A novel combination of fipronil and permethrin (Frontline Tri-Act®/Frontect®) reduces risk of transmission of *Babesia canis* by *Dermacentor reticulatus* and of *Ehrlichia canis* by *Rhipicephalus sanguineus* ticks to dogs

Frans Jongejan¹²*, Christa de Vos³, Josephus J. Fourie³ and Frederic Beugnet⁴

**Abstract**

**Background:** The ability of Frontline Tri-Act®/Frontect®, a topical ectoparasiticide containing fipronil and permethrin for dogs, to prevent the transmission of *Babesia canis* as well as *Ehrlichia canis* was evaluated by infesting dogs with infected vector ticks.

**Methods:** For the *Babesia canis* study, 16 dogs were randomly allocated to two groups. Eight dogs were treated on day 0 with a topical spot-on formulation containing 6.76 % w/v fipronil plus 50.48 % w/v permethrin and eight dogs served as the untreated control group. *Dermacentor reticulatus* ticks, with a *B. canis* infection rate ranging between 2 and 10 %, were placed onto dogs on days 7, 14, 21 and 28. *In situ* tick counts were performed on Days 9, 16 and 23. Ticks were counted and removed on Day 30. Infection of the dogs with *B. canis* was monitored by rectal temperature readings, clinical examinations and blood smears as well as PCR and IFA (indirect fluorescent antibody assay). For the *Ehrlichia canis* study, another 16 dogs were allocated to two groups. Eight dogs were treated with the fipronil and permethrin combination on days 0 and 28 and eight dogs served as untreated controls. *Rhipicephalus sanguineus* ticks, carrying an infection rate of 13 % for *E. canis*, were released in the sleeping kennels of the dogs on days 7, 14, 21, 28, 35, 42, 49 and 56. Ticks were counted *in situ* on the dogs on a weekly basis. All ticks were removed and counted on the final assessment day 58. Infection of the dogs with *E. canis* was monitored by rectal temperature, clinical examinations, and testing of blood samples by PCR, IFA and platelet counts.

**Results:** *B. canis* was transmitted by *D. reticulatus* ticks to all eight untreated control dogs and to one treated dog, which was confirmed by blood smears, PCR and IFA. *E. canis* was transmitted by *R. sanguineus* ticks to all eight untreated control dogs. Two of the dogs in the treated group were found positive based on PCR and/or IFA.

**Conclusions:** Frontline Tri-Act®/Frontect® significantly lowered the risk for dogs to acquire a *B. canis* infection by 87.5 % over a challenge period of 28 days. The risk for dogs to acquire *E. canis* was reduced by 75 % over a period of 56 days.

**Keywords:** Babesia canis, Ehrlichia canis, Dermacentor reticulatus, Rhipicephalus sanguineus, Fipronil, Permethrin, Transmission blocking studies

*Correspondence: F.Jongejan@uu.nl

¹Utrecht Centre for Tick-borne Diseases (UCTD), FAO Reference Centre for Ticks and Tick-borne Diseases, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584CL, Utrecht, The Netherlands

²Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort 0110, South Africa

Full list of author information is available at the end of the article

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Background

Hard ticks (Acarí: Ixodidae) infest dogs all over the world, causing direct damage due to high tick burdens. Their main importance, however, is related to their capacity to transmit a wide range of pathogenic micro-organisms [1, 2]. As a result, there is a continuous need to develop novel and/or combine existing tick control compounds for sustained tick control on companion animals, in particular dogs. Improved acaricidal formulations and combinations that are easy to administer, long acting, fast killing, and reduce infection by tick-borne diseases, e.g. babesiosis and ehrlichiosis, do provide value to veterinarians and their clients.

Canine babesiosis is caused by a number of different protozoan species of the genus Babesia, which vary in virulence and have expanded their distribution in recent years in particular in Europe [3].

Here the focus is on Babesia canis, where the occurrence largely coincides with the distribution of the ornate dog tick, Dermacentor reticulatus (Fabricius, 1794), a Palearctic species with a highly focal distribution pattern [4]. This tick occurs in foci in south-western England in the west all the way into Central Asia reaching the Yenisei river basin in Siberia in the east [5].

The second focus is on Ehrlichia canis, the causative agent of canine monocytic ehrlichiosis, which is transmitted by the brown dog tick, Rhipicephalus sanguineus (Latreille, 1806), and found worldwide anywhere between 50° N and 30° S [6].

Guidelines for conducting veterinary clinical studies have traditionally focussed on demonstrating acaricidal efficacy against ticks [7]. However, because of the importance of ticks as vectors of pathogens causing diseases in dogs and humans, there is an increasing demand for control methods that do not only kill ticks, but are also able to reduce the transmission of disease. Fipronil spotted onto dogs was shown to prevent infection with E. canis transmitted by R. sanguineus in Senegal [8]. Furthermore, application of amitraz-impregnated collars onto dogs in South Africa prevented infections with Babesia rossi transmitted by Haemaphysalis elliptica ticks [9]. In this particular study eight of 30 control dogs (26.6 %) became infected over a 6-month period compared to none of the 20 treated dogs. Field trials, however, depend on locally occurring challenge pressure, which often results in unpredictable numbers of untreated control animals contracting the tick-transmitted disease.

Over the past couple of years, laboratory models that allow for a more much standardised evaluation of the transmission blocking ability of acaricidal compounds have been developed both for B. canis [10] as well as for E. canis [11]. As a result, the WAAVP recognised this development and included in their recent guidelines that specific claims regarding the prevention or reduction of tick-borne pathogen transmission are now possible [12]. However, specific recommendations regarding the design of pathogen blocking studies have not yet been included in any of the regulatory guidelines [13].

Both transmission blocking models were initially used to determine the level of transmission blocking of B. canis-infected Dermacentor ticks and E. canis-infected Rhipicephalus ticks applied onto dogs treated with a combination of fipronil, amitraz and (s)-methoprene (CERTIFECT®) [10, 11]. Two additional studies were conducted with the E. canis blocking model; one study addressed the preventive capacity of a topical combination of imidacloprid and permethrin and the second study focussed on an imidacloprid and flumethrin collar for dogs [14, 15]. In addition to the topically active compounds [10] and slow release collar matrices [16], both recently discovered novel systemic compounds, afoxolaner [17] and fluralaner [18]) were also tested for their capacity to block transmission of Babesia [19, 20].

