

Efficacy of rifampicin in the treatment of experimental acute canine monocytic ehrlichiosis

Konstantina Theodorou¹, Mathios E. Mylonakis^{1*}, Victoria I. Siarkou², Leonidas Leontides³, Alexander F. Koutinas¹, Christos K. Koutinas¹, Maria Kritsepi-Konstantinou⁴, George Batzias⁵, Eugenia Flouraki¹, Osnat Eyal⁶, Vassilios Kontos⁷ and Shimon Harrus⁶

¹Companion Animal Clinic, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki (AUTH), Thessaloniki, Greece; ²Laboratory of Microbiology and Infectious Diseases, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki (AUTH), Thessaloniki, Greece; ³Laboratory of Epidemiology, Biostatistics and Animal Health Economics, School of Veterinary Medicine, University of Thessaly, Karditsa, Greece; ⁴Diagnostic Laboratory, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki (AUTH), Thessaloniki, Greece; ⁵Laboratory of Veterinary Pharmacology, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki (AUTH), Thessaloniki, Greece; ⁶Koret School of Veterinary Medicine, The Hebrew University of Jerusalem, Jerusalem, Israel; ⁷Department of Veterinary Public Health, National School of Public Health, Athens, Greece

*Corresponding author. Tel: +30-2310994495; Fax: +30-2310994516; E-mail: mmylonak@vet.auth.gr

Received 14 October 2012; returned 3 December 2012; revised 9 January 2013; accepted 24 January 2013

Objectives: To assess the efficacy of rifampicin in achieving clinical and haematological recovery and clearing infection in dogs with experimentally induced acute monocytic ehrlichiosis.

Methods: Five *Ehrlichia canis*-infected Beagle dogs were treated with rifampicin (10 mg/kg/24 h orally for 3 weeks), nine *E. canis*-infected dogs received no treatment (infected untreated dogs) and two dogs served as uninfected controls. Clinical score, platelet counts, immunofluorescent antibody titres and PCR detection of *E. canis*-specific DNA in blood, bone marrow and spleen aspirates were evaluated on post-inoculation days 21 (start of rifampicin), 42 (end of rifampicin) and 98 (end of the study).

Results: By day 21 post-inoculation, all infected dogs became clinically ill and thrombocytopenic, seroconverted and were PCR positive in at least one tissue. Clinical scores and antibody titres did not differ between the treated and infected untreated dogs throughout the study. The rifampicin-treated dogs experienced an earlier resolution of their thrombocytopenia (Kaplan–Meier survival plot, $P=0.048$), and the median platelet counts were significantly higher in the treated compared with the infected untreated dogs on post-inoculation days 42 ($P=0.0233$) and 98 ($P=0.0195$). At the end of the study, three treated and six untreated infected dogs remained PCR positive in one tissue each.

Conclusions: The rifampicin treatment regimen applied in this study hastened haematological recovery, but was inconsistent in eliminating the acute *E. canis* infection.

Keywords: dogs, *Ehrlichia canis*, therapy

Introduction

Tetracyclines are considered to be first-line drugs in the treatment of canine monocytic ehrlichiosis (CME) caused by *Ehrlichia canis*.^{1,2} However, only doxycycline has been critically evaluated in experimental or natural disease, and there are conflicting reports on the clearance of the infection following different doxycycline treatment protocols. In experimental acute or subclinical CME, it was very effective in eliminating the infection.^{3–6} In contrast, in other experimental or clinical studies, treatment with various doxycycline regimens failed to eradicate the infection

in 25%–100% of the acutely, subclinically or chronically infected dogs, despite their clinical and haematological recovery.^{7–11} Collectively, current evidence implies that doxycycline effectively ameliorates the *E. canis*-induced clinical and haematological abnormalities, but is not consistently effective in clearing the infection. In addition, some dogs may be intolerant to doxycycline,¹² thus justifying the evaluation of medications that could be used as alternatives to doxycycline.

Recent data in a limited number of dogs have indicated that rifampicin, an inhibitor of the B subunit of DNA-dependent RNA polymerase, may be partially effective in eliminating

ehrlichiaemia in dogs experiencing the subclinical or chronic phases of the disease.^{9,13} Of comparative interest, in human ehrlichiosis patients with a specific doxycycline contraindication, rifampicin is used as an alternative chemotherapeutic agent, affording rapid clinical recovery, although its efficacy in clearing the infection has not been evaluated.^{14,15} There are currently no data on the potential effectiveness of rifampicin in acute CME or on the elimination of *E. canis* from tissues other than blood.

This study was undertaken to evaluate the efficacy of rifampicin in clearing *E. canis* from blood, bone marrow (BM) and spleen aspirates, and in achieving clinical and haematological recovery in dogs with experimental acute CME.

Methods

Experimental dogs

Sixteen Beagle dogs, seven males and nine females, with an age ranging from 5 to 70 months (median 11 months), purchased from the School of Veterinary Medicine (University of Thessaly, Greece) experimental dog colony were used in the study. All dogs were fully vaccinated against canine parvovirus-2, canine adenovirus-2, canine distemper virus, *Leptospira icterohaemorrhagiae/Leptospira canicola* and rabies, and were subject to strict ectoparasite and endoparasite control measures. Before the experimental infection, they were healthy and had no haematological or biochemical abnormalities in serial evaluations over a 30 day acclimatization period. The dogs were seronegative to *E. canis*, *Babesia canis* (Fluo BABESIA canis; Agrolabo, Scarmagno, Italy; positive titre: $\geq 1/100$) and *Leishmania infantum* antibodies (Fluoleish BVT, Virbac, Carros, France; positive titre: $\geq 1/100$) by indirect fluorescence antibody (IFA) testing, as well as to *Dirofilaria immitis* antigens (Snap 3DX; IDEXX, Westbrook, ME, USA). All dogs were PCR negative for *E. canis* DNA in blood, spleen and BM aspirates before the inoculation with *E. canis*. The duration of the study after the experimental inoculation was 98 days (14 weeks). The study was approved by the Research and Ethics Committee, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki (458/23-6-2009) and the Directorate of Veterinary Services, Thessaloniki (13/3152, 16-03-2010), Greece. In every aspect, handling of the animals strictly adhered to the rules and regulations governing animal experimentation.

