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The prevention of transmission of *Babesia canis canis* by *Dermacentor reticulatus* ticks to dogs using a novel combination of fipronil, amitraz and (S)-methoprene

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ABSTRACT

Four groups of seven dogs were treated topically with a novel combination of fipronil, amitraz and (S)-methoprene in a spot-on formulation (CERTIFECT™, Merial Limited, GA, USA) on 28, 21, 14 and 7 days prior to tick infestation, respectively and acaricidal efficacy and transmission blocking compared with an untreated control group (seven dogs). All dogs were infested with adult *Dermacentor reticulatus* ticks harbouring *Babesia canis canis*.

Babesia canis canis was transmitted by *D. reticulatus* to all seven untreated control dogs, confirmed following demonstration of clinical signs, by the detection of *B. canis* parasites in thin blood smears and *B. canis canis* PCR-RLB DNA assay on blood and the development of *B. canis canis* antibody titres by 14–21 days after tick infestation. The majority of treated dogs remained sero-negative for 42 days after infestation. Therefore, the treatment of dogs with CERTIFECT applied up to 28 days prior to infestation with *D. reticulatus* harbouring *B. canis canis*, successfully prevented the development of clinical signs of canine babesiosis.

1. Introduction

Canine babesiosis is a clinically significant tick-borne protozoan disease, which occurs worldwide. Historically, *Babesia* parasites in dogs were divided into two morphological distinct groups, the larger *Babesia canis* and the smaller *Babesia gibsoni*. *B. canis* has been reclassified into three subspecies (*B. canis canis, B. canis rossi and B. canis vogeli*) on the basis of vector-specificity and cross-immunity and are now considered to be separate species, *B. canis*, *B. rossi* and *B. vogeli* (Uilenberg et al., 1989; Zahler et al., 1998; Carret et al., 1999). However, both species and sub-species names remain in use in the current literature. *Babesia canis canis* is widely distributed throughout Europe, where it is transmitted by adult *D. reticulatus* and ticks (Matjila et al., 2005; Bourdoiseau, 2006; Cardoso et al., 2008; Cassini et al., 2009; Beugnet and Marié, 2009). The clinical signs of babesiosis in dogs vary from a mild transient illness to acute disease due to severe haemolysis that rapidly results in death. Clinical findings include anorexia, pale mucus membranes, icterus, pyrexia, and splenic and hepatic enlargement (Jorgensen, 2005; Bourdoiseau, 2006).

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Globally, companion animals, in particular dogs, are exposed to a broad range of protozoan and bacterial pathogens transmitted through the bite of infected vector ticks. In particular, canine babesiosis, anaplasmosis and monocytic ehrlichiosis are the pre-eminent tick-borne diseases of dogs worldwide (Jongejan and Uilenberg, 2004). A recent increase in the incidence of canine tick-borne diseases is due largely to changes in the ecology (landscape changes with consequent increased wildlife host abundance), climate change (increased tick survival and abundance), human behaviour (recreation and travel with pets) among other factors (Beugnet and Marié, 2009; Blagburn and Dryden, 2009; Gray et al., 2009). As a result, there is a strong need for effective ectoparasiticides to control ticks on dogs.

The majority of published studies aimed to demonstrate the utility of tick control compounds on dogs have been focused on their acaricidal efficacy against the ixodid tick species. However, relatively little research has been carried out to determine the ability of such compounds to prevent transmission of tick-borne diseases to dogs. Guidelines for evaluating the efficacy of ectoparasiticides for the treatment, prevention and control of tick infestations on dogs do not include suggestions to quantify the dynamics of transmission of tick-borne pathogens (Marchiondo et al., 2007). However, several studies have been conducted that suggest that topically applied tick control compounds such as permethrin and imidacloprid in combination, fipronil and (S)-methoprene in combination, fipronil alone, and amitraz alone can aid in the prevention of the transmission of specific tick-borne pathogens including *Anaplasma phagocytophilum* (granulocytic anaplasmosis), *Borrelia burgdorferi* (Lyme disease), *Ehrlichia canis* (monocytic ehrlichiosis) and *B. canis rossi* (canine babesiosis) to dogs by ixodid ticks (Elfassy et al., 2001; Davoust et al., 2003; Spencer et al., 2003; Blagburn et al., 2004; Jacobson et al., 2004; Last et al., 2007; Otranto et al., 2008; Otranto et al., 2010). From this data in the literature it is evident that the development of a transmission blocking model may be feasible. Such a model would have to include a sufficient number of treatment groups to test the duration of preventive activity, plus an un-treated control group wherein the majority of dogs become infected by the tick-borne pathogen.

