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New method using quantitative PCR to follow the tick blood meal and to assess the anti-feeding effect of topical acaricide against *Rhipicephalus sanguineus* on dogs

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ABSTRACT

A 28-day study was conducted to assess the dynamic of blood feeding by *Rhipicephalus sanguineus* ticks on dogs treated or not with a novel topical combination of fipronil, amitraz and (*S*)-methoprene. Dogs were infested weekly through exposure to ticks in crates for 4 h. Ticks were then counted in the crates at 2 h and 4 h post dog exposure. Ticks were also counted and removed from the dogs at 2 h, 4 h, 6 h, 12 h and 24 h post tick exposure. The inhibition of blood feeding was assessed by both tick quantification and designing and performing a quantitative PCR (qPCR) to detect the canine hydroxymethylbilane synthase (HMBS) gene in ticks. The percentage of repellency *sensu lato* based on the ticks collected in crates at 2 h varied from 4.7% at day 28 to 48.3% at day 7. The immediate mortality rate of the ticks expelled at 2 h varied from 1.5% at day 21 to 31.7% at day 7. The efficacy calculation showed that the acaricidal combination started to kill ticks in as little as 2 h. The average efficacy reached 90.0% at 12 h post crate challenges and 100% at 24 h post exposure in crates. The inclusion of an internal amplification control was used to ensure that no significant template-derived PCR inhibition ($\leq$6.2%) affected the overall results. The reduction of blood feeding was significant at 4 h (>80.0%) and >99.0% at 24 h post tick exposure in the crate. The high repellency rate and the lethal efficacy of CERTIFECT® resulted in significantly fewer live attached ticks, consequently reducing blood intake and fluid exchanges.

1. Introduction

Tick infestations and transmitted diseases are an emerging threat for both humans and pets, especially dogs [1]. Amongst all tick species, *Rhipicephalus sanguineus* is the most widely distributed tick species on dogs and a vector of several pathogens such as *Ehrlichia* spp., *Rickettsia* spp., *Hepatozoon* spp. and *Babesia* spp. [1]. The control of tick infestations is mainly based on regular treatment of dogs with acaricidal formulation, the majority being formulated as topical spot-on treatments [2]. The classical approach to assessing acaricidal efficacy is to count and categorise ticks on dogs after infestations. Differentiation between live and dead ticks, attached or not, engorged or not, on both treated and untreated animals allows the calculation of acaricidal efficacy but also the prevention of the attachment or detachment effect [3–5]. A combination of fipronil, amitraz and (*S*)-methoprene has been launched in 2011 for the purpose of both tick and flea control on dogs [6]. It has been demonstrated that amitraz has a behavioural effect on ticks, inducing their detachment or preventing their attachment [7]. The new fipronil plus

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amitraz combination has shown this characteristic both in *in vitro* studies and studies on dogs [8–10]. The categorisation of tick engorgement remains qualitative and is subject to variations between technicians and techniques used. One possibility for assessing the prevention of attachment and of the risk to transmit diseases could be to quantify the blood meal taken by ticks. Male and female ticks start to ingest tissues and blood subsequent to attachment, resulting in accumulation of canine DNA in their gut [11]. We hypothesised that the quantity of dog DNA ingested by the tick would increase over time during the blood meal. Therefore, a treatment having a negative impact on the tick meal would induce a lower quantity of host DNA present in the tick. In order to test this hypothesis, we designed and employed a quantitative PCR assay specifically to detect the number of canine HMBS gene copies present in the sample. The specificity of the assay was validated through analysis of DNA isolated from rabbit- or rat-fed *Ctenocephalides felis*, *Dermacentor variabilis*, *Dermacentor reticulatus* and *R. sanguineus* where no canine HMBS gene signal should be detected. The use of PCR and qPCR on DNA isolated from fed ticks is subject to well-described inhibition [12–15]. Qiagen supplementary protocol is advising to refrain from using fed ticks as DNA source for qPCR quantification. An internal amplification control (as mimic of the HMBS) was employed to all samples in order to identify any significant qPCR inhibition that could influence the results.