Group allocation and treatment protocol

The dogs were randomized into three groups by utilizing a table of random numbers. Treated dogs ($n=5$) included *E. canis*-infected dogs that were treated with rifampicin (Rifadin) at 10 mg/kg/24 h, orally, for 3 weeks. All treated dogs were also given dexamethasone (0.5 mg/kg dexamethasone sodium, intravenously) once, 15 days after the completion of rifampicin, to precipitate any latent *E. canis* infection. Infected untreated dogs ($n=9$) included *E. canis*-infected dogs that received no treatment. Two Beagles served as uninfected controls. Administration of rifampicin started on post-inoculation day (PID) 21, when all treated dogs matched the following criteria: development of thrombocytopenia, seroconversion to *E. canis* and positive PCR in at least one of the tissues tested (blood, BM or spleen), with or without clinical manifestations.

The rifampicin dosage applied in the present study was based on previous suggestions¹⁶ and a preliminary pharmacokinetic evaluation conducted 2 months prior to this study on eight healthy Beagles, in which a high serum concentration of rifampicin (mean C_{max} 0.01554 ± 0.00183 mg/L and mean concentration of 0.00431 ± 0.00056 mg/L after 24 h) was achieved following a single oral dose of 10 mg/kg of

the product used in the present study (G. Batzias, K. Theodorou and M. E. Mylonakis, unpublished results).

Experimental infection

Thirteen dogs were inoculated intravenously with 5 mL of heparinized *E. canis*-infected blood that contained $\sim 12.5 \times 10^4$ 16S rDNA copies, quantified by an *E. canis*-specific real-time PCR.¹⁷ The blood was drawn from an acutely ill reservoir Beagle, artificially infected with the Israeli *E. canis* strain 611 (propagated in DH82 cell culture),^{18,19} donated by the Koret School of Veterinary Medicine, The Hebrew University of Jerusalem, Israel, as soon as the dog became thrombocytopenic (platelet count $44 \times 10^9/L$) and seroconverted to *E. canis* (IFA titre 1/1600) and *E. canis* DNA could be detected in blood and spleen samples on PID 19. The two uninfected dogs were inoculated with 5 mL of heparinized blood from a healthy uninfected Beagle.

Clinical and haematological examination

Each dog had serial clinical examinations performed on days 30, 14, 8, 6, 4 and 2 before and on the day of inoculation (day 0); thereafter, this was carried out every 2 days until PID 42 and weekly until PID 98. Physical examinations were performed by two assessors (M. E. M. and C. K. K.) who had no knowledge of the group allocation. A clinical evaluation sheet was filled out qualitatively (i.e. 0='absence' and 1='presence' of the clinical finding). For each dog, the cumulative clinical score per examination was calculated. Clinical manifestations systematically evaluated included depression/lethargy, fever ($>39.5^\circ C$), lymphadenomegaly, bleeding tendency, mucosal pallor and palpable splenomegaly. Anorexia was assessed by the primary caregiver of the dogs (K. T.) in an open-label manner and was also included in the cumulative clinical score.

Complete blood counts were performed on an Advia Hematology Analyzer (Advia 120 Haematology System; Bayer, Tarrytown, NY, USA). All dogs were tested 2 weeks before and on inoculation day. Thereafter, treated and infected untreated dogs were tested on PIDs 7, 14, 21, 28, 35, 42, 70, 84 and 98, while uninfected dogs were tested on PIDs 7, 14, 35, 70 and 98. Giemsa-stained blood smears were reviewed to confirm the presence of thrombocytopenia throughout the study. Aliquots of EDTA-treated blood samples were stored at $-20^\circ C$ until molecularly analysed.

BM and spleen sampling

BM and spleen aspirates (0.5–1 mL) were obtained as previously described^{20,21} under short-term sedation induced by acetylpromazine (0.05 mg/kg acepromazine, intravenously) and thiopental sodium (Pentothal; 5 mg/kg, intravenously). The aspirates were collected in EDTA-treated tubes and stored at $-20^\circ C$ until molecularly analysed. Giemsa-stained cytology smears were reviewed after each aspiration to confirm the adequacy of the samples. Aspiration of BM and spleen was performed for all dogs 2 days prior to the experimental inoculation; thereafter, treated and infected untreated dogs were sampled on PIDs 7, 21, 42, 70 and 98, and uninfected dogs on PIDs 42 and 98.

Serological testing for *E. canis*

An IFA test was used to detect anti-*E. canis* IgG antibodies on 15-well slides using *E. canis* acetone-fixed antigen suspension (Fluo EHRLICHIA canis; Agrolabo). Serial 2-fold dilutions of serum samples from the dogs were reacted with fluorescein-conjugated rabbit anti-canine immunoglobulin G (anti-dog IgG; Sigma-Aldrich, St Louis, MO, USA) with an end-titre of 1/1600. A titre equal to, or higher, than 1/100 was considered positive. Paired serum samples (14 days prior to inoculation and on the day of inoculation) were initially tested for all dogs; thereafter,

treated and infected untreated dogs were tested on PIDs 7, 21, 42, 70 and 98, while uninfected dogs were tested on PIDs 7, 70 and 98.

