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Doxycycline-Chloroquine Combination for the Treatment of Canine Monocytic Ehrlichiosis

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ABSTRACT

***Background*:** Ehrlichiosis in dogs is a vector borne disease caused by *Ehrlichia canis*, from the Anaplasmataceae family, capable of causing multisystemic disorders. Following an incubation period of 8-20 days, acute, subclinical and chronic forms of the disease may develop and affected dogs frequently showed hemorrhage, lymphadenopahty, splenomegaly, hepatomegaly, along with cardiac/renal disorders and myelosupression. Most of the untreated dogs spontaneously recover form the acute phase and enters the subclinical phase, in which some of them may develop chronic phase characterized by bone marrow aplasia. Ehrlichial organisms are commonly susceptible to tetracycline derivatives, and doxycycline is probably most commonly used for treatment of the infection. Doxycycline may be quite effective for clearing parasitemia in acute *E. canis* infection. Clinical recovery may be observed within 2-3 days, besides treatment should be continued for 3 weeks, as some cases may remain carriers even if short treatment protocole is administered. Most dogs infected with CME usually recover from the acute and subclinical phases when treated with doxycycline or other tetracyclines. Some dogs enter the chronic phase of the disease for which the prognosis is grave. The purpose of the present study was to report the presence of *E. canis* infection in dogs in Aydin, with a special reference to the efficacy of combined doxycycline and chloroquine therapy.

***Materials, Methods & Results*:** A propective study was carried out on dogs referred and 12 dogs were diagnosed as canine monocytic ehrlichiosis within traditional buffy coat smear within giemsa staining, Snap 4dx test and nested PCR applications. Data on rectal temperature, clinical haematology and serum biochemistry (involving vbALT, AST and BUN) were registered before and after treatment in both groups. A total of 6 dogs with a diagnosis of CME were enrolled in each group. Group CD (n = 6) received doxycycline (10 mg/kg perorally twice a day for 2 weeks) and chloroquine (2.5 mg/kg perorally twice a day for 2 weeks) and group D received solely doxycycline (10 mg/kg perorally twice a day for 2 weeks). The clinical haematology and biochemistry results of canine ehrlichiosis before (day 0) and after treatment (day 30) for both gorups were compared. Among relevant parameters of aforementioned groups, no statistically significant differences were detected ($P > 0.05$). Following treatment in both groups the clinical signs were dinished, body temperature of the dogs returned to physiological levels in both groups. Lymphadenopathy was regressed in 1 week, and 1 month later the clinical examination was repeated in which all dogs in combined treated group showed complete clinical recovery, whereas lymphadenopathy was stil evident in some of the dogs in chloroquine group.

***Discussion*:** Doxycycline has still been the first line-drug option for the treatment of acute canine monocytic ehrlichiosis, however for the subclinical and chronic phases of the diseases its effectiveness is controversial. Further research are warranted to investigate any adjuvant or combined therapy may be an alternatives. In this clinical trial combined doxycycline and chloroquine therapy were used for dogs with active *Ehrlichia canis* infection (PCR + and *E. canis* seroactive). Although there were no statistically significant hematological and serum biochemical differences among dogs treated with doxycycline and chloroquine in contrast to dogs treated alone with doxycycline, clinical recovery was impressive in dogs with combined treatment. These observations demonstrate that chloroquine may have helped hastening the relevant clinical signs of canine monocytic ehrlichiosis and clinical improvement.

Keywords: canine, monocytic, ehrlichiosis, doxycycline, chloroquine.

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INTRODUCTION

Ehrlichia canis causes canine monocytic ehrlichiosis (CME), which was first described in Algeria in 1935 [7]. CME is currently reported throughout the world but at higher frequencies in tropical and subtropical regions [13,16,22,26,31,32]

E. canis infection among dogs in Turkey was previously detected by serological methods [3,6]. Currently, the disease may be found throughout the country and is considered to be endemic in Eagean regions especially in Aydin province [15,28,30]. In a previous national/local research project regarding vector borne diseases in Eagean region, in which *E. canis* was detected, within rapid in-house diagnostic test kits, in a vey high dog population examined. Although the results were not published a seroprevalence rate of 25-30% were reported [Unpublished data]. Another study reported a seroprevalence of 36.2% *E. canis* seropositivity among 224 dogs [28] and with nested PCR 41.5%.

CME is a multisystemic disease manifesting in acute, subcinical or chronic forms. *Ehrlichia canis* may infect all breeds of dogs but some af the breeds might be more susceptible, showing severe clinical signs [19]. The acute form is characterized by a high fever, depression, lethargy, anorexia, lymphadenomegaly, splenomegaly and hemorrhagic tendencies [12,17] and thrombocytopenia [10]. The chronic phase symtoms similar to those of observed in the acute phase, but with a greater severity [12,19]. Ehrlichial organisms are commonly susceptible to tetracycline derivatives, and doxycycline is probably most commonly used for treatment of the infection. In the present study the aim was to emphasize, with a special reference to, the efficacy of combined doxycycline and chloroquine therapy.

MATERIALS AND METHODS

Study design and data collection

A propective study was carried out on dogs referred to the Department of Internal Medicine, Faculty of Veterinary, Adnan Menderes University between January and october 2011. Twelve dogs were diagnosed as canine ehrlichiosis within traditional buffy coat smear and giemsa staining, snap 4dx test and PCR applications. Data on rectal temperature, clinical haematology and serum biochemistry (involving vbALT, AST and BUN) were registered before and after treatment in both groups.