Recently, a combination of fipronil and permethrin (Frontline Tri-Act®/Frontect®) was tested for its acaricidal efficacy against D. reticulatus ticks [21] and also against R. sanguineus ticks [22]. Fipronil is a phenylpyrazole, which has been widely used as an acaricide/insecticide [23]. Permethrin, is a synthetic pyrethroid with a residual acaricidal activity as well as repellency effect sensu lato against ticks.

The studies reported in this paper assessed whether this combination is capable of preventing the transmission of Babesia as well as Ehrlichia using established transmission blocking models.

Methods

Study design and treatments

Both studies were conducted in compliance with the South African animal welfare legislation, the Good Clinical Practice guideline (Veterinary International Conference on Harmonization GL9) and the European Medicines Agency guidelines for testing and evaluation of the efficacy of anti-parasitic substances for treatment and prevention of tick and flea infestation in dogs and cats (EMEA/CVMP/005/ 2000-Rev.2). The studies employed a parallel group design, randomised and blinded. The dogs were ranked, within gender in descending order of individual body weight on Day -7. All dogs, identifiable by a microchip number, were individually housed in tick-proof kennels and observed daily throughout the study duration. Persons involved in the post-treatment assessments and observations were different from those that performed the treatments with the active ingredients in order to eliminate bias.

Both studies were conducted on two groups of eight dogs each. The dogs had not been treated with any ectoparasiticide for 12 weeks prior to the start of the study. For the B. canis study, the dogs were tested sero-negative for B. canis by IFA and negative for Babesia DNA by PCR. A further 16 dogs were randomly allocated to one of two
groups for the *Ehrlichia* study. They were admitted to the study after they were confirmed sero-negative for ehrlichiosis by IFA as well as PCR negative for *Ehrlichia* DNA.

The treatment consisted of 6.76 mg/kg fipronil and 50.48 mg/kg permethrin applied by parting the hair and applying the acaricide directly onto the skin along the midline of the neck. The total amount was divided into two fractions: one was applied between the shoulders and one at the base of the skull. Dogs were observed hourly for 4 h following treatment administration.

**Tick challenge on dogs**

A laboratory-bred *Dermacentor reticulatus* tick strain, originating from France, naturally infected with *B. canis*, was used. Ticks from the above mentioned strain were infected with *B. canis* by acquisition feeding on a dog with confirmed acute babesiosis. Unfed adult ticks with a balanced gender ratio (50 % female: 50 % male) were used for the dog infestations. A sample of 50 *D. reticulatus* ticks taken from the batch of ticks to be used was confirmed positive by PCR analysis (rate ranging between 2 and 10 %). Each dog was infested on days 7, 14, 21 and 28 with 50 (±5) viable ticks applied directly onto the back of the dog. During this process dogs were restrained for 10 min in an infestation crate. Gloved fingers were used to facilitate the ticks through the dog’s hair coat in order to reach the skin. Any tick that was found dislodged during the first ten min was placed back onto the dog.

In the second study, a laboratory-bred *R. sanguineus* tick strain, originating from France, naturally infected with *E. canis*, was used for the dog infestations. Ticks from the above mentioned strain were infected with *E. canis* by acquisition feeding on a dog with confirmed acute ehrlichiosis. A sample of 50 *R. sanguineus* ticks from the batch used for infestation was confirmed infected with *E. canis* with an infection rate of 13 % by PCR analysis. Fifty (±5) unfed adult ticks of equal gender were released in the sleeping kennels of the dogs on days 7, 14, 21, 28, 35, 42, 49 and 56. Once a dog became infected with *E. canis* and was subsequently rescue-treated, adult *R. sanguineus* ticks from a pathogen-free batch were used for the artificial infestations (on Days 49 and 56) for a comparison of the acaricidal efficacy between groups. Additionally, the pens of the dogs were inspected daily from Day 14 onwards for engorged detached ticks. These ticks were collected and preserved in 70 % ethanol from each individual animal.

**Tick counts on dogs**

In the *Babesia* study, *in situ* thumb counts were performed approximately 48 h after each tick challenge (Days 9, 16 and 23). Ticks were removed only 48 h after the last infestation (Day 30). In the *Ehrlichia* study, ticks were counted approximately 48 h post-application on the dogs without removing them (Days 9, 16, 23, 30, 37, 44, and 51). All ticks were removed on the final assessment day (Day 58). During the *in situ* thumb counts, sexes were not distinguished. The ticks counted and removed on Day 58 were categorized within sex (male/female) as free or attached and dead or alive following the recommendations recently updated by the WAAVP [12].

**Methods for calculating the acaricidal efficacy**

Efficacy against ticks was calculated from the total count of live ticks counted on the dogs 48 h after each infestation, or removed. Efficacy calculations based on arithmetic and geometric means of the tick counts was calculated using Abbott’s formula: Efficacy (%) = 100 × (C – T) / C, whereby: C = Mean live tick count on the control group; T = Mean live tick count on the treated group. Statistical analysis were carried out using the chi-square test or Fisher’s exact test as applicable using software package SAS * version 9.3. The level of significance of the tests was set at 5 %.

**Monitoring of Babesia and Ehrlichia infections**

*Babesia*

Scheduled clinical examinations were conducted on Days −7, 7, 14, 21, 28, 35, 42, 49 and 56. The clinical examination included general appearance, respiration rate, heart rate and body temperature. Additional examinations were conducted on all dogs displaying clinical signs associated with babesiosis, which included fever, depression, anorexia, lethargy, anaemia, haematuria and icterus. Blood smears were prepared from dogs displaying abnormally high body temperatures (>39.4 °C) and examined for *B. canis* infection in erythrocytes. Treatment for babesiosis consisted of 1 ml/20 kg body weight diminazene followed by 1.2 ml/kg imidocarb dipropionate 24 h later.

Blood was collected for PCR analysis from all dogs prior to the start of the study and on Days 14, 21, 28, 35, 42 and 56 and on any dog at the time of diagnosis with babesiosis prior to rescue treatment. Blood was also collected for serology on the same days as for PCR analyses, and additionally on Day 7 prior to the tick challenge. EDTA blood samples collected for PCR analysis were collected in EDTA tubes and total genomic DNA extracted using a commercial kit. A fragment of approximately 300 bp from the 18S internal transcribed spacer-1 gene of *Babesia* was PCR amplified using methods originally published by Duarte et al. [24] and subsequently modified by Beugnet et al. [19]. Positive, negative, no template as well as internal amplification controls were included in each run.