PCR amplification and analysis of *E. canis* DNA

Two PCR assays were carried out in this study in two independent laboratories for confirmation of ehrlichial infection and response to treatment. They were performed with whole-genome DNA extracted by use of a commercial kit according to the manufacturer's protocol (NucleoSpin Tissue kit; Macherey-Nagel, Dueren, Germany), from 200 μ L of blood samples and 50 μ L of BM and spleen aspirates, and stored at -20°C until analysed.

An *E. canis*-specific nested PCR assay detecting the *E. canis* 16S rRNA gene was performed at the Laboratory of Microbiology, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki, Greece, using the primers ECC 5'-AGAACGAACGCTGGCGCAAGCC-3' and ECB 5'-CGTATTACCGCGCTGCTGGCA-3' for the primary amplification, and 'canis' 5'-CAATATTTATAGCCTCTGGCTATAGGA-3' and HE3 5'-TATAGGTACCGTCATTATCTCCCTAT-3' for the secondary amplification, as previously described,^{11,22} except that Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA) was used for optimum amplification. In each set of amplifications, two positive controls (blood and BM DNA from a molecularly confirmed *E. canis*-infected dog) and a negative control (without DNA template) were included. The identity of 'canis'/HE3 PCR amplicons (389 bp) as *E. canis* was established by subsequent sequencing of both strands of the purified amplicons performed by VBC-BIOTECH Service GmbH (Vienna, Austria) using the representative PCR primers. Amplicon sequences were compared with the reference '611' (accession number U26740) by multiple sequence alignment by use of the ClustalW program (Conway Institute, Dublin, Ireland).²³

Molecular evidence of infection was also assessed by the Koret School of Veterinary Medicine, which performed an *E. canis*-specific real-time PCR assay targeting a 123 bp fragment from a single copy of *E. canis* 16S rRNA gene, as previously described.¹⁷ Primers used were Ec-16S-F 5'-TCGCTATTAGATGAGCCTACGT and Ec-16S-R 5'-GAGTCTGGACCGTATC TCAG. The reactions were carried out using Thermo Start Master Mix (Thermo Scientific, Germany) and SYTO 9 fluorescent dye (Thermo Scientific) in a Corbett Research Rotor-Gene 6000 (Corbett Research Pty Ltd, Sydney, Australia). The protocol used was the same as that described in Peleg *et al.*,¹⁷ with a minor change to the start-up temperature: 15 min at 95°C instead of 7 min, owing to the use of a different enzyme. Sequence analysis and comparisons were conducted using the forward primer Ec-16S-F and performed at the Hebrew University Sequencing Core. Sequences were evaluated with ChromasPro software version 1.33 (Technelysium Pty Ltd, South Brisbane, Australia) and compared with sequence data available from GenBank using the BLAST 2.2.9 program (<http://www.ncbi.nlm.nih.gov/BLAST/>).

All 16 dogs were tested by nested PCR assay of blood, BM and spleen prior to *E. canis* inoculation. Thereafter, treated and infected untreated dogs were tested by the nested PCR assay on PID 7, and by both nested and real-time PCR on PIDs 21, 42, 70 and 98, while uninfected dogs were tested on PIDs 42 and 98. With the exception of the *E. canis*-infected blood of the reservoir dog, which was quantified by the real-time PCR, both nested and real-time PCR results were recorded as either negative or positive.

Statistical analysis

The Wilcoxon–Mann–Whitney test was used to compare the median values of the clinical score during the pre-treatment (PIDs 1–21), treatment (PIDs 22–42) and post-rifampicin treatment (PIDs 43–98) periods, and the median values of clinical score, platelet counts and *E. canis* IFA titres between treated and infected untreated dogs at

rifampicin initiation (PID 21), rifampicin completion (PID 42) and the end of the study (PID 98). A Kaplan–Meier survival plot was developed to compare the cumulative probability of resolution of thrombocytopenia with time during rifampicin treatment between treated and infected untreated dogs. The survival curves were evaluated with a log-rank test. All tests were done using Stata Statistical Software Release 9.2 (College Station, TX, USA: StataCorp LP, 2006) and evaluated for significance at the 5% level.

Results

Clinical and haematological findings

Post-inoculation, all 14 infected dogs experienced clinical manifestations (median 3, range 2–4) compatible with CME, including fever (13, 92.9%), palpable splenomegaly (10, 71.4%), lymphadenomegaly (8, 57.1%), anorexia (4, 28.6%), depression (4, 28.6%), pallor (2, 14.3%) and mucosal petechiae (1, 7.1%). The uninfected dogs remained healthy during the whole study period. No difference was found in the median clinical score between treated and infected untreated dogs throughout the study period (Table 1).