In this paper a transmission blocking model is presented, wherein topical formulations can be evaluated for their ability to prevent dogs from becoming infected with babesiosis. Specifically we tested the ability of CERTIFECT™ (Merial Limited, GA, USA), a novel combination of fipronil, amitraz and (S)-methoprene in a spot-on formulation, to prevent transmission of *B. canis canis* to dogs artificially infested with *D. reticulatus*. Fipronil, which belongs to the phenylpyrazole family, and (S)-methoprene, an insect growth regulator, have been used in combination for several years for the treatment and control of ticks, fleas and lice (FRONTLINE Plus® for dogs or FRONTLINE Combo® Spot-on Dog (Merial, GA, USA)(Dryden, 2005). Amitraz is a formamidine which kills ticks by inhibition of monoamine oxidase and it also has been reported as a tick repellent and tick detachment drug (Folz et al., 1986; Taylor, 2005).

2. Materials and methods

2.1. Study design

The study was conducted according to the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products Guideline 9: Good Clinical Practice (Anon, 2000) and in compliance with local animal welfare legislation. The study employed a controlled, blinded, randomized block design and utilized full grown, healthy, mongrel dogs. All dogs were individually penned in tick-proof kennels, managed similarly and observed twice daily for health abnormalities throughout the study. When health abnormalities were detected between the scheduled physical examinations, additional examinations were conducted. The 13 male and 22 female dogs, all negative for *B. canis* antibodies by indirect fluorescent antibody assay (IFA), were randomly divided into five equal groups. Group 1 was designated untreated control. The other four groups (treatment groups 2–5) were treated once with the novel combination of fipronil, amitraz and (S)-methoprene spot-on at respectively 28, 21, 14 or 7 days before artificial challenge with *B. canis canis*-infected *D. reticulatus* ticks.

Treatments were applied topically to deliver at least 6.7 mg fipronil/kg bodyweight (bw), 8.0 mg amitraz/kg bw and 6.0 mg (S)-methoprene/kg bw by simultaneously applying two separate formulations from dual pipettes, one containing 10% (w/v) fipronil plus 9% (w/v) (S)-methoprene and the other 20% (w/v) amitraz. Each formulation was applied directly onto the skin divided equally between two spots on the dorsal midline, one in front of the shoulder blades and one at mid neck. Care was taken when handling dogs for study procedures to prevent drug cross contamination between treatment groups by changing protective clothing and changing/cleaning equipment as necessary.

2.2. Monitoring

After all treatments were completed physical examinations of all dogs were conducted on the day before infestation and for each dog remaining without signs of babesiosis at 14, 21 and 28 days after infestation, as a means of monitoring the establishment of babesiosis. The physical examination included measurement of clinical variables – body temperature, heart rate and respiration rate. In addition, daily body temperature measurements of all dogs were recorded from 6 to 13 days after infestation, when the risk of clinical signs of babesiosis occurring was considered highest, in order to rapidly detect the onset of babesiosis in individual dogs. When a dog's body temperature was recorded above normal (>39.4 °C), a thin blood smear was prepared, stained (Kyro-Quick Romanowski stain – Kyron Laboratories Pty Ltd, Benrose, South Africa), and examined microscopically for the presence of *B. canis* species (*canis canis, canis rossi* and *canis vogeli)* parasites in red blood cells. Dogs that were positive for *B. canis* spp. in blood smears were blood sampled for *B. canis canis* DNA assay using Polymerase Chain Reaction-Reverse Line Blot (PCR-RLB) to confirm the infection. Any dog positive for *B. canis* on blood smear was consequently treated with diminazene

aceturate to prevent fatal babesiosis, and no further clinical data was collected from these dogs except for *B. canis* serology.

