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Prevention of transmission of *Ehrlichia canis* by *Rhipicephalus sanguineus* ticks to dogs treated with a combination of fipronil, amitraz and (S)-methoprene (CERTIFECT®)

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ABSTRACT

The ability of CERTIFECT® (a combination of fipronil, amitraz and (S)-methoprene) to prevent transmission of *Ehrlichia canis* was studied in two groups of eight dogs. One group was treated with CERTIFECT while the other group remained untreated. All dogs were exposed to *E. canis*-infected *Rhipicephalus sanguineus* ticks on Days 7, 14, 21 and again on day 28 post-treatment by releasing ticks into the kennels of the dogs to simulate the natural way of infestation. Animals were examined *in situ* for ticks on Days 9, 16 and 23 and any ticks present were counted and removed on Day 30. The efficacy of CERTIFECT against *R. sanguineus* was 100%, since no ticks were found on the treated dogs at any time. Clinical examinations (including monitoring body temperature and blood collections for PCR analysis and serology) were performed at intervals throughout the study until Day 56. Five out of 8 untreated control dogs (62.5%) became infected with *E. canis*, as demonstrated by detection of specific *E. canis* antibodies and the presence of *E. canis* DNA in blood samples by PCR. These dogs displayed fever and thrombocytopenia and were rescue-treated with doxycline. None of the 8 dogs treated with CERTIFECT became infected with *E. canis*, in comparison to the 5/8 control dogs, as confirmed by the lack of specific antibodies and absence of any ehrlichial DNA in blood samples by PCR. CERTIFECT prevented transmission of *E. canis* and effectively provided protection against monocytic ehrlichiose for at least 4 weeks post treatment.

1. Introduction

Globally, dogs are exposed to a broad range of protozoan and bacterial pathogens transmitted through the bite of infected vector ticks (Chomel, 2011). In particular, canine babesiosis (*Babesia* spp.), granulocytic anaplasmosis (*Anaplasma phagocytophilum*), Lyme Disease (*Borrelia burgdorferi*) and canine monocytic ehrlichiosis (*Ehrlichia canis*) are the most common tick-borne diseases of dogs (Shaw et al., 2001; Jongejan and Uilenberg, 2004).

Relatively little research has been carried out to determine the ability of tick control products to prevent the transmission of tick-borne diseases to dogs. Most published studies are aimed at demonstrating the acaricidal product's efficacy against the range of ixodid tick species. Guidelines for evaluating the efficacy of ectoparasiticides on dogs and cats focus on treatment, prevention and

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control of ticks only (EMA, 2007; Marchiondo et al., 2007). Hence, the development of models wherein the blocking of pathogen transmission can be demonstrated is a relatively new area of research. Studies that have been conducted suggest that topically applied tick control compounds can aid in the prevention of the transmission of specific tick-borne pathogens. For instance, the ability of fipronil to prevent transmission of *B. burgdorferi* by field-collected *Ixodes scapularis* ticks was reported several years ago by Jacobson et al. (2004).

Recently, CERTIFECT® (Merial, GA, USA), a combination of fipronil, amitraz and (S)-methoprene in a spot-on formulation. It has been published that fipronil and amitraz act synergistically in this formulation, providing a high speed of kill but also a good prevention of tick attachment (Pfister, 2011; Prullage et al., 2011). These two properties may explain a good indirect prevention of pathogen transmission by the ticks. This formulation was therefore shown to protect dogs from *B. burgdorferi* and *A. phagocytophilum* infections transmitted by field-collected *I. scapularis* ticks (Pfister, 2011; McCall et al., 2011). Moreover, a laboratory transmission blocking model was developed recently to evaluate the ability of topical formulations to prevent *Babesia canis* transmission in dogs. In this model, treatment of dogs with CERTIFECT applied up to 28 days prior to infestation with adult *Dermacentor reticulatus* ticks infected with *B. canis* successfully prevented the development of clinical signs of canine babesiosis (Jongejan et al., 2011).