The median values of haematocrit, leucocytes and platelets after *E. canis* inoculation are depicted in Figure 1. By PID 14, 12/14 (86%) of the infected dogs (four treated and eight untreated dogs) developed mild-to-moderate anaemia; on PID 21, only five untreated dogs remained anaemic, and by PID 42, none of the infected dogs was still anaemic. By PID 14, 13/14 (93%) of the infected dogs (five treated and eight untreated dogs) became mildly leucopenic; on PID 21, only two treated and two untreated dogs remained leucopenic, and by PID 42, none of the dogs was still leucopenic. By PID 21, all infected dogs developed thrombocytopenia (median $72.5 \times 10^9/\text{L}$, range $44\text{--}101 \times 10^9/\text{L}$, reference intervals $200\text{--}500 \times 10^9/\text{L}$). On PID 42, one treated dog and six untreated dogs were still thrombocytopenic, while on PID 98, all treated dogs had normal platelet counts and four untreated dogs were still thrombocytopenic. On PID 21, median platelet counts did not differ between treated and untreated dogs; however, treated dogs had significantly higher platelet counts compared with the untreated infected dogs on PID 42 ($251 \times 10^9/\text{L}$ versus $168 \times 10^9/\text{L}$, $P=0.0233$) and 98 ($254 \times 10^9/\text{L}$ versus $205 \times 10^9/\text{L}$, $P=0.0195$). The Kaplan–Meier survival curves showing the probability of resolution of thrombocytopenia with time (PIDs 21–42) differed ($P=0.048$) between treated and untreated dogs (Figure 2). Among the treated dogs, the median time for normalization of platelets was PID 28, whereas among the untreated ones, resolution of thrombocytopenia was not observed during the period analysed.

E. canis serology

By PID 21, all infected dogs had seroconverted to *E. canis* antigens (median IgG titres 1/200, range 1/200–1/1600) and remained seropositive throughout the study period. The two uninfected dogs remained seronegative during the same period. No difference was found in the median anti-*E. canis* titres between treated and infected untreated dogs on PIDs 21, 42 and 98.

Table 1. Clinical score in 14 dogs experimentally infected with *E. canis* treated or not treated with rifampicin

PID(s)	Median clinical score (range)	
	treated (n=5)	untreated (n=9)
1–21	7 (6–13)	9 (4–12)
21	1 (1–2)	1 (0–4)
22–42	0 (0–3)	1 (0–9)
42	0 (0–0)	0 (0–1)
43–98	0 (0–2)	1 (0–11)
98	0 (0–0)	0 (0–1)

PID(s): 1–21, before rifampicin treatment; 21, start of rifampicin; 22–42, rifampicin treatment; 42, end of rifampicin; 43–98, post-rifampicin follow-up period; 98, end of study.

PCR-based evaluation of *E. canis* infection and response to treatment

The PCR (nested and real-time assays) results before and after (7, 21, 42, 70 and 98 days post-inoculation) inoculation with *E. canis*, with regard to timepoint and tissue tested are presented in Table 2. On PID 21, all treated (median positive tissues 2, range 1–2) and untreated (median positive tissues 2, range 1–3) dogs became PCR positive. Upon termination of the study (PID 98), three treated and six untreated dogs were still PCR positive in one tissue each. The three PCR-positive treated dogs were clinically healthy and had normal haematological values; one of them, however, had been persistently thrombocytopenic from PID 42 to 84. Of the six PCR-positive untreated dogs, two were still thrombocytopenic (one with splenomegaly) and one had splenomegaly, but no thrombocytopenia. Two more untreated dogs were thrombocytopenic on PID 98 (one with splenomegaly), but PCR of the tissues was negative. Uninfected dogs remained PCR negative throughout the study. Overall, among the tissues tested post-inoculation until the completion of the study for the 14 infected dogs (a total of 208 PCR-examined samples, irrespective of the PCR assay applied), a positive PCR outcome was documented in 14/70 (20%), 30/70 (42.9%) and 17/68 (25%) blood, BM and spleen aspirates, respectively. The 16S rDNA sequences of the PCR amplicons derived from both PCR assays presented 100% homology with *E. canis* strain 611.

Discussion

Previous reports indicated that rifampicin may be an alternative chemotherapeutic agent to doxycycline for the treatment of CME.^{13,24} In a study on antibiotic susceptibilities, rifampicin demonstrated an anti-*E. canis* activity comparable to that of doxycycline, with a very low MIC (0.00003–0.00006 mg/L).²⁴ When rifampicin was given (15 mg/kg/12 h orally for 7 days) to two subclinically infected, moderately pancytopenic dogs, pancytopenia was resolved and *E. canis* DNA was cleared from the blood, as documented by PCR.¹³ The same rifampicin regimen, given to two asymptomatic *E. canis*-infected dogs

700 days post-inoculation, after an ineffective course with doxycycline, appeared to clear the infection in one of the two dogs, based on xenodiagnosis with ticks.⁹ To the best of our knowledge, this is the first study evaluating the efficacy of rifampicin in acute CME in a controlled clinical trial, expanding the range of PCR-tested tissues to include BM and spleen, in addition to blood. As administered in the current study, rifampicin objectively hastened resolution of thrombocytopenia, but was inconsistent in eliminating the acute *E. canis* infection.

The rifampicin regimen applied in the current study differed substantially from that previously reported in CME^{9,13} in terms of dosing and duration. The high bioavailability of rifampicin in the dog when given orally, once daily, at 10 mg/kg,¹⁶ coupled with our preliminary pharmacokinetic study, the very low MIC for *E. canis*²⁴ and the fact that higher doses are more likely to be associated with side effects,²⁵ heavily affected the dosing scheme in this study; on the other hand, the failure of a dog to be cleared of the *E. canis* infection when treated with rifampicin for 1 week dictated towards a longer treatment duration.⁹ However, even with the present regimen, clearance of the infection was only inconsistently achieved (2/5 dogs).