Blood samples for *B. canis* antibody serology using *B. canis canis* antigen were collected from all dogs prior to tick infestation on the day of infestation and at 14, 21 and 28 days and, except for controls, at 42 days after infestation.

2.3. Infestation by Dermacentor reticulatus

All *D. reticulatus* were provided from a laboratory-maintained population derived from wild ticks collected in Europe. All dogs were infested on the same day, either 28, 21, 14 or 7 days after treatment administration, while under sedation, with 25 male and 25 female adults, unfed ticks of which 33% were harbouring *B. canis canis* as inferred from other ticks sampled from the same batch.

2.3.1. Infection of ticks by Babesia canis canis

The *D. reticulatus* ticks were infected with *B. canis canis* by feeding them on dogs infected with a *B. canis canis* strain isolated from a *D. reticulatus* female collected from a dog in France. *B. canis canis* infestation of ticks was determined by PCR-RLB DNA assay on a sample of ticks (51) taken from the infestation batch and ticks collected from study dogs on Day 6 post-infestation.

2.3.2. Tick counts

Following infestation, ticks on all dogs, including untreated controls, were counted *in situ* at 2, 3, 4 and 5 days and removed at Day 6 post-infestation in order to determine the efficacy of the combination of fipronil, amitraz and (S)-methoprene at preventing the establishment of tick infestation. Tick counts were performed and recorded by tick categories (Table 1).

2.3.3. Laboratory analyses

Blood samples for serology were assayed for *B. canis* antibodies using an indirect fluorescent antibody (IFA) assay with *B. canis canis* antigen as the substrate and carried out as described by Uilenberg et al. (1989). This test cross reacts with antibodies of *B. canis rossi* and *B. canis vogeli*. However, homologous species antigen and antibody combinations (*B. canis canis* antibodies with *B. canis canis* antigen) will cause a stronger reaction (higher titre) than heterologous species combinations such as and *B. canis rossi* antibodies and *B. canis canis* antigen where the reaction will be weak. In this study where *B. canis* sero-negative ticks were infected with *B. canis canis* it is assumed that all positive *B. canis* titres reflected *B. canis canis* antibodies. For screening purposes the sera were diluted at 1:80, and results were expressed as positive (fluorescence at dilution 1:80) or negative (no fluorescence). Positive samples collected at 21, 28 and 42 days after infestation were additionally serially two-fold diluted starting at 1:80 through 1:2560 to determine the *B. canis canis* antibody titre of each one.

Parasite DNA extraction from blood or ticks, PCR amplification and reverse line blot (RLB) hybridization for simultaneous detection and differentiation of *Babesia* species was carried out as described previously (Gubbels et al., 1999; Matjila et al., 2004). Briefly, QIAAMP® (QIAAMP is a registered trademark of Qiagen GmbH in the United States of America and elsewhere) blood and tissue extraction kits were used for DNA extraction, following the manufacturer's protocols. PCR was performed with primers RLB-F2 and RLB-R2 amplifying a fragment of 460–540 bp from the 18S rRNA gene spanning the V4 region (Gubbels et al., 1999). Reverse line blot hybridization was performed on amplified PCR products using an improved protocol published by Matjila et al. (2005). The protocol was improved by the addition of an internal quality plasmid control, which was used to check whether all *Babesia* species-specific oligonucleotides were attached correctly to the RLB membrane and functioning properly. The RLB probe (TGCGTTGACGGTTTGAC) employed for *B. canis canis* had been shown previously to be species-specific (Matjila et al., 2005).

2.4. Statistical analysis

The primary variable for determining the ability of the novel combination to prevent transmission of *B. canis canis* to dogs was the antibody titre. At 14 days post-infestation the results were reported as positive (titre $\geq$1:80 dilution) or negative, whereas no end-point titres were determined. Because very few dogs in treatment groups 2–5 developed titres at 21, 28 or 42 days post-infestation, analysis of differences in titres between these groups was not possible.