For serology, serum samples were examined for the presence of *B. canis*-specific antibodies using IFA according to the instructions of the manufacturer (MegaCor Diagnostik, Austria).
**Ehrlichia**

Infection with *E. canis* was monitored by rectal temperature records, clinical examinations and platelet counts, as well as by testing blood samples by PCR and IFA. Dogs displaying clinical signs usually based on an elevated body temperature >39.4 °C for 2 consecutive days received appropriate concomitant treatment with doxycycline and dexamethasone. EDTA blood samples were collected for PCR from all dogs prior to the first infestation and on Days 21, 28, 35, 42, 49, 56, 63, 70, 77 and 84, as well as any dogs that developed fever.

For PCR analysis of *E. canis*, a specific primer set was used for amplification of a fragment of the dsb gene (ECAdsbF: 5′- GCAAGTGGGGCAGAGAATGAAG-3′; ECAdsbR: 5′- GTATCCCTACTATGATAGGCAGG AGTGC-3′). The amplified product was subjected to agarose gel electrophoresis for confirmation. Up to 400 ng isolated DNA served as template for PCR amplification of the target region in a 20 µl reaction volume using Phire HotStart II DNA polymerase. A PCR product of approximately 500 bp confirmed the presence of the *E. canis* dsb target region in the sample. Positive, negative, no template as well as internal amplification controls were included in each run.

For serology, serum was recovered and frozen at ~20 °C until assayed for *E. canis* antibodies using an IFA assay for the detection of specific *E. canis* antibodies using a commercial test kit (MegaCor Diagnostik, Austria). An additional serum sample was collected three weeks after the last scheduled serum collection on Day 84, to confirm the results for dog 4FA 06A that had not sero-converted by the end of study.

**Methods for calculating the Babesia/Ehrlichia blocking efficacy**

An efficacy failure (successfully infected with *Babesia*) was defined as a dog in the treatment group that tested serologically positive for *B. canis* antibodies or positive for *B. canis* DNA by PCR analysis. An efficacy failure (successfully infected with *Ehrlichia*) was defined as a dog in the treatment group that tested serologically positive for *E. canis* antibodies or tested positive for *E. canis* DNA by PCR analysis. Any treated dog that met either one of the above criteria was considered infected. Percentage blocking efficacy for the treatment group was calculated as follows: Efficacy (%) = 100 × (Tc − Ti)/Tc, whereby Tc = Total number of infected dogs in the negative control group, and, Ti = Total number of infected dogs in the treatment group.

As proposed recently by Navarro et al. 2015 [25], the percentage of protection may also be calculated in comparison to the number of infective challenges, and not in relation to the number of infected dogs in the control groups.

Protection (%) = 100 (IcC − IcT)/IcC whereby IcC is the number of infective tick challenges conducted in the control group that lead to positive infection and IcT the number of infective tick challenges in the treated dogs that lead to infection. This % of protection provides a better view of the risk reduction provided by the treatment to dogs that will face infected tick challenges.

**Results**

In general, clinical signs, fever and reduced platelet counts, observed in dogs enrolled in the studies could be linked to the tick-transmitted *Babesia* or *Ehrlichia* infections, and there were no adverse reactions noted in response to the treatment.

**Efficacy on ticks**

The acaridical efficacy of Frontline Tri-Act®/Frontect® against *D. reticulatus* and *R. sanguineus* ticks is summarised in Table 1 and Table 2, respectively. The efficacy against *D. reticulatus* ticks was 98.3 % on Day 9 and increased to 100 % on Day 16 (Table 1). Any dog that tested positive for *B. canis* parasites in stained blood smears was not challenged any further with infected *Dermacentor* ticks. As a result, meaningful statistical comparison was limited to Days 9 and 16 (Table 1).

Live *R. sanguineus* ticks were only found on Day 9 with a corresponding efficacy of 99.0 % for the treatment group (Table 2). The control group carried statistically (*p < 0.05*) more ticks compared to the treated group on all assessment

### Table 1: Acaridical efficacy based on geometric and arithmetic means against *Dermacentor reticulatus* ticks

| Day   | Geometric means          | Arithmetic means          |
|-------|--------------------------|---------------------------|
|       | Control group            | Treated group             |                  |
| Mean  | Mean (Efficacy %)        | P-value                   | Mean  | Mean (Efficacy %) | P-value |
| Day 9 | 23.1                     | 0.4 (98.3 %)              | <.0001 | 24.5             | 0.9 (96.4 %) |
| Day 16| 30.6                     | 0.0 (100.0 %)             | <.0001 | 32.7             | 0.0 (100.0 %) |
| Day 23| NA                       | 0.5                       |        | 1.4              |        |
| Day 30| NA                       | 0.3                       |        | 0.6              |        |

*P*-value: One-way ANOVA test

Dogs were treated once on day 0

NA Not Applicable, dogs removed from the study after babesiosis diagnosis
days with a group average of 5.6 to 20.9 (Table 2). After Day 9, no more live ticks could be found on the treated dogs on any of the assessment days (i.e. 100 % efficacy).

**Babesia canis blocking efficacy**

The infection rate of ticks used for infestation on day 7 was 2 %, whereas those used on Days 21 and 28 carried an infection rate of 10 and 8 %, respectively.

Blood smears were prepared and examined for the presence of *B. canis* for all dogs from Day 14 onwards when pyrexia (>39.4 °C) was present. *B. canis* was observed in blood smears of all control dogs on at least one occasion (Table 3). By Day 28, all the dogs in the control group were positive for *Babesia*, and therefore for those animals tick challenges were discontinued. For all treated dogs, tick challenges were continued up to Day 28, except for dog B2A 234 from the treatment group, confirmed positive on Day 22.