Similar to other studies,^{6,9,13} all dogs experienced typical CME-associated clinical manifestations post-inoculation that had abated irrespective of treatment status prior to the institution of rifampicin (PID 21). It appeared, therefore, particularly challenging to substantiate a rifampicin-mediated clinical improvement in the acute phase of CME, which frequently demonstrates a rapid spontaneous clinical recovery.¹ Similarly, although anaemia and leucopenia occurred in several of the 14 infected dogs by PID 14 (12 and 13 dogs, respectively), only a few dogs remained anaemic and leucopenic 1 week later, which also hindered their evaluation as treatment response indicators. In contrast, all the infected dogs remained persistently thrombocytopenic until initiation of rifampicin. The time for normalization of platelet counts was significantly shorter in the rifampicin-treated compared with the untreated dogs, and the median platelet counts of the former dogs were significantly higher on PID 42 (the end of rifampicin treatment) and PID 98 (the end of the study), implying that rifampicin affords haematological recovery in acute CME, similar to what was previously documented in two subclinically infected dogs.¹³

Although the results of several studies have suggested that the sustained post-treatment resolution of thrombocytopenia is correlated reasonably well with the elimination of *E. canis* infection^{3–6,26} this was not supported by the results of this study. At the end of the study, the three treated PCR-positive dogs had normal platelet counts, an experience shared by other authors,^{7,9} implying that normal platelet counts, even long after treatment, do not invariably indicate clearance of *E. canis* infection. The high percentage of infected untreated dogs (4/6) that experienced normal platelet counts at the end of the study despite being carriers of *E. canis* further underlines the suboptimal performance of platelet counts as an infection clearance indicator. All infected dogs remained seropositive during the whole study period, and no difference was found in the median anti-*E. canis* titres between the treated and untreated dogs on PIDs 21, 42 and 98, suggesting that, similar to platelet counts, serology may be of limited value as a post-treatment monitoring tool.¹

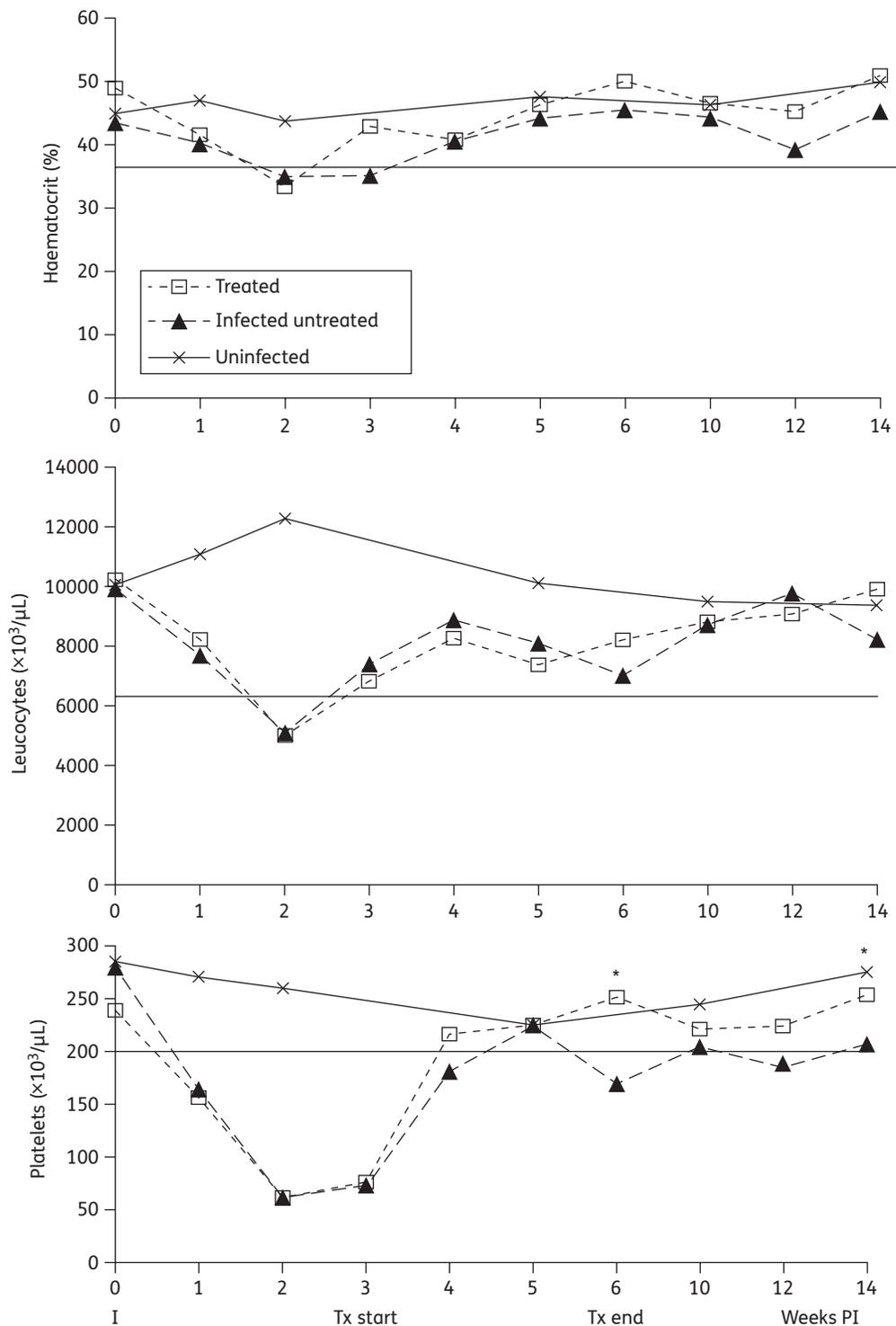


Figure 1. Median haematocrit, leucocyte and platelet values prior to and post-inoculation (PI) with *E. canis* in 14 dogs that were treated or not treated with rifampicin; uninfected dogs served as controls. I, inoculation day; Tx start, start of rifampicin treatment; Tx end, end of rifampicin treatment. *Significantly higher median platelet counts.