For clinical variables, body temperature, heart rate and respiration rate measured at 14, 21 and 28 days post-infestation, the pre-infestation (baseline) responses measured the day before infestation were analyzed as an analysis of variance using the Mixed procedure with the effects sex, treatment, and sex-by-treatment interaction as the fixed effects. Replicate was the random effect. To detect responses to *B. canis canis* infection by each treated group (groups 2–5) in the absence of post-infestation data for the untreated controls, the change from pre-infestation baseline was analyzed for each treated group.

In order to determine the effectiveness of the novel combination of fipronil, amitraz and (S)-methoprene in preventing establishment of tick infestation, for each dog at each counting time, the total number of ticks that were assigned to categories 1, 2, 3, and 6 (live free, attached/unengorged and attached/engorged and dead attached/engorged) were transformed to the natural logarithm of (count + 1) for calculation of geometric means. For the treated groups, the percent reduction in tick counts compared to group 1 (untreated control) was computed using the formula $100 \times (1 - T/C)$, wherein T and C were the geometric means of the particular treated and control group 1, respectively. For all ticks within each counting time, the expected tick counts of the treated groups were compared with the expected tick count of the control group using the Friedman rank test.

All analyses were conducted using SAS® Version 9.1.3 (SAS Institute Inc., Cary, NC, USA), and all statistical comparisons were made using a (two-sided) 5% significance level.

Table 1
Count categories of live and dead ticks by attachment status.

Category[a]	General findings	Attachment status	Interpretation
1	Live	Free	Acaricidal effect NOT demonstrated
2	Live	Attached; unengorged	Acaricidal effect NOT demonstrated
3	Live	Attached; engorged[b]	Acaricidal effect NOT demonstrated
4	Dead	Free	Acaricidal effect demonstrated
5	Dead	Attached; unengorged	Acaricidal effect demonstrated
6	Dead	Attached; engorged	Acaricidal effect NOT demonstrated

[a] Adapted from Marchiondo et al. (2007).
[b] Engorged tick: a tick with a conspicuous enlargement of the alloscutum that has blood in its digestive tract.

3. Results

3.1. Treatment tolerance and animal health

The novel combination of fipronil, amitraz and (S)-methoprene administered concurrently as topical solutions to 28 dogs was well-tolerated by all animals. No significant health abnormalities, other than babesiosis in controls, were detected during the study.

3.2. Tick counts

Tick counts for each group by tick category (1–6) and day (2, 3, 4, 5, and 6 days after infestation) are summarized in Table 2. The geometric mean of live plus dead engorged (categories 1, 2, 3 and 6) tick counts per day for each treatment group is listed in Table 3. Percent reductions in tick counts for each treated group compared to the untreated control are also listed in Table 3. The treatment rapidly reduced the number of adult *D. reticulatus* by more than 98% within 2 days after infestation and to 100% within 3 days.

3.3. Challenge with Babesia-infected ticks

The ticks sampled from the infestation batch contained 17 out of 51 (33.3%) *B. canis canis* infected tick. For ticks collected on Day 6 post-infestation, all from untreated control animals, 55 out of 122 (45.1%) were found to be infected with *B. canis canis*. Of those ticks from the infestation batch, 20% of 25 males and 46% of 26 females were infected while of ticks collected on Day 6, 50% of 42 males and 42% of 80 females were infected.

3.4. Babesia canis canis transmission blocking

All dogs were sero-negative for *B. canis* prior to tick infestation. All the dogs in group 1 (untreated controls) became positive for *B. canis* on thin blood smears collected after body temperatures were measured at >39.4 °C. These dogs were consequently treated with diminazene aceturate to prevent fatal babesiosis. Five of the seven untreated control dogs were sero-positive for *B. canis canis* on Day 14 post-infestation, and all seven developed antibody titres ranging from 160 to ≥2560 on Day 21 and Day 28 post-infestation (Tables 4 and 5).