Blood smear examination was followed up by PCR and IFA analysis. All eight untreated dogs were confirmed positive by PCR on the same day as their positive blood smear (Table 4). By Day 28 all untreated dogs had seroconverted and displayed specific *B. canis* antibodies (Table 5). One of the dogs (B2A 234) in the treated group was found positive for babesiosis based on blood smear examination (Day 22) (Table 3), PCR (Day 21) (Table 4) and IFA (Day 42) (Table 5). Overall the effectiveness of Frontline Tri-Act®/Frontect® in reducing *Babesia* transmission was 87.5 % over the challenge period of 28 days compared to control dogs (P-value: 0.0014). When calculating the protection conferred against infective tick challenges, the percentage of protection was 94.3 % ([8/15 − 1/31]/8/15] = 1 infection in 31 infective challenges in treated dogs compared to eight infections in 15 challenges in control dogs).

**Ehrlichia canis blocking efficacy**

Fifty adult *R. sanguineus* ticks were taken from the batch of ticks used for challenging the dogs and confirmed PCR positive (13 %). In both groups, four dogs were observed with elevated body temperatures (>39.4 °C) (Table 6). Reduced platelet counts (< 200 × 10⁹/l) were observed in five untreated dogs, but also in four of the treated dogs (Table 6). *Ehrlichia canis* infection was confirmed by PCR in all untreated dogs (Table 7) and they also all seroconverted (Table 8). *Ehrlichia* DNA was detected in two treated dogs (4DA C4C and 4FA 06A on Day 70 and Day 77, respectively) (Table 7). However, only one of the PCR positive dogs in the treated group was confirmed by IFA (Table 6). Additional serum samples collected from dog 4FA 06A after Day 84 were also sero-negative. Overall, Frontline Tri-Act®/Frontect® effectively reduced transmission of *E.canis* to dogs by 75 % over the challenge period of 56 days compared to control dogs (P-value: 0.0070). When calculating the protection conferred against infective tick challenges, the percentage of protection was 85.15 % (two infections based on PCR in 64 infective challenges compared to eight infections in 38 challenges in control dogs).

### Discussion

**Acaricidal efficacy**

Topical administration of a combination of fipronil and permethrin onto eight dogs enrolled in each of the clinical studies included in this paper did not induce any adverse reactions. Any clinical signs observed were linked to either *B. canis* infection or to *E.canis* infection.

The advantage of combining 6.76 % fipronil and 50.48 % permethrin is their different mode of action. Permethrin has a pronounced repellency effect related to irritant effect by contact, and then is followed by a killing effect. Fipronil
induces a progressive onset of tick mortality [23]. “Synergistic” effects, or at least additive effects, by combining both topical compounds into a single formulation, as discovered for the combination of fipronil and amitraz [26], have not been reported but are probable.

### Speed of transmission
Pathogen transmission depends on the duration of attachment required by ticks to transmit specific pathogens such as *B. canis* and *E. canis*. In general, protozoan *Babesia* parasites require several days (36 to 72 h) for transmission.

### Table 3 Rectal temperature records and detection of *Babesia canis* in blood smears from dogs challenged with infected *Dermacentor* ticks

| Group   | Animal ID | Body temp range (°C) | Blood smear preparation and examination day |
|---------|-----------|-----------------------|---------------------------------------------|
|         |           | Min | Max          | 14  | 15  | 16  | 17  | 20  | 21  | 22  | 28  | 35  | 42  | 49  | 56  |
| Control | 4F3 1A0   | 37.7 | 40.3 | -    | POS | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   |
|         | B25 46D   | 37.9 | 39.1 | POS  | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   |
|         | B29 74B   | 38.2 | 39.7 | ND   | POS | -   | -   | ND  | -   | ND  | ND  | ND  | ND  | ND  | ND  |
|         | B2C 449   | 37.8 | 38.8 | -    | -   | -   | -   | -   | POS | -   | -   | -   | -   | -   | -   |
|         | C00 CE3   | 37.6 | 39.6 | ND   | ND  | POS | -   | -   | ND  | -   | ND  | ND  | ND  | ND  | ND  |
|         | C22 25E   | 37.6 | 39.2 | -    | -   | -   | POS | -   | -   | -   | -   | -   | -   | -   | -   |
|         | C22 726   | 37.5 | 40.1 | ND   | POS | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   |
|         | E9E 126   | 37.9 | 39.8 | POS  | ND  | -   | -   | -   | ND  | ND  | ND  | ND  | ND  | ND  | ND  |
| Treated | B2A 234   | 37.7 | 40.5 | ND   | -   | -   | -   | ND  | ND  | POS | ND  | ND  | ND  | ND  | ND  |
|         | B2B 68D   | 38.4 | 39.2 | -    | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   |
|         | D6F 46F   | 37.8 | 38.8 | -    | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   |
|         | D6F 576   | 38.0 | 39.0 | ND   | -   | -   | -   | -   | ND  | ND  | ND  | ND  | ND  | ND  | ND  |
|         | D6F 725   | 37.4 | 39.1 | ND   | -   | -   | -   | -   | -   | ND  | ND  | ND  | ND  | ND  | ND  |
|         | D7F 38    | 37.6 | 38.4 | -    | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   |
|         | E15 564   | 37.5 | 38.4 | -    | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   |
|         | E46 966   | 37.3 | 39.2 | ND   | -   | -   | -   | -   | -   | ND  | ND  | ND  | ND  | ND  | ND  |

**ND** not detected, **POS** positive, - = no blood smear prepared

### Table 4 Detection of *Babesia canis* DNA using a PCR assay in dogs challenged with infected *Dermacentor* ticks

| Animal ID | DAY | 14 | 15 | 16 | 17 | 20 | 21 | 22 | 28 | 35 | 42 | 49 | 56 |
|-----------|-----|----|----|----|----|----|----|----|----|----|----|----|----|
| Control   | 4F3 1A0 | -  | POS | -  | -  | -  | ND | -  | -  | -  | -  | -  | -  | -  |
|           | B25 46D | POS | -  | -  | -  | -  | ND | -  | -  | -  | -  | -  | -  | -  |
|           | B29 74B | -  | POS | -  | -  | -  | ND | -  | -  | -  | -  | -  | -  | -  |
|           | B2C 449 | -  | -  | -  | POS | -  | -  | -  | -  | -  | -  | -  | -  | -  |
|           | C00 CE3 | -  | POS | -  | -  | ND | -  | -  | -  | -  | -  | -  | -  | -  |
|           | C22 25E | -  | -  | POS | -  | ND | -  | -  | -  | -  | -  | -  | -  | -  |
|           | C22 726 | -  | POS | -  | -  | ND | -  | -  | -  | -  | -  | -  | -  | -  |
|           | E9E 126 | POS | -  | -  | -  | ND | -  | -  | -  | -  | -  | -  | -  | -  |
| Treated   | B2A 234 | -  | -  | -  | -  | POS | POS | -  | -  | -  | -  | -  | -  | -  |
|           | B2B 68D | -  | -  | -  | ND  | ND  | ND  | ND  | ND  | ND  | -  | -  | -  | -  |
|           | D6F 46F | -  | -  | -  | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND  |
|           | D6F 576 | -  | -  | -  | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND  |
|           | D6F 875 | -  | -  | -  | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND  |
|           | D7F 38  | -  | -  | -  | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND  |
|           | E15 564 | -  | -  | -  | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND  |
|           | E46 966 | -  | -  | -  | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND  |