In the present study, three untreated dogs experienced spontaneous clearance of infection, as evidenced from the negative PCR assays in all three tissues tested, similar to what has been

documented previously in 25%–50% of subclinically or chronically infected dogs; this supports the possibility of self-elimination of *E. canis* infection in nature irrespective of the clinical phase of

the disease.^{3,4,27} Alternatively, the negative PCR status of the treated and untreated dogs in the present study may be due to the suppression of ehrlichiaemia and tissue bacterial loads to a level not detectable by the applied assays. This possibility was recently clearly demonstrated in *E. canis*-infected dogs treated with doxycycline, where xenodiagnosis was superior to PCR in demonstrating ehrlichiaemia of low level.⁹ The two PCR-negative untreated dogs that remained thrombocytopenic on PID 98 (one with splenomegaly) may support the possibility of persistent infection.

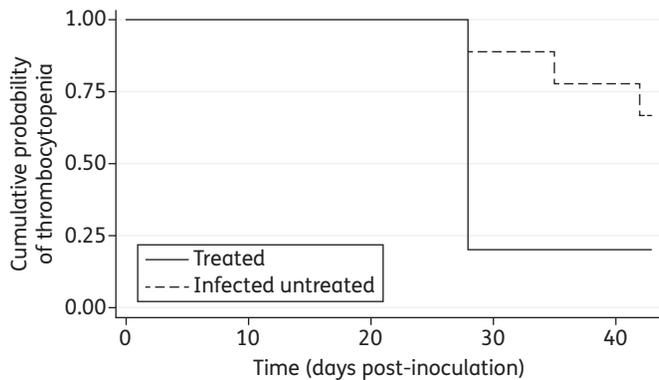


Figure 2. Kaplan–Meier survival curves showing the cumulative probability of resolution of thrombocytopenia with time (PIDs 21–42) in five rifampicin-treated and nine untreated *E. canis*-infected dogs.

The results of the present study emphasize the importance of assessing the response to treatment in CME by applying molecular testing in a range of tissues rather than solely the blood, and for a sufficient time after cessation of treatment. Upon completion of rifampicin, two treated dogs were still PCR positive, but this number increased to four and three dogs, 4 and 8 weeks post-treatment, respectively, presumably due to a dexamethasone-induced recrudescence of latent *E. canis* infection. Of note, one treated dog that was found to be positive at 4 weeks post-treatment became negative at 8 weeks post-treatment, possibly indicating that its final clearance may have not been mediated by rifampicin. Surprisingly, 7/9 untreated dogs were PCR negative on PID 42, but this number diminished to 3/9 by the end of the study. These data suggest that the molecular assessment of infection clearance in CME should be extended for at least 2 months post-treatment.

In the current study, BM aspirates had the highest sensitivity for determining the clearance status of the infection by PCR at the end of the study. Surprisingly, all treated and untreated dogs were blood PCR negative at the end of the study, suggesting that blood should not be the sole tissue PCR-tested post-treatment, as it may lead to an overestimation of the clearance rate. Previously, spleen aspirates had been shown to be of higher sensitivity than blood and BM in assessing clearance of infection,^{6,27,28} while in another study, blood, BM and spleen were of equal value in post-treatment PCR testing.⁴ Collectively, these data suggest that blood, BM and spleen may be a reasonable combination of materials for the PCR-based assessment of *E. canis* infection clearance in the clinical setting.

Table 2. Nested and real-time PCR results for blood, BM and spleen before and after inoculation of 14 dogs with *E. canis*; treated dogs were given rifampicin, untreated dogs received no treatment and uninfected dogs served as controls

Tissue	Overall PCR-positive/tested dogs (tested by nested/real-time PCR)					
	pre-inoculation	7 DPI	21 DPI	42 DPI	70 DPI	98 DPI
Treated (n=5)						
blood	0/5 (0/ND)	0/5 (0/ND)	1/5 (1/1)	2/5 (0/2)	3/5 (0/3)	0/5 (0/0)
BM	0/5 (0/ND)	3/5 (3/ND)	5/5 (5/4)	0/5 (0/0)	0/5 (0/0)	2/5 (0/2)
spleen	0/5 (0/ND)	3/5 (3/ND)	2/5 (1/2)	0/5 (0/0)	1/5 (1/0)	1/5 (1/0)
Untreated (n=9)						
blood	0/9 (0/ND)	1/9 (1/ND)	5/9 (1/5)	1/9 (0/1)	1/9 (1/0)	0/9 (0/0)
BM	0/9 (0/ND)	3/9 (3/ND)	9/9 (9/7)	1/9 (1/0)	2/9 (2/2)	5/9 (4/4)
spleen	0/9 (0/ND)	2/9 (2/ND)	6/9 (6/3)	0/7 (0/0)	1/9 (0/1)	1/9 (0/1)
Uninfected (n=2)						
blood	0/2 (0/ND)	ND	ND	0/2 (0/0)	ND	0/2 (0/0)
BM	0/2 (0/ND)	ND	ND	0/2 (0/0)	ND	0/2 (0/0)
spleen	0/2 (0/ND)	ND	ND	0/2 (0/0)	ND	0/2 (0/0)
Group	PCR-positive dogs irrespective of tissues tested or PCR assay applied					
	pre-inoculation	7 DPI	21 DPI	42 DPI	70 DPI	98 DPI
Treated	0/5	5/5	5/5	2/5	4/5	3/5
Untreated	0/5	4/9	9/9	2/9	3/9	6/9
Uninfected	0/2	ND	ND	0/2	ND	0/2

DPI, days post-inoculation; ND, not done.