All treated dogs (groups 2–5) were sero-negative for *B. canis canis* at 14, 21, and 28 days post-infestation, except for two dogs positive at 28 days in group 5 (treated 7 days prior to infestation) (Tables 4 and 5). On Day 42 post-infestation, the same two dogs were positive again, as well as two additional dogs in group 2 (treated 28 days prior to infestation). The four dogs in treated groups 2 and 5 displayed low titres ranging between 80 and 160 and did not show any clinical signs of babesiosis including raised body temperature (>39.4 °C) throughout the study. The two dogs in group 5 were also negative for *B. canis* on additional blood smear examinations and *B. canis canis* on PCR-RLB DNA assay conducted 43 days post-infestation.

For clinical variables, body temperature, heart rate and respiration rate, the pre-infestation (baseline) responses measured the day before infestation showed no significant ($P > 0.05$) differences between groups. For body temperature there was no statistical evidence ($P > 0.05$) that the change from pre-infestation baseline interacted with any treatment effect in the treated groups (2–5). Several variations up and down in heart rate and respiration rate were observed that represented significant ($P < 0.05$) changes from baseline observations, but these fluctuations were not considered clinically relevant as they were intermittent and not related to treatment interval.

4. Discussion

In order to be able to conduct the study, it was required to generate a large batch of ticks with an adequate *Babesia* infection rate. The sample of ticks from the challenge batch of infected ticks taken on the day of infestation contained 33.3% infected ticks while of those ticks collected on Day 6 post-infestation from untreated control animals, 45.1% were found positive. On each occasion some male and female ticks were infected although the proportion of infected males was much lower than females in the sample from the infestation batch. It is likely that both male and female ticks facilitated transmission of *B. canis .canis,* but a separate study with only male or female ticks will be required to investigate this further. Regardless of which sex or if both sexes transmit the *B. canis canis,* the infection rate in the ticks was sufficiently high to successfully transmit the infection to 7 out of 7 untreated (control) dogs.

The study was designed to detect exposure to *Babesia* infection in dogs through an economical use of available diagnostic techniques. The detection and confirmation of *B. canis canis* infection was based on an initial three stage approach: (1) regular monitoring of body temperature of all dogs; (2) thin blood smear examination for *B. canis* parasites in red blood cells of pyrexic dogs (>39.4 °C); (3) molecular assay for detection of *B. canis canis* DNA by PCR-

Table 2
Tick counts for each treatment group by tick category (1–6) and count day (2, 3, 4, 5, and 6 days after infestation).

Tick category ►	1. Live free					2. Live attached unengorged					3. Live attached engorged				
Days after infestation ►	2	3	4	5	6	2	3	4	5	6	2	3	4	5	6
Group[a]															
1	1	1	3	5	3	173	162	121	67	41	0	0	32	61	75
2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
5	2	0	0	0	0	8	0	0	0	0	0	0	0	0	0

Tick category ►	4. Dead free					5. Dead attached unengorged					6. Dead attached engorged				
Days after infestation ►	2	3	4	5	6	2	3	4	5	6	2	3	4	5	6
Group[a]															
1	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0
2	3	0	0	0	0	0	1	0	0	0	0	0	0	0	0
3	8	0	0	0	0	2	1	0	0	0	0	0	0	0	0
4	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	3	0	1	1	0	1	5	0	0	0	0	0	0	0	0

[a] Group 1 = untreated control; group 4 = treated 14 days before infestation; group 2 = treated 28 days before infestation; group 5 = treated 7 days before infestation. Group 3 = treated 21 days before infestation.

RLB on blood samples from dogs with a positive *B. canis* blood smear to confirm the infection. Unfortunately, with this approach the DNA assay was not used on all dogs, a shortcoming that may have resulted in subclinical infections being undetected. However, serological testing was performed on samples collected from all dogs irrespective whether the dogs were pyrexic. The use of serology on all dogs to detect specific *B. canis* antibodies did correctly detect infections in all dogs showing clinical signs of babesiosis (i.e. pyrexia). Moreover, serology did detect

Table 3
Geometric mean tick counts (tick categories 1, 2, 3, and 6) by treatment group and percent reductions in counts compared to untreated control (treatment group 1).