2. Materials and methods

2.1. Experimental design

Twelve mongrel dogs over 6 months of age, 6 males and 6 females, were infested weekly with 50 *R. sanguineus* ticks (25 males and 25 females). The dogs were from ClinVet colony, bred at the facility. Ticks were from a South African colony known to be susceptible to acaricides and maintained on dogs at Clinvet. Larvae and nymphs were fed on rabbits or rats in order to avoid the presence of any canine genetic material. Dogs were not treated with ectoparasiticides or insect growth regulators (either topical or systemic) within 3 months before the start of the study.

Based on tick infestation at day −7 and tick counts at day −5, 12 dogs out of 14 were randomly allocated into 2 groups; one untreated control and one CERTIFECT treated group. From 0 to 2 h and then 2 to 4 h, dogs were placed in individual crates, where it was possible to collect and assess ticks on the ground. At days 1, 4, 7, 14, 21 and 28, 50 ticks were put in the bottom of the crates and not directly on dogs (Table 1). Then the dogs were moved at 4 h in individual cages.

The ticks were mechanically removed from the dogs at different times post exposure in the crates. The collection was done using a specific rotation plan (Table 2).

2.2. Methodology for tick collection/count: rotation plan (Table 2)

Based on the product label and publication, the efficacy against ticks is maintained during 5 weeks [16]. The dogs were infested 6 times, at days 1, 4, 7, 14, 21 and 28 (Table 2). All infestations were done by putting the ticks in a crate and introducing a dog in the crate for 2 h. The ticks were assessed on the ground of the crate at 2 and 4 h after each infestation. They were counted on the dogs at either2 h, 4 h, 6 h, 12 h, 24 h or 72 h post exposure, based on a rotation plan. This rotation plan was designed in order to get a unique removal time after each infestation for each dog, in order to collect ticks from each dog at each time point (Table 2). This rotation plan was designed to accommodate dog variability in relation to timing after tick exposure.

Table 1
Schedule of operations.

Physical examinations	Day −7
Pre-infestation 50 ticks	Day −7
Tick count at 48 h	Day −5, allocation of 12 dogs with highest counts in 2 groups of 6 dogs
Treatment	Day 0 for 6 dogs in the CERTIFECT group
General health observations	Daily from day −7 to +28
Tick infestations	50 *Rhipicephalus sanguineus* (25 females and 25 males) Ticks deposited in the crates, not directly on dogs Days −7, +1, +4, +7, +14, +21 and +28
Tick removal and counts	Day −5 for allocation See specific design below for each collection after infestation
PCR analysis	DNA extraction from all removed ticks and PCR analysis on pools of 5 female or 5 male ticks

In the analysis the focus was put on the tick counts and on the percentage reduction in HMBS gene copy numbers observed in ticks from the control and treated groups at the different time points after removal. The weeks of infestation were not considered to be of significance based on the fact that both treated and control dogs followed the same design and rotation plan.

The study was blinded and technicians doing the tick infestations and removals were not aware of the treatment status.

The two variables studied were the tick counts and the qPCR results obtained from the pools of the removed live ticks.

2.3. Quantitative PCR assay development

Pools of 5 female ticks and pools of 5 male ticks were constituted to minimise standard deviation. DNA extracts were prepared for each pool of 5 ticks. Each DNA extract was then quantitatively evaluated by real-time PCR for the presence of the canine HMBS gene.

When it was possible, in relation to the number of ticks collected, a maximum of six pools were done for each dog (3 pools of female ticks and 3 of male ticks), at each time-point, based on Table 2. Therefore corresponding to a maximum of 36 pools per time-points (2 h, 4 h, 6 h, 12 h, 24 h and 72 h), 6 pools per dog (three of female ticks and three of males). For each dog and time-point, the

Table 2
Rotation plan for tick removal after each tick infestation.