**POS** Positive, **ND** Not detected; - = No sample tested
their sporoblasts to mature into infective sporozoites within the tick’s salivary glands before they can be transmitted [27]. Bacterial pathogens, such as *Anaplasma phagocytophilum*, require 24 to 36 h to be transmitted by nymphal *Ixodes scapularis* ticks. [28, 29]. In a recent study, the time that an infected *R. sanguineus* tick had to be attached before it could transmit *E. canis* was determined in vivo as well as in vitro [30]. The study revealed that transmission of *E. canis* starts within a few hours (3 h on dogs and 8 h on artificial membranes), an interval considerably shorter than presumed previously. These findings highlight the need for further research.

Table 5 Detection of *Babesia canis* antibodies by Indirect Fluorescent Antibody assay in dogs challenged with infected *Dermacentor* ticks

| Animal ID | DAY Pre-infestation Day -7 | 7 | 21 | 28 | 35 | 42 | 49 | 56 |
|-----------|---------------------------|---|----|----|----|----|----|----|
| Control   |                           |   |    |    |    |    |    |    |
| 4F3 1A0   | NEG                       | NEG POS | - | - | POS | POS | POS |
| B2S 46D   | NEG                       | NEG POS | - | - | POS | POS | POS |
| B29 74B   | NEG                       | NEG NEG POS | - | POS | POS | POS |
| B2C 449   | NEG                       | NEG NEG POS | - | POS | POS | POS |
| C0 CE3    | NEG                       | NEG NEG POS | - | POS | POS | POS |
| CC2 25E   | NEG                       | NEG POS | - | - | POS | POS | POS |
| CC2 726   | NEG                       | NEG POS | - | - | POS | POS | POS |
| E9E 126   | NEG                       | NEG NEG POS | - | POS | POS | POS |
| Treated   |                           |   |    |    |    |    |    |    |
| B2A 234   | NEG                       | NEG NEG NEG | NEG | NEG | NEG | POS | POS |
| B2B 68D   | NEG                       | NEG NEG NEG | NEG | NEG | NEG | NEG | NEG |
| DF6 4EF   | NEG                       | NEG NEG NEG | NEG | NEG | NEG | NEG | NEG |
| DF6 576   | NEG                       | NEG NEG NEG | NEG | NEG | NEG | NEG | NEG |
| DF6 725   | NEG                       | NEG NEG NEG | NEG | NEG | NEG | NEG | NEG |
| DF7 D38   | NEG                       | NEG NEG NEG | NEG | NEG | NEG | NEG | NEG |
| E15 564   | NEG                       | NEG NEG NEG | NEG | NEG | NEG | NEG | NEG |
| E46 966   | NEG                       | NEG NEG NEG | NEG | NEG | NEG | NEG | NEG |

POS Positive, NEG Negative; - = No sample tested

Table 6 Rectal temperature records and platelet counts in dogs challenged by *Ehrlichia canis*-infected *Rhipicephalus* ticks

| Animal ID | Body temp range (°C) | Platelet count and examination Day |
|-----------|----------------------|-----------------------------------|
|           | Min | Max | -6 | 21 | 28 | 35 | 36 | 42 | 49 | 56 | 63 | 70 | 77 | 84 |
| Control   |     |     |    |    |    |    |    |    |    |    |    |    |    |    |    |
| CC5 CDA   | 37.5 | 39.7 | 285 | 275 | 292 | 284 | - | 221 | - | - | - | - | - | - | - |
| CD6 3F9   | 37.5 | 38.8 | 242 | 218 | 189 | - | - | - | - | - | - | - | - | - |
| EA1 FF0   | 37.7 | 39.8 | 245 | 171 | - | 246 | - | - | - | - | - | - | - | - |
| 4F1 4AF   | 38.1 | 40.1 | 494 | 415 | 519 | 449 | - | 459 | 383 | 388 | 274 | - | - | - | - |
| 4F0 57A   | 37.8 | 39.4 | 253 | 282 | 279 | 197 | - | - | - | - | - | - | - | - | - |
| 4F6 87C   | 38.2 | 39.8 | 333 | 327 | 194 | 179 | - | - | - | - | - | - | - | - | - |
| 286 FFE   | 38.4 | 39.4 | 485 | 478 | 512 | 505 | - | 495 | 406 | 143 | - | - | - | - | - |
| 964 441   | 37.8 | 39.1 | 377 | 349 | 359 | 353 | - | 269 | - | - | - | - | - | - | - |
| Treated   |     |     |    |    |    |    |    |    |    |    |    |    |    |    |    |
| CBD 700   | 37.3 | 38.6 | 213 | 238 | 216 | - | 209 | 221 | 210 | 193 | 208 | 213 | 185 | 193 |
| B2C 3F0   | 38.2 | 39.6 | 261 | 192 | 192 | 169 | - | 153 | 181 | 198 | 216 | 170 | 204 | 180 |
| 28A 3C2   | 38.1 | 39.8 | 363 | 343 | 307 | 380 | - | 370 | 348 | 351 | 376 | 349 | 325 | 308 |
| E18 F40   | 38.0 | 39.2 | 480 | 228 | 304 | 250 | - | 299 | 280 | 280 | 288 | 312 | 275 | 277 |
| 4F0 890   | 38.2 | 39.6 | 347 | 367 | 332 | 330 | - | 372 | 341 | 204 | 378 | 349 | 339 | 332 |
| 4DA C4C   | 38.2 | 39.2 | 425 | 385 | 402 | 360 | - | 299 | 320 | 291 | 323 | 163 | - | - |
| DF7 4DB   | 37.7 | 39.1 | 248 | 273 | 268 | 255 | - | 244 | 248 | 259 | 314 | 252 | 265 | 244 |
| 4FA 06A   | 37.6 | 39.7 | 249 | 281 | 256 | 250 | - | 244 | 269 | 235 | 181 | 228 | 202 | - |

Normal range for platelet count is between $200 \times 10^9/l$ and $500 \times 10^9/l$ (below normal range indicated in bold)
- = no platelets were counted
concerning the actual speed of transmission of tick-borne pathogens.