This paper focuses on the prevention of canine monocytic ehrlichiosis, which is caused by the rickettsial pathogen *E. canis*, is of worldwide importance, and originally reported from Algeria (Donatien and Lestoquard, 1935). *E. canis* is transmitted transstadially and intrastadially by the brown dog tick, *Rhipicephalus sanguineus* (Bremer et al., 2005). The majority of cases of monocytic ehrlichiose are seen in sub-tropical regions hospitable to *R. sanguineus* ticks. Due to the host preference of *R. sanguineus*, dogs serve both as a reservoir and a domestic animal host for *E. canis* (Stich et al., 2008). In dogs, *E. canis* develops in monocytes and macrophages, whereas in ticks the infection can be localized in the midgut and salivary glands. Monocytic ehrlichiosis is characterized by thrombocytopenia, leukopenia, fever, depression and bleeding tendencies (Harrus et al., 1999; de Castro et al., 2004).

Here, we tested the ability of a spot-on formulation combining fipronil, amitraz and (S)-methoprene, to prevent transmission of *E. canis* to dogs exposed to infected *R. sanguineus* ticks.

2. Material and methods

2.1. Study design

This study was carried out according to the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products Guideline: Good Clinical Practice (EMA, 2000) and in compliance with appropriate animal welfare requirements. The study employed a controlled, blinded, randomized block design with 6 males and 10 females fully grown, healthy, Beagle dogs. All dogs were managed similarly in individually tick-proof kennels, and, throughout the study, they were observed twice daily for health abnormalities. When health abnormalities were observed between the scheduled physical examinations, additional full examinations were conducted. The dogs, all negative for *E. canis*, prior to treatment and/or first tick challenge, as shown by the absence of specific antibodies in the indirect fluorescent assay (IFA), were divided randomly into two equal groups. Group 1 was designated the untreated controls and Group 2 dogs were treated once with the combination of fipronil, amitraz and (S)-methoprene spot-on on Day 0.

CERTIFECT was topically applied on each treated dog in two spots, as per the label 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. The pipette size was based on the weight of the dogs as per product labelling.

2.2. Infection of ticks by E. canis

A laboratory-bred *R. sanguineus* strain of French origin was used as a source to create an *Ehrlichia* infected population and subsequently to do the artificial challenges in a kennel environment. The ticks had been maintained for several generations under laboratory conditions. The immature and adult stages of the ticks in the breeding program were fed on rabbits, not previously treated with any acaricides. *E. canis* was isolated from a local case of canine monocytic ehrlichiosis (Bloemfontein, South Africa) by subinoculation of blood into a susceptible laboratory-bred Beagle dog. *R. sanguineus* nymphs fed on this recipient dog acquired the infection, and, after moulting, the adult ticks were used as a basis for the current study. The identity of *E. canis* was confirmed by comparing the partial sequence of the p36 gene of *E. canis* with that of a number of other *E. canis* isolates, whereby the South African isolate appeared closely related and formed a clade with several Asian isolates (Fourie, unpublished data).

Prior to the study a sample of ticks from the challenge batch of infected ticks was tested for *E. canis*, and 38% of ticks were found infected. In addition, a further sample of 50 ticks (25 males and 25 females) from the same batch was pre-tested by feeding on a susceptible naive dog and resulted in transmission of *E. canis*. Hence, a rate of 38% of *R. sanguineus* ticks infected with *E. canis* was considered adequate under the conditions of the model and represented a realistic challenge.

2.3. Infestations by R. sanguineus ticks

On Days 7, 14, 21 and 28, 50 ticks were released into each individual kennel of each dog to simulate the natural exposure to ticks. The potentially *Ehrlichia* infected adult ticks, which were used in the artificial challenges, were unfed, at least one week old, and had a balanced sex ratio of 50% female and 50% male ticks.

2.4. Monitoring of dogs

All dogs were observed daily (from Days −7 to 56) for general health condition and clinical signs or adverse reactions to the treatment. The dogs (both groups) were

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observed approximately every hour for 4 h after initiation of the treatment. Rectal body temperatures were recorded daily from Days 17 to 51 and from Days 54 to 56. All dogs were subjected to a clinical examination on Days −7, 21, 28, 35 42, 49 and 56. Additional clinical examinations were conducted on dogs displaying an abnormally high body temperature (>39.5 °C). Clinical examinations included general appearance, respiration rate, heart rate and body temperature. Particular attention was given to clinical manifestations of monocytic ehrlichiosis, which include fever, depression, anorexia, haemorrhages and epistaxis.