Control dog/treated dog	2 h pi[a]	4 h pi	6 h pi	12 h pi	24 h pi	72 h pi
C1/T1	Day +1 2 separate groups: – Ticks on dogs – Ticks in the crate	Day +4	Day +7	Day +14	Day +21	Day +28
C2/T2	Day +28	Day +1 2 separate groups: – Ticks on dogs – Ticks in the crate	Day +4	Day +7	Day +14	Day +21
C3/T3	Day +21	Day +28	Day +1	Day +4	Day +7	Day +14
C4/T4	Day +14	Day +21	Day +28	Day +1	Day +4	Day +7
C5/T5	Day +7	Day +14	Day +21	Day +28	Day +1	Day +4
C6/T6	Day +4	Day +7	Day +14	Day +21	Day +28	Day +1

Dogs are distributed by pair, one control and one treated together in the rotation plan.
[a] pi, post-infestation by ticks in the crate; C1, control dog 1; T1, treated dog 1.

qPCR results were averaged for the pool processed, keeping female and male ticks separately.

In parallel, three pools of unfed females and three of unfed male ticks were done in order to get the zero baseline of the PCR.

It was not in the regular standard of qPCR ran in triplicates, but the risk of errors was diluted by the repetition of the pools per dog per time-points during the month in accordance to the rotation plan.

2.4. Canine HMBS qPCR detection assay design

Wang et al. [15] used quantitative PCR to target the single-copy hydroxymethylbilane synthase (HMBS) gene present in the dog genome in order to quantify flea feeding patterns on dogs. The present study also targeted the canine HMBS gene region for quantification of canine DNA present in total DNA isolated from feeding parasites.

The canine HMBS gene (NCBI accession number: XM_546491) was submitted to the Sigma–Aldrich OligoArchitect™ primer and probe design programme. Two primer and probe sets were selected from the output file and synthesised by Sigma–Aldrich. The primer and probe sets were evaluated during qPCR analysis using isolated canine DNA as well as DNA isolated from rabbit- or rat-fed *C. felis*, *R. sanguineus*, *D. variabilis* and *D. reticulatus* samples. Results obtained indicated that primers Canine HMBS-2F (5′-ACTGCTGACTGAGGTGATC-3′) and Canine HMBS-2R (5′-GTGGCTTGAACTTTCTCTAAAGA-3′) together with the HMBS2F2Rp probe ([6FAM]5′-CGGCAGCAAGGCACTCTTCACAGC-3′[BHQ1]) exhibited the highest specificity and sensitivity towards the canine HMBS gene target. The linear range and sensitivity of the assay was determined using isolated canine DNA (ranging from 143 ng to 14.3 pg/20 μl qPCR) in conjunction with the Thermo Scientific Maxima Probe/ROX qPCR master mix containing 300 nM of each primer and 200 nM probe in a final volume of 20 μl (Fig. 1). All thermal cycling and detection was performed using an Applied Biosystems StepOne thermal cycler employing a thermal profile of 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The qPCR product is 133 bp in size with a 53.4% GC content.

2.5. Internal amplification control (IAC) qPCR assay design

Sigma–Aldrich OligoArchitect™ was used to design a primer pair and probe combination based on a preselected region of lambda DNA. Lambda DNA served as the template for amplification of a target region using primers qPCR HMBS-1F (5′-ACTGCTGACTGAGGTGATCGAGCG-GAAAGAGCATTATTCAGC-3′) and qPCR HMBS-1R (5′-GTG-GCTTGAACTTTCTCTAAAGAGCATTTCGTAGCGGTCCAGC-3′) to incorporate the hybridisation sites for Canine HMBS-2F and Canine HMBS-2R primer pair into the resulting PCR product. Primers Canine HMBS-2F and Canine HMBS-2R was used in combination with probe qPCR-IACp ([JOE]5′-TCCTGACCGTGTGGCTTACCTGACC-3′[BHQ1]) to detect 100 ag template (obtained from qPCR HMBS-1F and qPCR HMBS-1R amplification from lambda DNA) in the presence of the DNA isolated from the tick samples as described in 1.1. The IAC product is 133 bp in size with a 54.9% GC content.