As a result, the preventive efficacy of ecto-parasiticides with respect to blocking pathogen transmission has become an important issue in advice from veterinarians towards pet owners.

**Table 7** Detection of *Ehrlichia canis* DNA using a PCR assay in dogs challenged with infected *Rhipicephalus* ticks

| Animal ID | Day-6 | 21 | 28 | 35 | 42 | 49 | 56 | 63 | 70 | 77 | 84 |
|-----------|-------|----|----|----|----|----|----|----|----|----|----|
| Control   |       |    |    |    |    |    |    |    |    |    |    |
| CC5 CDA   | ND    | ND | ND | ND | POS| -  | -  | -  | -  | -  | -  |
| CD6 3F9   | ND    | ND | ND | POS| -  | -  | -  | -  | -  | -  | -  |
| EA1 FF0   | ND    | POS| -  | ND | -  | -  | -  | -  | -  | -  | -  |
| 4F1 4AF   | ND    | ND | ND | ND | ND | ND | ND | POS| -  | -  | -  |
| 4F0 57A   | ND    | ND | ND | POS| -  | -  | -  | -  | -  | -  | -  |
| 4F6 87C   | ND    | ND | POS| POS| -  | -  | -  | -  | -  | -  | -  |
| 286 FFE   | ND    | ND | ND | ND | ND | ND | POS| -  | -  | -  | -  |
| 964 441   | ND    | ND | ND | ND | ND | POS| -  | -  | -  | -  | -  |

**Treated**

| CBD 700   | ND    | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| B2C 3F0   | ND    | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 28A 3C2   | ND    | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| E18 F40   | ND    | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 4 F0 890  | ND    | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 4DA C4C   | ND    | ND | ND | ND | ND | ND | ND | POS| -  | -  | -  |
| DF7 4DB   | ND    | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 4FA 06A   | ND    | ND | ND | ND | ND | ND | ND | ND | POS| -  | -  |

POS Positive, ND Not detected; - = Not tested

**Table 8** Detection of *Ehrlichia canis* antibodies by Indirect Fluorescent Antibody assay in dogs challenged with infected *Rhipicephalus* ticks

| Animal ID | DAY | 7  | 21 | 28 | 35 | 42 | 49 | 56 | 63 | 70 | 77 | 84 |
|-----------|-----|----|----|----|----|----|----|----|----|----|----|----|
| Control   |     |    |    |    |    |    |    |    |    |    |    |    |
| CC5 CDA   | NEG | NEG| NEG| NEG| POS| POS| POS| POS| POS| POS| POS| POS|
| CD6 3F9   | NEG | NEG| NEG| POS| POS| POS| POS| POS| POS| POS| POS| POS|
| EA1 FF0   | NEG | POS| NEG| NEG| POS| POS| POS| POS| POS| POS| POS| POS|
| 4F1 4AF   | NEG | NEG| NEG| NEG| NEG| NEG| NEG| NEG| POS| POS| POS| POS|
| 4F0 57A   | NEG | NEG| NEG| POS| POS| POS| POS| POS| POS| POS| POS| POS|
| 4F6 87C   | NEG | NEG| NEG| POS| POS| POS| POS| POS| POS| POS| POS| POS|
| 286 FFE   | NEG | NEG| NEG| NEG| NEG| POS| POS| POS| POS| POS| POS| POS|
| 964 441   | NEG | NEG| NEG| POS| POS| POS| POS| POS| POS| POS| POS| POS|

**Treated**

| CBD 700   | NEG | NEG| NEG| NEG| NEG| NEG| NEG| NEG| NEG| NEG| NEG| NEG |
| B2C 3F0   | NEG | NEG| NEG| NEG| NEG| NEG| NEG| NEG| NEG| NEG| NEG| NEG |
| 28A 3C2   | NEG | NEG| NEG| NEG| NEG| NEG| NEG| NEG| NEG| NEG| NEG| NEG |
| E18 F40   | NEG | NEG| NEG| NEG| NEG| NEG| NEG| NEG| NEG| NEG| NEG| NEG |
| 4F0 890   | NEG | NEG| NEG| NEG| NEG| NEG| NEG| NEG| NEG| NEG| NEG| NEG |
| 4DA C4C   | NEG | NEG| NEG| NEG| NEG| NEG| NEG| POS| POS| POS| POS| POS|
| DF7 4DB   | NEG | NEG| NEG| NEG| NEG| NEG| NEG| NEG| NEG| NEG| NEG| NEG |
| 4FA 06A   | NEG | NEG| NEG| NEG| NEG| NEG| NEG| NEG| NEG| NEG| NEG| NEG |

POS Positive, NEG Negative

**Transmission blocking efficacy**

The blocking capacity of various acaricidal compounds against infected *D. reticulatus* ticks has been evaluated in a series of clinical laboratory studies [10, 16, 19, 20, 25]. For instance, the ability to block transmission of *B. canis* by *D. reticulatus* to dogs was recently demonstrated for...
afloxolane [19] as well as for fluralaner [20]. Moreover, blocking of the transmission of E. canis has also been evaluated in a number of similar studies with other acaricidal molecules [11, 14, 15].

Recently, prevention of B. canis by a fixed combination of permethrin and fipronil (Effitix®) using the blocking model with infected D. reticulatus ticks was reported [25].

When calculating the protection conferred against Babesia-infected tick challenges, the percentage of protection was 93.4% (one infection in 31 infective challenges versus eight infective challenges in 15 in control dogs). Likewise, the percentage of protection against Ehrlichia-infected tick challenges was 85.15% (two infections based on PCR in 64 infective challenges compared to eight infections in 38 challenges in control dogs). This approach allowed for a more realistic estimate of the repeated tick challenge of the dogs without the need for additional dogs.