Blood samples for serology, platelet counts and PCR were collected on Days −7, 7, 21, 28, 35, 42, 49 and Day 56. All samples collected, including those taken from dogs with suspected ehrlichiosis (*e.g.*, abnormally low platelet count) and/or dogs that tested positive for *E. canis* antibodies using the IFA, but also asymptomatic dogs from both groups, were PCR assayed (Table 3).

Dogs with abnormally low platelet counts or fever (>39.5 °C) were suspected of starting clinical ehrlichiosis and rescue treated with doxycycline *per os* for 21 days.

2.5. Laboratory assays

Sera were assayed for *E. canis* antibodies using a commercial IFA test (IGG IFA, Fuller Laboratory) performed according to the manufacturer descriptions at the Department of Veterinary Tropical Diseases (DVTD), Faculty of Veterinary Science, University of Pretoria, South Africa. EDTA blood samples for platelet counts were examined at Pathcare Veterinary Laboratory, Bloemfontein, South Africa. In addition, DNA was extracted from EDTA blood samples using a commercial genomic DNA isolation kit. Isolated DNA was used as a template for a PCR that employed sequence specific primers for the detection of *E. canis* target DNA. Primer pair ECAdsbF (5′-GCAAGTGCGGGCAGAGAATGAAG-3′) and ECAdsbR (5′-GTATCCCCTACTATGATAGCAGGAGTGC-3′) was used to amplify the disulphide oxidoreductase gene (AF403710). PCR mixtures were separated electrophoretically using agarose gel electrophoresis and visualized under UV excitation after staining with ethidium bromide. Necessary controls (positive control, negative control, no template control, as well as an internal amplification control to validate the reaction) were included for each batch. An amplified fragment of 500 bp indicated the presence of *E. canis* DNA in the sample.

2.6. Tick counts

Tick thumb counts were performed on dogs 48 hours (h) after each exposure, except on Day 30 when all ticks were counted and removed. To simulate natural exposure to the ticks, it was decided not to remove ticks from dogs until the end of the study. Tick counts were recorded according to the following six categories: 1 = live and free; 2 = live, attached and unengorged (no filling of the alloscutum evident); 3 = live, attached and engorged (obvious or conspicuous filling of the alloscutum); 4 = killed and free; 5 = killed, attached and unengorged; 6 = killed, attached and engorged.

During the thumb counts on dogs, genders were not distinguished, but ticks were categorized. The ticks counted and removed on Day 30 were categorized within gender (male/female) according to categories 1–6. Additionally, the pens of the dogs were inspected daily from Day 14 up to Day 30 for engorged detached ticks. For each dog these ticks were collected and preserved in 70% ethanol.

2.7. Statistical analysis

Any dog serologically positive for *E. canis* antibodies and positive for *E. canis* by PCR analysis was regarded as infected. To determine the effectiveness of the treatment, the total number of ticks that were assigned to categories 1, 2, 3, and 6 were transformed to the natural logarithm of (count + 1) for calculation of geometric means. For the treated group, the percent reduction in tick counts compared to the 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, respectively. The proportions of the animals infected in each group were compared by a Chi-square test. SAS® Version 8 (Release 8.02 TS Level 02M0) was used for all the statistical analyses. The level of significance of the formal tests was set at 5%.

3. Results

3.1. Tick counts

The arithmetic and geometric mean tick counts recorded for the two study groups are summarized in Table 1. On all assessment days, statistically significant ($p < 0.0006$) fewer ticks were recorded 48 h after each weekly exposure on the treated dogs compared to the untreated control dogs. Efficacy values (%) based on arithmetic mean tick counts for the group treated once with the combination of fipronil, amitraz and (S)-methoprene spot-on are summarized in Table 1. The combination fipronil, amitraz and (S)-methoprene was fully effective (100%) against all weekly exposures to *R. sanguineus* ticks up to at least four weeks post treatment.