2.6. DNA isolation and PCR methodology

Total genomic DNA was isolated from ticks using the QIAGEN DNeasy Blood & Tissue Kit according to the manufacturer's recommendations. Male and female ticks were separated and samples contained pools of DNA from 5 ticks of the same gender. The ticks were cut open longitudinally to expose the gut. Isolated DNA was eluted using 100 μl elution buffer. A total of 5 μl isolated DNA (ranging from 7 ng to 2.67 μg) was added to the qPCR mix as described, followed by thermal cycling and detection. A second run was performed as described above, with the HMBS2F2Rp replaced by the qPCR-IACp, and the reaction was supplemented with 100 ag IAC template (approximately 700 copies). All PCR results were calculated and normalised using the white blood cell counts obtained for individual dogs prior to infestation in order to account for any variability in white blood cell counts at the time of infestation, thereby ensuring a more accurate comparison of the target present between dogs in different groups

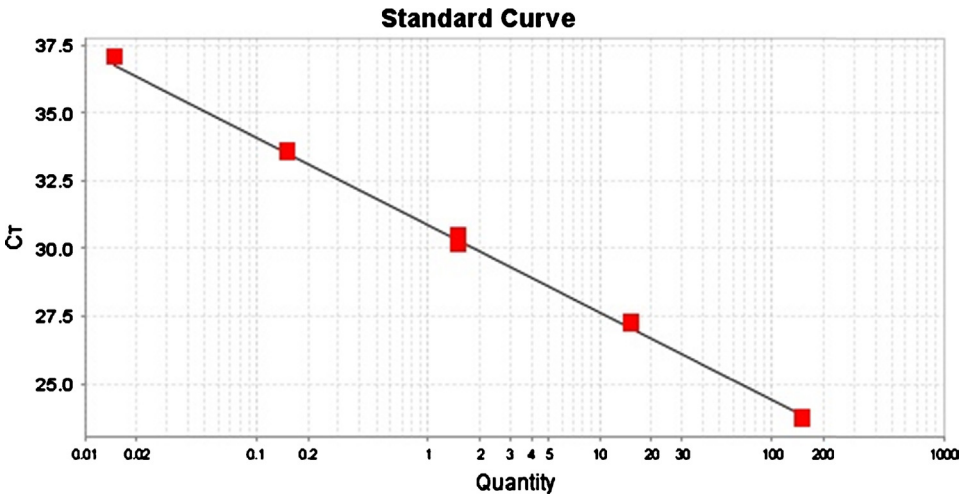

Fig. 1. The linear range of the HMBS qPCR assay developed. Each PCR run data is supported by a standard curve with slopes and PCR efficiencies within the recommended range. Quantities are given in ng units.

3. Results and discussion

3.1. Assay optimisation and copy number determination

The HMBS2F2R qPCR assay was reliably able to amplify purified canine genomic DNA ranging from 14.3 pg to 143 ng (approximately 6–60,000 copies calculated using a genome size of 2.53×10^9 bp) within the linear range (Fig. 1) [13]. DNA extraction efficiency for the specific canine whole blood sample used for standard DNA preparation yielded 28.6 ng canine genomic DNA/μl whole blood processed, resulting in 14.3 pg DNA being represented by approx. 0.5 nl extracted venous whole blood (white blood cell count dependent on dogs and sample sites). This range served as qPCR standard for each qPCR run in order to quantify the HMBS copy numbers present in the isolated DNA samples.