Another interesting issue is the definition of an efficacy failure or success when executing blocking models. Per definition transmission blocking implies the prevention of any babesial sporozoites or ehrlichial organisms from passing from the tick vector to the host. A dog that has sero-converted and/or tested positive for B. canis or E. canis DNA by PCR is therefore regarded as an efficacy failure, irrespective of any clinical disease manifestation [13]. However, it can also be argued that successful transmission of a pathogen should result in clinical disease. It is possible that a dog that sero-converted or tested positive by PCR did not develop any clinical signs due to insufficient challenge. An acaricidal product can potentially disrupt the feeding process sufficiently to prevent transmission of a viable infection load of either B. canis or E. canis. In that case, prevention of disease transmission should be calculated in regard to the number of dogs developing clinical signs and confirmed by either PCR or serology. Nevertheless, it is the opinion of the authors that the definition of a successfully infected B. canis dog was used by Navarro et al. [25], stating that infected dogs must be PCR positive and seropositive is not acceptable. We consider that in terms of infection, PCR is a proof that the pathogen has been inoculated, as well as seropositivity. Therefore, it is our opinion that one or the other should be regarded as an efficacy failure [10].

Another improvement of the protocol for these models includes the way ticks are brought into contact with the dogs. R. sanguineus ticks were placed in the dog’s kennel [11], whereas D. reticulatus was placed directly onto the dogs. This is considered in line with differences in host seeking behaviour of both tick species. Moreover, re-infestation with non-infected ticks after a dog has become positive was introduced in the protocol of the Ehrlichia study, which resulted in a meaningful statistical comparison between groups throughout the study (Table 2).

In the Ehrlichia study, dogs were monitored for thrombocytopenia by determining platelet counts in non-infected dogs ranging between 200 × 10^9/l and 500 × 10^9/l. Platelet counts below 200 × 10^9/l were detected in both groups as different time points and did not correlate with an elevated body temperature (Table 6). In fact, in the control group there were only two dogs (EA1 FF0 and 4F6 87C) with fever and low platelet counts, whereas in the treated group there were also two dogs (B2C 3F0 and 4FA 06A) with fever and lower platelet values (Table 6). Clearly, seroconversion and PCR positivity are better criteria than platelet counts. Nevertheless, thrombocytopenia is a characteristic of monocytic ehrlichiosis, but differs between individual dogs and between time points collected from the same dogs [31].

In these studies, dogs were challenged with either D. reticulatus ticks with Babesia infection between 2 and 10% with B. canis or with R. sanguineus ticks carrying an Ehrlichia infection rate of around 13%. Tick infection rates in field collections vary between publications and depend upon which publication is cited. However, the challenge load in both models appears fairly realistic when compared to an E. canis incidence risk in dogs in southern Europe of 11% [32] and with an infection rate in D. reticulatus field ticks of 1.64% recently determined in the Netherlands [33].

Conclusions

The findings presented here demonstrate that a combination of 6.76% w/v fipronil and 50.48% w/v permethrin was able to reduce transmission of B. canis as well as E. canis to dogs.

Competing interests

This research was funded by Merial S.A.S., a Sanofi company, France, of which FB is an employee. Frontline®/Frontline Tri-Act® and Certifect® are registered trademarks of Merial. Any references in this article to these trademarks are informative only and not intended for commercial purposes.

Authors’ contributions

FJ, CDV, JIF and FB contributed to the design and protocols of two different blocking studies targeting B. canis and E. canis. CDV carried out the study, whereas CDV and JIF compiled and analysed the data. The results of both studies were integrated into one document by FJ, who also wrote the first draft of the manuscript. This version of the manuscript was subsequently revised and improved by all authors and resulted in the final version, which was approved by all authors.

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Author details

1Utrecht Centre for Tick-borne Diseases (UCTD), FAO Reference Centre for Ticks and Tick-borne Diseases, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584CL, Utrecht, The Netherlands. 2Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort 0110, South Africa. 3ClinVet
International (Pty) Ltd, P.O. Box 11186Universitas, Bloemfontein 9321, Republic of South Africa. *Meriel S.A.S., 29 Av Tony Garnier, 69007 Lyon, France.