3.2. Transmission blocking of E. canis

There were no adverse effects of the treatment administered as a topical solution. During the study, no health abnormalities, other than signs of ehrlichiosis in controls, were observed. Five untreated control dogs developed fever (>39.5 °C) between Day 28 and Day 35 and received doxycycline 10 mg/kg treatment for 21 days starting on Day 30 (4 dogs) or on Day 35 (one dog). All of these dogs recovered fully. Low platelet counts ($<200 \times 10^9$/L) were observed in the same five untreated control dogs but not in any of the treated dogs. Thrombocytopenia was evident with platelets counts as low as 2 and 4×10^9/L were recorded on Day 28 and Day 35, respectively. These values returned to normal between 200 and 500×10^9/L on Day 42 as a result of the doxycycline treatment (Table 2). None of

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Table 1
Tick counts on dogs 48 h after exposure in crates.

Day	Group 1 – Untreated control		Group 2 – (CERTIFECT® spot-on dogs)	
	Arithmetic mean	Geometric mean	Arithmetic mean	Geometric mean (% efficacy)
9[a]	10.1	9.5	0.0	0.0 (100%)
16[a]	12.9	11.5	0.0	0.00 (100%)
23[a]	13.0	10.7	0.0	0.00 (100%)
30	23.0	20.4	0.0	0.00 (100%)

[a] *In situ* counts.

the CERTIFECT treated animals developed illness, although one dog displayed a transient fever on Day 21 but platelet counts were normal. No rescue treatment was started on this dog as the temperature subsided and its serological and PCR status remained negative during the whole study.

The IFA assay results are summarized in Table 3. All dogs tested negative for *E. canis* antibodies prior to the first tick challenge. Five untreated control dogs (Group 1) developed specific *E. canis* antibodies detected first on Day 21 (3 dogs) or Day 28 (2 dogs) and remained positive through the end of the study. The same untreated control dogs that developed specific *E. canis* antibodies were confirmed PCR positive. None of the treated dogs showed seroconversion neither PCR positive result (Table 3).

In total, 5 of 8 dogs (62.5%) became infected with *E. canis* in the untreated control group and none in the CERTIFECT treated group ($p = 0.007$).

4. Discussion

The control of infestations of *R. sanguineus* using CERTIFECT on dogs has been reported previously, whereby an efficacy of (98.4–100%) was achieved in the first 48 h post treatment (Hunter et al., 2011). In addition, the improved speed-of-kill for this combination of fipronil and amitraz has been demonstrated on dogs experimentally infested by *R. sanguineus* ticks with an acaricidal efficacy > 96% as early as 18 h following infestations for at least 35 days (Hunter et al., 2011). It has also been demonstrated that this combination was able to prevent the attachment of *R. sanguineus* ticks during a full month after treatment, which indirectly reduce the risk of pathogen transmission (Prullage et al., 2011).

As far as the prevention of transmission of tick-borne pathogens is concerned, field studies conducted in West Africa revealed that fipronil by itself could reduce the transmission of *E. canis* to dogs (Davoust et al., 2003). Furthermore, it has been demonstrated that the application of a combination of 10% imidacloprid/50% permethrin reduced *E. canis* infection in dogs under field conditions in southern Italy (Otranto et al., 2008). McCall et al. (2011) studied the transmission blocking capacity of fipronil, amitraz and (S)-methoprene under laboratory conditions using field-collected *I. scapularis* ticks. The latter authors demonstrated that CERTIFECT was able to protect dogs against *B. burgdorferi* and *A. phagocytophilum* infections. In addition, a transmission blocking model of *B. canis* was developed recently based on experimental batches of infected *D. reticulatus* ticks. In this study it was shown that CERTIFECT applied up to 28 days prior to infestation with *B. canis* infected *D. reticulatus* ticks successfully prevented the development of clinical signs of canine babesiosis (Jongejan et al., 2011).

Here we show that using a tick exposure study model is successful in demonstrating prevention of transmission of *E. canis* by infected *R. sanguineus* ticks. Both of these transmission blocking study models could be used to test other tick control compounds. Furthermore, adaptations of these studies to measure the transmission blocking of other tick-borne pathogens, such as

Table 2
Thrombocyte counts in treated and control dogs.