No detectable HMBS2F2Rp probe hydrolysis could be observed using isolated DNA from rabbit- or rat-fed *C. felis*, *R. sanguineus*, *D. variabilis* and *D. reticulatus* as template during the qPCR assay over 40 cycles (Fig. 2). No significant difference could be observed in HMBS copy number determination for samples containing 60 copies of canine DNA in the presence of 100; 200; 400 and 800 ng *D. reticulatus* DNA in 20 μl PCR volume when compared to the well-well cycle threshold (CT) uniformity of the thermal cycler specific to this assay.

Any significant template-derived inhibition of the PCR was excluded using the IAC qPCR in a duplicate run for each sample. The IAC was designed to yield a PCR product that is the same size (133 bp) and has similar GC content (54.9% *vs.* 53.4%) when compared to the HMBS PCR product [14]. It also employs the same primer set for amplification when compared to the HMBS PCR. The introduction of lambda DNA region flanked by the primer sites in the IAC created a unique DNA sequence where the hydrolysis probe could be based, thereby limiting possible cross reaction probe hydrolysis when using a wide variety of host and parasite DNA. This allows detection of inhibition affecting the primers and polymerase during product formation.

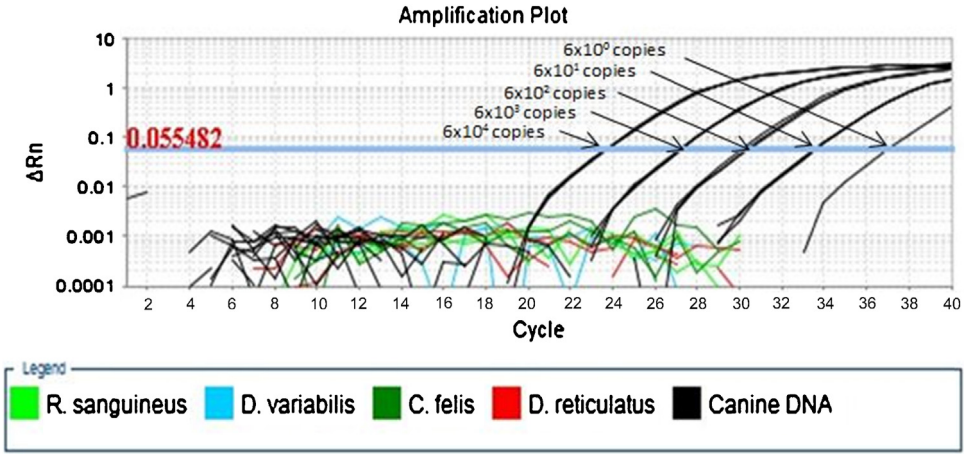

Fig. 2. Amplification of the HMBS from canine DNA used as standards with no detectable amplification for any of the rabbit- or rat-fed parasites. The number of copies/reaction (6×10^4–6×10^0) as well as the fluorescence threshold level (0.055482) is indicated.

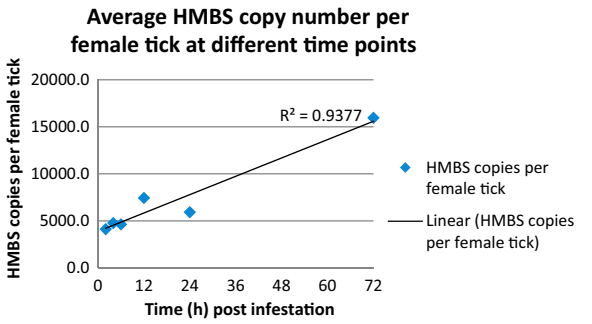

Fig. 3. Detection of canine HMBS copies over time for attached live female ticks showing the dynamic of blood feeding represented by a single female *R. sanguineus* tick.

Well-well CT uniformity for this assay was determined to be a maximum of 0.186 cycles, where IAC qPCR CT maximum change was <0.362 cycles. This result indicates that the worst inhibition effect detected translates to a 6.2% detectable change in copy number per qPCR when using an average HMBS CT value of 32.291.