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References

1. Jongejan F, Uilenberg G. The global importance of ticks. Parasitology. 2004; 129(Suppl):S53–S66.
2. Irwin PJ. It shouldn’t happen to a dog … or a veterinarian: clinical paradigms for canine vector-borne diseases. Trends Parasitol. 2014;30:104–12.
3. Beugnet F, Marié J-L. Emerging arthropod-borne diseases of companion animals in Europe. Vet Parasitol. 2009;163:298–305.
4. Estrada-Peña A, Farkas R, Jaensen TGT, Koens F, Madder M, Pascucci I, et al. Association of environmental traits with the geographic ranges of ticks (Acari: Ixodidae) of medical and veterinary importance in the western Palearctic: A digital data set. Exp Appl Acarol. 2013;59:351–66.
5. Dautel H, Dippel C, Oehme R, Hartelt K, Schettler E. Evidence for an increased geographical distribution of Dermacentor reticulatus in Germany and detection of Rickettsia sp. Rp484. Int J Med Microbiol. 2006;296(Suppl):149–56.
6. Walker AR, Bouattour A, Camisas J-L, Estrada-Peña A, Horak IG, Latif AA, Pegram RG, Preston PM. Ticks of domestic animals in Africa: a Guide to identification of species. 2003. University of Edinburgh. http://www. alanwalker.co/assets/PDF/tickguide-africa.pdf
7. Marchiondo AA, Holdsworth PA, Green P, Blagburn BL, Jacobs DE. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.). guidelines for evaluating the efficacy of parasiticides for the treatment, prevention and control of flea and tick infestation on dogs and cats. Vet Parasitol. 2007;145:332–44.
8. Davoust B, Marié J-L, Mercier S, Boni M, Vandeweghe A, Parzy D, et al. Assessment of fipronil efficacy to prevent canine monocytic ehrlichiosis in endemic areas. Vet Parasitol. 2003;11291–100.
9. Last RD, Hill JM, Matjila PT, Rème CA. A field trial evaluation of the prophylactic efficacy of amitraz-impregnated collars against canine babesiosis (Babesia canis) in South Africa. J S Afr Vet Assoc. 2007;78:685–5.
10. Jongejan F, Fourie JJ, Chester ST, Manavela C, Mallook Y, Poolmeier MG, et al. The prevention of transmission of Babesia canis by Dermacentor reticulatus ticks to dogs using a novel combination of fipronil, amitraz and (S)-methoprene. Vet Parasitol. 2011;179:343–50.
11. Fourie JJ, Ollagnier C, Beugnet F, Luus HG, Jongejan F. Prevention of transmission of Ehrlichia canis by Rhipicephalus sanguineus ticks to dogs treated with a combination of fipronil, amitraz and (S)-methoprene (CERTIFECT®). Vet Parasitol. 2013;193:223–8.
12. Marchiondo AA, Holdsworth PA, Fourie LJ, Rugg D, Hellmann K, Snyder DE, et al. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) second edition: guidelines for evaluating the efficacy of parasiticides for the treatment, prevention and control of flea and tick infestations on dogs and cats. Vet Parasitol. 2013;194:84–97.
13. Fourie JJ. Integrated Control of Ticks and Fleas on Dogs with Particular Reference to the Prevention of Vector-Borne Diseases. PhD thesis, Utrecht University. 2015; 1-180. ISBN: 978-90-393-6407-9.
14. Fourie JJ, Luus HG, Staneck D, Jongejan F. The efficacy of Advantix® to prevent transmission of Ehrlichia canis to dogs by Rhipicephalus sanguineus ticks. Parasite. 2013;20:36.
15. Staneck D, Fourie JJ. Imidacloprid 10 % / flumethrin 4.5 % collars (Seresto®, Bayer) successfully prevent long-term transmission of Ehrlichia canis by infected Rhipicephalus sanguineus ticks to dogs. Parasitol Res. 2013;112:32–32.
16. Fourie JJ, Staneck D, Jongejan F. Prevention of transmission of Babesia canis by Dermacentor reticulatus ticks to dogs treated with an imidacloprid/ flumethrin collar. Vet Parasitol. 2013;192:273–8.
17. Shoop WL, Hartline EJ, Gould BR, Waddell ME, McDowell RG, Kinney JB, et al. Discovery and mode of action of afloxicin, a new isoxazolyl parasiticide for dogs. Vet Parasitol. 2014;201:179–89.
18. Gassel M, Wolf C, Noack S, Williams H, Jrg T. The novel isoxazolyl ectoparasiticide fluralaner: selective inhibition of arthropod γ-aminobutyric acid- and L-glutamate-gated chloride channels and insecticidal/ acaridical activity. Insect Biochem Mol Biol. 2014;45:111–24.
19. Beugnet F, Halos L, Larsen D, Labuschagné M, Erasmus H, Fourie J. The ability of an oral formulation of afloxicin to block the transmission of Babesia canis by Dermacentor reticulatus ticks to dogs. Parasite Vectors. 2014;7:283.
20. Taender J, Liebenberg J, Roepke RKA, Heckeroth AR. Prevention of transmission of Babesia canis by Dermacentor reticulatus ticks to dogs treated orally with fluralaner chewable tablets (Bravecto®). Parasite Vectors. 2015;8:305.
21. Dumont P, Fourie JJ, Soll M, Beugnet F. Repellency, prevention of attachment and acaricidal efficacy of a new combination of fipronil and permethrin against the main vector of canine babesiosis in Europe, Dermacentor reticulatus ticks. Parasite Vectors. 2015;8:50.
22. Dumont P, Chester TS, Gale B, Soll M, Fourie JJ, Beugnet F. Acaricidal efficacy of a new combination of fipronil and permethrin against Ixodes ricinus and Rhipicephalus sanguineus ticks. Parasite Vectors. 2015;8:51.
23. Beugnet F, Franc M. Insecticidal and acaricidal molecules and/or combinations to prevent pet infestation by ectoparasites. Trends Parasitol. 2012;28:267–79.
24. Duarte SC, Linhares GFC, Romanovsky TN, da Silveira Neto OJ, Borges LMF. Assessment of primers designed for the subspecies-specific discrimination among Babesia canis canis, Babesia canis vogeli and Babesia canis rossi by PCR assay. Vet Parasitol. 2008;152:16–20.
25. Navarro C, Reynold N, Fourie J, Hellmann K, Bonneau S. Prevention of Babesia canis in dogs: efficacy of a fixed combination of permethrin and fipronil (Effitix®) using an experimental transmission blocking model with infected Dermacentor reticulatus ticks. Parasite Vectors. 2015;8:32.
26. Prullage JB, Cawthorne WJ, Le Hir de Fallis LP, Timmons PR. Synergy between fipronil and amitraz in a rhipicephaline sanguineus tick residual contact test. Exp Appl Acarol. 2011;54:173–6.
27. Pesman J, Spielman A. Human babesiosis on Nantucket Island: prevalence of Babesia microti in ticks. Am J Trop Med Hyg. 1980;29:742–6.
28. Des Vignes F, Pesman J, Heffernan R, Schulze TL, Stafford KC, Fish D. Effect of tick removal on transmission of Borrelia burgdorferi and Ehrlichia phagocytophilia by ixodes scapularis nymphs. J Infect Dis. 2001;183:773–8.
29. Katawolos P, Armstrong PM, Dawson JE, Telford SR. Duration of tick attachment required for transmission of granulocytic ehrlichiosis. J Infect Dis. 1998;177:1422–5.
30. Fourie JJ, Staneck D, Luus HG, Beugnet F, Wijnveld M, Jongejan F. Transmission of Ehrlichia canis by Rhipicephalus sanguineus ticks feeding on dogs and on artificial membranes. Vet Parasitol. 2013;197:595–603.
31. Fourie JJ, Horak I, Crafford D, Erasmus HL, Botha OJ. The efficacy of a generic doxycycline tablet in the treatment of canine monocytic ehrlichiosis. J S Afr Vet Assoc. 2013;84:E10.
32. René-Martellet M, Lebert I, Chêne J, Massot R, Leon M, Leal A, et al. Diagnosis and incidence risk of clinical canine monocytic ehrlichiosis under field conditions in Southern Europe. Parasite Vectors. 2015;8:3.
33. Jongejan F, Ringenier M, Putting M, Berger L, Burgers S, Konteka R, et al. Novel foci of Dermacentor reticulatus ticks infected with Babesia canis and Babesia caballi in the Netherlands and in Belgium. Parasite Vectors. 2015;8:232.