومة المبيدات المستعملة، كما يعد القراد وسيطا ناقلا للكثير من المسببات المرضية للحيوان والإنسان. لقد ازداد في الآونة الأخيرة استخدام المبيدات في المجال البيطري بشكل كبير بالعالم مما أدى ذلك الى ظهور مشكلة مقاومة تلك المبيدات من قبل القراد ولذلك فان تطوير تقنية تفاعل سلسلة البلمرة اللحظي لتقييم قياس مستوى التعبير الجيني لعامل الهستامين ضروري في إجراء المزيد من الدراسات المتعلقة بهذا الجانب خصوصا تلك المتعلقة بدراسة مقاومة القراد للمبيدات. لقد تم أيضا استخدام تقنية تفاعل سلسلة البلمرة التقليدي لتأكيد التشخيص لنوع القراد المدروس من خلال إجراء فحص التسلسل الجيني ولإجراء ذلك تم تصميم ثلاثة بوادر جينية لاختيار الأفضل من خلال إجراء تجارب المعايرة قبل إجراء التجربة. أظهرت نتائج التعبير الجيني وجود زيادة مستوى التعبير الجيني لعامل الهستامين في القراد المتعرض لمبيد الدلتامثرين بمستوى تعبير ٤,٣±٠,٥ بالمقارنة مع مجموعة السيطرة التي أظهرت مستوى منخفض مقداره ١,٦٩±٠,٤. كما أظهرت النتائج أن النوع المدروس والمنتشر في المنطقة المدروسة من نوع الهايلوما اناتولكم.

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