3.2. Blood feeding and qPCR results

After attachment, ticks start to exchange fluids with their host. They secrete proteases, other enzymes and cytokine-like molecules to create a specific zone of lysis. As a result, they start to ingest this digested area of the skin, containing tissue and blood cells [12]. Tick bites attract white blood cells to the inflammatory site, explaining why the copy number of the canine HMBS gene would increase the more they ingest. Looking at the live female ticks removed from the dogs, the quantitative PCR showed a clear tendency for an increased number of copies during this time period, in relation to the engorgement of the ticks (Fig. 3).

The average HMBS gene copies detected using qPCR results were not significantly different between live female ticks removed from the control and treated dogs, indicating that all attached ticks behave in the same way. Nevertheless, the number of ticks removed from the dogs was very different between groups and this variable should be integrated when calculating total tick feeding inhibition. This observation resulted in the proposed method of analysis, which would only include the total number of HMBS copies detected per group per time point, for comparison between groups.

3.3. Tick killing efficacy

The comparison of the total number of ticks (males and females) collected in the crates at 2 h post exposure allows for the calculation of the prevention of dog infestation, which can be defined as the percentage repellency *sensu lato*, as well as of the immediate mortality rate of the expelled ticks (Table 3). The repellency percentage observed at 2 h varied from 4.7% at day 28 to 48.3% at day 7. This repellency has been called "expellency" and defined by Halos et al., 2012 as the sum of the repelled ticks which never infested the host, and the ticks that infested but fell off quickly due to irritant or behavioural effects of acaricidal molecules [17]. The amitraz effect seems to be more behavioural, through increased motility, while other compounds such as permethrin have a more repellent and irritant effect [9,18,19]. Published data have shown that the repellency *sensu lato* effect of CERTIFECT increases over time and it was found to be up to 100% at 24 h [9]. The immediate mortality rate of the ticks expelled at 2 h varied from 1.5% at day 21 to 31.7% at day 7. The mortality of expelled ticks was not assessed after incubation. Published data have shown that it could range from 90 to 100% mortality after 24 h in an insectarium [9].

Published data also suggests that the efficacy is sustained for 5 weeks, which allows the combination of tick counts from different days on different dogs [16,20,21]. The efficacy calculation showed that CERTIFECT started killing ticks in as little as 2 h, with an acaricidal efficacy of 27.5%, reaching 90% efficacy at 12 h and 100% at 24 h post exposure in crates (Table 4). The comparison of attached ticks on control and treated dogs also allowed the calculation of the prevention of attachment induced by the treatment (Table 4), which was significant during the full study.

3.4. Inhibition of blood feeding relative to tick burdens

The reduction in blood feeding was calculated as the percentage difference between the total HMBS gene copies detected in attached ticks from the treated group and in attached ticks from the control group at specific time points. This approach automatically takes into account the variation in tick burdens and reports only on the amount of detectable blood (as a function of detectable HMBS copy numbers) consumed between groups and time points. Results indicate significant reduction in blood feeding (>80.0%) and attached tick counts (>70.0%) as early as 4 h post infestation (Fig. 4).

Table 3
Repellency and mortality of ticks at 2 h.

Ticks in crate at 2 h	Day 1	Day 4	Day 7	Day 14	Day 21	Day 28
Ticks in crate, Control dogs	14	16	6	6	8	15
% Total ticks (/300)	4.7	5.3	2.0	2.0	2.7	5.0
Mortality rate (%)	0	0	0	0	0	0
Ticks in crate, treated dogs	104	159	151	88	65	29
% Total ticks (/300)	34.7	53	50.3	29.3	21.7	9.7
% Repellency at 2 h	30*	47.7*	48.3*	27.3	19.0	4.7
Mortality rate (%)	31.7*	32.1*	29.8*	6.8	1.5	13.8
p-Value	0.00152	0.00152	0.00152	0.28	0.69	0.38

* Significant, $p > 0.05$ (Mann–Whitney *Z*-score, 2 sides).