Days after infestation	Group[a]	*n*	Geometric mean	Percent reduction[b]	*P*-value[c]
2	1	7	24.5	–	–
	2	7	0.1	99.6	0.008
	3	7	0.1	99.6	0.008
	4	7	0.1	99.6	0.008
	5	7	0.4	98.3	0.008
3	1	7	23.0	–	–
	2	7	0.0	100.0	0.008
	3	7	0.0	100.0	0.008
	4	7	0.0	100.0	0.008
	5	7	0.0	100.0	0.008
4	1	7	21.9	–	–
	2	7	0.0	100.0	0.008
	3	7	0.0	100.0	0.008
	4	7	0.0	100.0	0.008
	5	7	0.0	100.0	0.008
5	1	7	18.2	–	–
	2	7	0.0	100.0	0.008
	3	7	0.0	100.0	0.008
	4	7	0.0	100.0	0.008
	5	7	0.0	100.0	0.008
6	1	7	14.6	–	–
	2	7	0.0	100.0	0.008
	3	7	0.0	100.0	0.008
	4	7	0.0	100.0	0.008
	5	7	0.0	100.0	0.008

[a] Group 1 = untreated control; group 2 = treated on Day 0; group 3 = treated on Day 7; group 4 = treated on Day 14; group 5 = treated on Day 21, all groups infested with ticks on Day 28.
[b] Percent reduction in tick count compared to untreated control = $100 \times (1 - T/C)$, where T and C are the geometric means of the treated and untreated control groups, respectively.
[c] Two-sided *P*-values comparing the expected tick counts using Friedman rank test.

Table 4
Ratio of sero-positive dogs to the total number of dogs based on *Babesia canis canis* antibodies according to treatment group.

Days after infestation	Group 1	Group 2	Group 3	Group 4	Group 5
−43	0/7[a]	0/7	0/7	0/7	0/7
0	0/7	0/7	0/7	0/7	0/7
14	5/7	0/7	0/7	0/7	0/7
21	7/7	0/7	0/7	0/7	0/7
28	7/7	0/7	0/7	0/7	2/7
42	–[b]	2/7	0/7	0/7	2/7

[a] Number of sero-positive dogs (=IFA titre of ≥1:80)/total number of dogs.
[b] Samples were not collected.

four other dogs with low antibody titres but without clinical signs and indeed the two of these that were DNA tested on Day 43 were in fact negative for *B. canis canis* DNA using PCR-RLB. Thus the combination of thin blood smear and DNA assay on only pyrexic dogs with the use of serology on all dogs was considered an effective means of documenting exposure to *B. canis canis* in the study dogs (Uilenberg et al., 1989).

Six out of the seven control dogs, which developed antibody titres increasing from 80 on or soon after Day 14 to 320 up to ≥2560 in 7 days or less, demonstrated strong sero-conversion. However, the titre of the remaining control dog went up to only 160. The low titre first measured at 21 days post-infestation in this control dog was due probably to the early administration of the anti-babesial treatment at Day 7 post-infestation, despite *B. canis canis* infection, as for all other controls, being confirmed by positive blood smear and PCR results on samples collected on the day a raised body temperature was detected.

The late stage weak sero-conversion by Days 28–42 post-infestation in four of the treated dogs indicated a low level transmission of *B. canis canis* sporozoites. Two of these dogs (in group 5) and the other two (in group 2) were challenged with ticks at 7 and 28 days post-treatment, respectively. Transmission is likely to have taken place during a brief attachment by a small number of ticks without fever or other signs of babesiosis being evident to prompt blood smear collections from these dogs. It was noted that one dog in each of the treated groups (2–5), but not the same dogs positive for *B. canis canis* antibodies in these groups, had relatively small numbers (1 or 8) of live attached, unengorged ticks present at the 2-day tick count but none thereafter (Table 2). Although treatment with the novel combination did not completely block the transmission of *B. canis canis* in a small proportion of dogs (14%), it was 100% successful in preventing the development of clinical signs of babesiosis in all dogs up to 42 days after tick infestation.

The results obtained in controlled laboratory trials with *B. burgdorferi* are similar to those obtained in this study with *B. canis canis*. When the challenge infestation was conducted 28 days after treatment, fipronil/(S)-methoprene spot-on prevented all but 2 of 16 dogs from becoming infected with *B. burgdorferi* and was 97.6% effective against tick infestation 48 h after challenge (Jacobson et al., 2004). It therefore appears feasible to dramatically reduce the transmission of tick-borne pathogens with suitable anti-tick control compounds, although a 100% transmission blocking may be difficult to achieve.