In the present study, we cannot exclude the possibility that failure of rifampicin to eradicate *E. canis* infection was associated with an insufficient duration of treatment or, most importantly, with the emergence of resistance to rifampicin. In a recent study on *Staphylococcus pseudintermedius* isolates from dogs, rifampicin treatment resulted rapidly in *rpoB* gene mutations that conferred resistance to rifampicin.²⁹ Future studies may therefore be warranted to investigate whether the efficacy of rifampicin could be improved when given for a longer period of time, or whether *rpoB* gene-mediated resistance emerges in rifampicin-treated *E. canis* infection. In the latter case, the combination of rifampicin with another drug with known *in vitro* efficacy against *E. canis* might be able to diminish this resistance potential. In this respect, doxycycline could be paired with rifampicin, similar to what has been suggested for feline and canine bartonellosis and *Brucella canis*-induced endophthalmitis in the dog.^{30–33} Only a few other drugs have been critically evaluated for the treatment of CME. Imidocarb dipropionate and fluoroquinolones, initially thought to be efficacious in achieving clinical remission,^{34–36} were subsequently found to be ineffective in eliminating acute *E. canis* infection.^{26,37}

In conclusion, rifampicin was partially effective in eliminating acute *E. canis* infection, but it significantly hastened the resolution of thrombocytopenia. It may therefore be considered as an alternative antibacterial treatment in CME, especially in dogs intolerant to doxycycline. The importance of assessing the response to treatment in CME by performing molecular testing in a range of tissues including blood, BM and spleen, at least 2 months after the end of treatment, is also emphasized. Platelet counts and anti-*E. canis* IgG antibody titres were of limited value in the evaluation of the post-treatment clearance of *E. canis* infection.

Acknowledgements

Part of this work was presented as an oral presentation at the Twenty-second Congress of the European College of Veterinary Internal Medicine-Companion Animals (ECVIM-CA), Maastricht, The Netherlands, 2012, and an abstract is included in the proceedings (Abstract IM-0-1, pp. 232–3).

Funding

This work was supported by the Companion Animal Clinic, School of Veterinary Medicine, Aristotle University of Thessaloniki, Greece, and the Koret School of Veterinary Medicine, The Hebrew University of Jerusalem, Israel.

Transparency declarations

None to declare.

Author contributions

Principal investigator, with full access to the data of the study, ensuring the accuracy of the data analysis: K. T. Study concept and design: M. E. M., V. I. S., G. B. and A. F. K. Acquisition of data: K. T., M. E. M., C. K. K., M. K.-K., E. F., V. K., O. E. and V. I. S. Statistical expertise: L. L. Analysis and interpretation of the data: K. T., M. E. M., V. I. S., S. H. and L. L. Study supervision: M. E. M., V. I. S., A. F. K. and

G. B. Critical revision of the manuscript for important intellectual content: all authors.

References

- Harrus S, Waner T, Neer TM. *Ehrlichia canis* infection. In: Greene CE, ed. *Infectious Diseases of the Dog and Cat*. St Louis, MO: Elsevier, 2012; 227–38.
- Neer TM, Breitschwerdt EB, Green RT *et al*. Consensus statement on ehrlichial disease of small animals from the Infectious Disease Study Group of the ACVIM. *J Vet Intern Med* 2002; **16**: 309–15.
- Breitschwerdt EB, Hegarty B, Hancock S. Doxycycline hyclate treatment of experimental canine ehrlichiosis followed by challenge inoculation with two *Ehrlichia canis* strains. *Antimicrob Agents Chemother* 1998; **42**: 362–8.
- Eddlestone SM, Diniz PPVP, Neer TM *et al*. Doxycycline clearance of experimentally induced chronic *Ehrlichia canis* infection in dogs. *J Vet Intern Med* 2007; **21**: 1237–42.
- Gaunt SD, Beall MJ, Stillman BA *et al*. Experimental infection and coinfection of dogs with *Anaplasma platys* and *Ehrlichia canis*: hematologic, serologic and molecular findings. *Parasit Vectors* 2010; **3**: 33.
- Harrus S, Kenny M, Miara L *et al*. Comparison of simultaneous splenic sample PCR with blood sample PCR for diagnosis and treatment of experimental *Ehrlichia canis* infection. *Antimicrob Agents Chemother* 2004; **48**: 4488–90.
- Harrus S, Waner T, Aizenberg I *et al*. Therapeutic effect of doxycycline in experimental subclinical canine monocytic ehrlichiosis: evaluation of a 6-week course. *J Clin Microbiol* 1998; **36**: 2140–2.
- Iqbal Z, Rikihisa Y. Reisolation of *Ehrlichia canis* from blood and tissues of dogs after doxycycline treatment. *J Clin Microbiol* 1994; **32**: 1644–9.
- McClure JC, Crothers ML, Schaefer JJ *et al*. Efficacy of a doxycycline treatment regimen in three different phases of experimental ehrlichiosis. *Antimicrob Agents Chemother* 2010; **54**: 5012–20.
- Schaefer JJ, Needham GR, Bremer WG *et al*. Tick acquisition of *Ehrlichia canis* from dogs treated with doxycycline hyclate. *Antimicrob Agents Chemother* 2007; **51**: 3394–6.
- Wen B, Rikihisa Y, Mott JM *et al*. Comparison of nested PCR with immunofluorescent-antibody assay for detection of *Ehrlichia canis* infection in dogs treated with doxycycline. *J Clin Microbiol* 1997; **35**: 1852–5.
- Schultz BS, Hupfauer S, Ammer H *et al*. Suspected side effects of doxycycline use in dogs – a retrospective study of 386 cases. *Vet Rec* 2011; **169**: 229.
- Schaefer JJ, Kahn J, Needham GR *et al*. Antibiotic clearance of *Ehrlichia canis* from dogs infected by intravenous inoculation of carrier blood. *Ann NY Acad Sci* 2008; **1149**: 263–9.
- Ismail N, Bloch KC, McBride JW. Human ehrlichiosis and anaplasmosis. *Clin Lab Med* 2010; **30**: 261–92.
- Krause PJ, Corrow CL, Bakken JN. Successful treatment of human granulocytic ehrlichiosis in children using rifampin. *Pediatrics* 2003; **112**: 252–3.
- Frank LA. Clinical pharmacology of rifampin. *J Am Vet Med Assoc* 1990; **197**: 114–7.
- Peleg O, Baneth G, Eyal O *et al*. Multiplex real-time qPCR for the detection of *Ehrlichia canis* and *Babesia canis vogeli*. *Vet Parasitol* 2010; **173**: 292–9.
- Dawson JE, Rikihisa Y, Ewing SA *et al*. Serologic diagnosis of human ehrlichiosis using two *Ehrlichia canis* isolates. *J Infect Dis* 1991; **163**: 564–7.