Group	Animal ID	Day 21 ($\times 10^9$/L)	Day 28 ($\times 10^9$/L)	Day 35 ($\times 10^9$/L)	Day 42 ($\times 10^9$/L)
Control*	CC3 3F3	226	**7**	234	365
	9AF 5EA	336	327	332	304
	958 EB5	301	**2**	448	496
	8AF 142	345	**18**	293	408
	6DF 0EF	458	210	**4**	367
	958 B91	216	**2**	214	308
	B8C B85	600	557	518	442
	CCF C02	361	376	376	366
CERTIFECT treated group	E9E E23	399	341	375	383
	E9F F45	261	348	360	354
	CD0 108	315	279	324	291
	9AE D8D	333	292	365	367
	DF4 8A3	370	359	276	414
	9B0 374	395	391	446	384
	954 C83	544	538	550	462
	DF6 ADF	450	289	460	407

A rescue treatment was started on all dogs with a platelet count <200 $\times 10^9$/L.
* Platelet counts normal range 200–500 $\times 10^9$/L.

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Table 3
Ehrlichia canis antibodies determined by IFA test and PCR results.

Group	Animal ID	<Day −7	Day 7[a]	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
Control	CC3 3F3	neg/neg	neg/neg	neg/neg	**POS/neg**	**POS/POS**	**POS/POS**	**POS/neg**	**POS/neg**
	9AF 5EA	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg
	958 EB5	neg/neg	neg/neg	**POS/neg**	**POS/neg**	**POS/POS**	**POS/neg**	**POS/neg**	**POS/neg**
	8AF 142	neg/neg	neg/neg	**POS/neg**	**POS/neg**	**POS/POS**	**POS/POS**	**POS/neg**	**POS/neg**
	6DF 0EF	neg/neg	neg/neg	neg/neg	**POS/POS**	**POS/POS**	**POS/POS**	**POS/neg**	**POS/neg**
	958 B91	neg/neg	neg/neg	**POS/neg**	**POS/neg**	**POS/POS**	**POS/neg**	**POS/POS**	**POS/neg**
	B8C B85	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg
	CCF C02	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg
CERTIFECT treated group	E9E E23	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg
	E9F F45	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg
	CD0 108	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg
	9AE D8D	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg
	DF4 8A3	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg
	9B0 374	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg
	954 C83	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg
	DF6 ADF	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg	neg/neg

[a] Prior to tick challenge. POS, positive for *E. canis* antibodies; neg, negative for *E. canis* antibodies; /POS, positive for PCR; /neg = negative for PCR.

Babesia vogeli transmitted by *R. sanguineus* ticks become feasible.

A prerequisite for the present study was the availability of a large batch of *R. sanguineus* ticks with an adequate level of infection with *E. canis*. An infection rate of 38% of the ticks was considered adequate under the conditions of the model and represented a realistic challenge. This conclusion is further supported by the fact that infection rates of *R. sanguineus* for *E. canis* in the field are usually lower than 38%. For instance, in northeastern Brazil the prevalence of *E. canis* in *R. sanguineus* ticks removed from dogs and analysed by PCR was 21.9% (Souza et al., 2010).

Reliable laboratory tests are required to confirm the diagnosis of monocytic ehrlichiosis, because clinical signs are themselves not specific enough (de Castro et al., 2004; Harrus et al., 1999). Nevertheless, thrombocytopenia ($<200 \times 10^9$/L), as observed in the five infected control dogs (Table 3), is considered to be a strong indicator of the infection (Harrus et al., 1999). The same five dogs developed specific *E. canis* antibodies and were PCR positive on several occasions (Table 3). The results obtained by platelet counts, IFA serology and PCR were in agreement. The conventional PCR test was previously developed to build the model and to assess infections in both tick and blood samples. It was considered as sensitive enough by the authors for the purpose of this study which was the assessment of infected dogs. A possible reason that three out of eight control dogs did not acquire infection with *E. canis* could be that the distribution of ehrlichial carrying ticks within batches was not homogeneous or that the numbers of ehrlichial organisms present in some ticks may have been too low. This hypothesis seems to be supported by the fact that number of ticks feeding on control dogs was comparable among the dogs. A real-time quantitative PCR approach would be a superior to the conventional PCR in order to study the number of *Ehrlichia* organisms in ticks in the future.

Conflict of interest

The work reported herein was funded by Merial SAS, Lyon, France. Two authors (Catherine Ollagnier and Frederic Beugnet) are currently employees of Merial, however, there were no conflicting interests that may have biased the work reported in this paper.

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