Table 4
Speed of kill and prevention of attachment on ticks calculated at different time points.

	2 h	4 h	6 h	12 h	24 h
Control live ticks on dogs	222	199	201	196	155
Average	74.0	66.3	67.0	65.3	51.7
CERTIFECT live ticks on dogs	161	47	34	19	0
Average	53.7	15. 7	11.3	6.3	0
% Efficacy	27.5	29.1	80.6	90.3	100
p-Value	0.33	0.0027	0.17	0.16	0.047
Attached ticks on control dogs	220	199	200	195	155
Average	36.67	33.17	33.33	32.5	25.83
Attached ticks on treated dogs	93	43	33	19	0
Average	15.5	7.17	5.5	3.17	0
% Prevention of attachment	57.73%*	73.38%*	83.50%*	90.24%*	100%*
p-Value	0.0083	0.00152	0.00152	0.00152	0.00152

* Significant, $p > 0.05$ (Mann–Whitney *Z*-score, 2 sides).

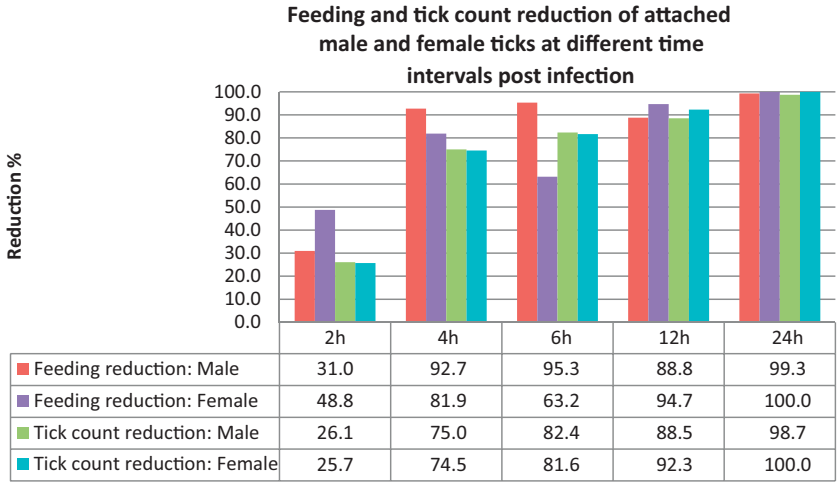

Fig. 4. Feeding and tick count reduction of attached male and female ticks at different time intervals post infestation. Reduction percentage was determined using the difference in values obtained for the control and CERTIFECT treated groups.

Feeding and tick count reduction of attached males and females at different time points post infestation were compared and indicated >99.0% feeding reduction and >98.0% attached tick count reduction at 24 h post infestation. A clear correlation between feeding reduction and tick reduction could be observed, especially from 6 h onward.

The reduction of blood feeding was significant at 6 h (77.3%) and complete at 24 h post tick exposure in the crate. It was a clear quantitative demonstration that the quantity of host DNA ingested by ticks is lower on dogs treated by CERTIFECT.

4. Conclusion

The quantitative study of canine DNA ingested by ticks may be used to determine the non-lethal effect of future acaricidal molecules. The same methodology could be applied to assess active feeding by flea (*e.g.* speed of bites). Future work will incorporate both the HMBS and the IAC in one duplex qPCR run with different probe hydrolysis signals without any cross-talk between detection channels. This will allow for *in vitro* reaction comparison and correction of the HMBS copy number based on each individual IAC response.

The expellency and the lethal efficacy of the novel topical formulation of CERTIFECT [fipronil/amitraz and (*S*)-methoprene] resulted in significantly fewer live attached ticks, consequently reducing blood intake and fluid exchanges. As a result, it reduces the risk of transmission of pathogens. The latter is supported by other studies showing the inhibition of transmission of *Babesia canis*, *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, or *Ehrlichia canis* [22,23,24].

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