- 19** Keysary A, Waner T, Rosner M *et al.* The first isolation, in vitro propagation, and genetic characterization of *Ehrlichia canis* in Israel. *Vet Parasitol* 1996; **62**: 331–40.
- 20** MacWilliams PS. The spleen. In: Cowell RL, Tyler RD, Meinkoth JH *et al.*, eds. *Diagnostic Cytology and Hematology of the Dog and Cat*. St Louis, MO: Mosby Elsevier, 2008; 330–8.
- 21** Mylonakis ME, Day MJ, Leontides LS *et al.* Type of smear may influence thrombopoietic cell counts in the bone marrow of clinically healthy dogs. *Vet Clin Pathol* 2005; **34**: 358–61.
- 22** Siarkou VI, Mylonakis ME, Bourtzi-Hatzopoulou E *et al.* Sequence and phylogenetic analysis of the 16S rRNA gene of *Ehrlichia canis* strain in dogs with clinical monocytic ehrlichiosis. *Vet Microbiol* 2007; **125**: 304–12.
- 23** Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994; **22**: 4673–80.
- 24** Branger S, Rolain JM, Raoult D. Evaluation of antibiotic susceptibilities of *Ehrlichia canis*, *Ehrlichia chaffeensis*, and *Anaplasma phagocytophilum* by real-time PCR. *Antimicrob Agents Chemother* 2004; **48**: 4822–8.
- 25** Greene CE, Calpin J. Antimicrobial drug formulary. In: Greene CE, ed. *Infectious Diseases of the Dog and Cat*. St Louis, MO: Elsevier, 2012; 1207–320.
- 26** Neer TM, Eddlestone SM, Gaunt SD *et al.* Efficacy of enrofloxacin for the treatment of experimentally induced *Ehrlichia canis* infection. *J Vet Intern Med* 1999; **13**: 501–4.
- 27** Harrus S, Waner T, Aizenberg I *et al.* Amplification of ehrlichial DNA from dogs 34 months after infection with *Ehrlichia canis*. *J Clin Microbiol* 1998; **36**: 73–6.
- 28** Baneth G, Harrus S, Ohnona FS *et al.* Longitudinal quantification of *Ehrlichia canis* in experimental infection with comparison to natural infection. *Vet Microbiol* 2009; **136**: 321–5.
- 29** Kadlec K, van Duijkeren E, Wagenaar JA *et al.* Molecular basis of rifampin resistance in methicillin-resistant *Staphylococcus pseudintermedius* isolates from dogs. *J Antimicrob Chemother* 2011; **66**: 1236–42.
- 30** Breitschwerdt EB, Chomel BB. Canine bartonellosis. In: Greene CE, ed. *Infectious Diseases of the Dog and Cat*. St Louis, MO: Elsevier, 2012; 552–63.
- 31** Guptill-Yoran L. Feline bartonellosis. In: Greene CE, ed. *Infectious Diseases of the Dog and Cat*. St Louis, MO: Elsevier, 2012; 545–52.
- 32** Ledbetter EC, Landry MP, Stokol T *et al.* *Brucella canis* endophthalmitis in 3 dogs: clinical features, diagnosis and treatment. *Vet Ophthalmol* 2009; **12**: 183–91.
- 33** Vinayak A, Greene CE, Moore PA *et al.* Clinical resolution of *Brucella canis*-induced ocular inflammation in a dog. *J Am Vet Med Assoc* 2004; **224**: 1804–7.
- 34** Kontos V, Athanasiou L. Use of enrofloxacin in the treatment of acute canine ehrlichiosis. *Canine Pract* 1998; **23**: 10–4.
- 35** Matthewman LA, Kelly PJ, Brouqui P *et al.* Further evidence for the efficacy of imidocarb dipropionate in the treatment of *Ehrlichia canis* infection. *J S Afr Vet Assoc* 1994; **65**: 104–7.
- 36** Sainz A, Tesouro MA, Amusatequi I *et al.* Prospective comparative study of 3 treatment protocols using doxycycline or imidocarb dipropionate in dogs with naturally occurring ehrlichiosis. *J Vet Intern Med* 2000; **14**: 134–9.
- 37** Eddlestone SM, Neer TM, Gaunt SD *et al.* Failure of imidocarb dipropionate to clear experimentally induced *Ehrlichia canis* infection in dogs. *J Vet Intern Med* 2006; **20**: 840–4.