The reported study indicates the importance of the speed of kill of anti-tick compounds with respect to the early removal of feeding ticks in order to successfully block transmission. The effect of early removal of feeding ticks on pathogen transmission depends on the tick attachment duration that is required for ticks to transmit the specific pathogen concerned. For instance, transmission of *Borrelia* spirochetes appears rare within the first 24 h, whereas in the same period most *A. phagocytophilum* rickettsiae

Table 5
Babesia canis canis antibody titres in individual dogs according to treatment group.

Group	Replicate	Infestation +21 days[c]	Infestation +28 days	Infestation +42 days
1[b]	1	≥1:2560[a]	≥1:2560	–[d]
	2	1:320	1:320	–
	3	1:640	1:320	–
	4	≥1:2560	1:1280	–
	5	1:160	1:160	–
	6	1:640	1:160	–
	7	1:1280	1:640	–
2	1	neg[e]	neg	1:80
	6	neg	neg	1:160
5	2	neg	1:80	1:80
	7	neg	1:160	1:80

[a] Positive = titre ≥1:80.
[b] Group 1 = untreated control; group 2 = treated 28 days before infestation; group 3 = treated 21 days before infestation; group 4 = treated 14 days before infestation; group 5 = treated 7 days before infestation.
[c] *B. canis* antibody titres were not determined on Day 14 after tick infestation.
[d] Samples were not collected.
[e] Samples negative for *B. canis canis*.

are already transmitted (Des Vignes et al., 2001). For most viral pathogens transmission appears to be rapid; for example, Powassan virus can be transmitted within 15 min after attachment of the vector tick (Ebel and Kramer, 2004).

Protozoan parasites require additional time, usually several days, for their sporoblasts to mature into sporozoites in the salivary glands of the tick before they can be secreted into the saliva and transmitted to the mammalian host. For instance, *Babesia microti* is transmitted by *Ixodes scapularis* between 36 and 48 h after tick attachment (Piesman and Spielman, 1980). The attachment duration required for *Rhipicephalus appendiculatus* ticks to transmit *Theileria parva* is at least 72 h (Konnai et al., 2007). The four dogs in this study that sero-converted despite treatment must have been bitten by ticks in which mature *B. canis canis* sporozoites were already present in the salivary glands. It can be speculated that the maturation of these sporozoites was induced by a high ambient temperature preceding the tick challenge of the study dogs, a phenomenon demonstrated for *Theileria annulata* sporozoites which mature more rapidly in *Hyalomma* ticks when incubated at a higher temperature (Samish, 1977).

The early events after tick attachment which lead to the actual transmission of pathogens are crucial and need to be studied in further detail in order to optimize the blocking of pathogen transmission. The dynamics of pathogen transmission may be easier to study in in vitro feeding assays, which have been developed for ixodid ticks, because the parasite-host-pathogen interactions do not have to be taken into account (Kröber and Guerin, 2007).

This same *B. canis canis* transmission blocking model for *D. reticulatus* ticks could also be used to test other combinations of tick control compounds and potentially adapted to study the blocking of transmission of other tick-borne pathogens, such as *B. canis vogeli* and *E. canis* transmitted by *R. sanguineus* ticks.

5. Conclusions

Using the transmission blocking model for *D. reticulatus* infected with *B. canis canis,* the treatment of dogs with the combination of fipronil, amitraz and (S)-methoprene as a spot-on topical formulation applied up to 28 days prior to infestation with *D. reticulatus* harbouring *B. canis canis*, successfully prevented the development of clinical signs of canine babesiosis up to 42 days after infestation in all dogs despite a low level of *B. canis canis* transmission in a small proportion.

Conflict of interest

The work reported herein was funded by Merial Limited, GA, USA.

Five authors (STC, CM, YM, MGP and DB) are current employees of Merial, and assisted with study design, data analysis and review of the manuscript, however there were no conflicting interests that may have biased the work reported in this paper.

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