All dogs had classical historical and clinical features of CME. Various purebred dogs (3 German shepherd, 2 golden retriever, 2 terrier, 1 boxer, doberman and miniature pinscher, respectively) and crossbred (n = 2) were represented, including 7 males and 5 females. Their ages ranged from 1 to 8 years. All dogs had prior contact with ticks observed, none of them had had antiparasitic drug applications, nor had prior therapy against any disease condition diagnosed.

Enrolled cases were forwarded to Department of Internal Medicine and sequentially randomized to treatment. A total of 6 dogs with a diagnosis of CME were enrolled in each group. Group CD (n = 6) received doxycycline hyclate[1] (10 mg/kg perorally twice a day for 2 weeks) and chloroquine[2] (2.5 mg/kg perorally twice a day for 2 weeks) and group D received solely doxycycline hyclate1 (10 mg/kg perorally twice a day for 2 weeks).

All dogs had classical historical and clinical features of CME (i.e. depression, lethargy, anorexia, pyrexia and lymphadenopathy). Various purebred dogs and mongrels were represented, including 11 males and 12 females (Table 1). Their ages ranged from 1.5 to 9.5 years, and they weighed between 6.8 and 52.7 kg. The duration of clinical signs related to CME ranged from 1 to 7.5 years.

DNA extraction and nested PCR Analysis

DNA extraction was performed according to commercial High Pure PCR Template Preparation Kit[3]. DNA was extracted from 600 mL of stored EDTA-whole-blood samples that had been frozen at -20°C . Amplification PCR as described previously by Breitschwewerd *et al*. [4]. Briefly, with a 50 mL reaction mixture containing 1 µg of template DNA; 200 mM (each) dATP, dTTP, dCTP, and dGTP; 0.05 pmol (each) of the outer primers designated EHR-OUT1 and EHR-OUT2 , 12.5 pmol (each) of the iner primers designated GE2f and EHRL3-IP2; 2 mM MgCl; and 2.5 U of Taq DNA polymerase[4] in a 1X reaction buffer (50 mM KCl, 10 mM Tris HCl [pH 8.3]). The first round of amplification included denaturation at 94°C for 45 s, annealing at 50°C and the extension at 72°C for 1.5 min. The PCR cycle was repeated 20 times. For the second round of amplification 1,5 µL of the primary PCR products were used as the template in a total volume of 25 µL. The cycle rounds were all the same as first PCR except this cycle was repeated 50 times and followed by a final extension of 5 min at

Table 1. Haematological and serum biochemical analysis and their descriptive statistics for doxycycline and doxycycline + chloroquine groups before and after therapy.

Parameters	Doxycycline		Doxycycline + chloroquine	
	Before treatment	After	Before treatment	After treatment
	Mean	SD	Mean	SD
PLT	91.83(±40.46)	293,83(±21,2)	95,33(±34.0)	353,50(±51,4)
RBC	4,31 (±0,4)	5,60 (±0,5)	4,60 (±0,3)	5,41 (±0,2)
Haemoglobin	9,35 (±1,3)	12,06 (±1,1)	9,57 (±0.8)	11,25 (±0,3)
PCV	28,31 (±3,6)	31,69 (±3,5)	28,66 (±2.0)	32,69 (±1,0)
MCV	64 (±2,0)	60,16 (±2,0)	62,50 (±4,9)	60,33 (±1,1)
MCHC	33 (±1,0)	32,96 (±0,6)	31.05 (±2,7)	33,75 (±0.4)
WBC	10,08 (±1,6)	12,34 (±1,9)	11,42 (±1,9)	16,05 (±1,4)
AST	36,83 (±4,6)	34,16 (±4,7)	40,16 (±10,7)	42,00 (±6,2)
ALT	34,5 (±2,9)	45,66 (±5,0)	46,83 (±8,9)	48,83 (±6,4)
BUN	25,16 (±4,1)	29,33 (±1,6)	30,33 (±4,6)	26,50 (±1,7)

$P > 0.05$. No statistically significant differences were detected among groups before and after therapy.

72°C. All PCR products were electrophoresed through 1% agarose gels in Tris-boric acid-EDTA buffer, and the DNA fragments were visualized by ethidium bromide staining under UV fluorescence. The sizes of the products produced were 152 bp with the *Ehrlichia* genus-specific primers

Statistical analysis

All related data were checked for normal distribution within Shapiro-Wilk test and by Levene'stest (in terms of homogenity of the variaces). Logarithmic transformation of data was performed in an attempt to normalize the distribution. Repeated measures by two-way analysis of variance was conducted to assess the effect of applications (treatment) and time. In case of interactions among treatment applications and time post hoc Tukey test was used in an attempt to compare therapy duration and different drug application. $P < 0.05$ level was accepted as significant. Results were expressed as mean ± standart error.

RESULTS

Regarding official registration and according to the physical and other relevant examinations of 12 dogs involved, the most commonly observed clinical signs were pale mucous membranes, lymphadnopathy, fever and splenomegaly. *Ehrlichia canis* seroactivity was determined by in-house diagnostic Snap 4dx test able to evaluate and diffferentiate antibodies against *E. canis*, *Anaplasma phagocytophilum* and *Borrelia burgdorferi* and antigen towards *Dirofilaria immitis*. All 12 dogs were seropositive by snap 4dx test against *Erlichia canis*.

Active infection was described by the presence of organism-specific DNA sequences in the whole blood. Based on PCR analysis and *E. canis* serology results allowed further classification of the dogs involved in this study in 2 different stages: acute *E. canis* infection (no detectable antibody by snap 4dx test indeed with detectable DNA [PCR+]; active infection (PCR + and *E. canis* seroactive [detectable antibody by snap 4dx test]) [Figure 1].

The clinical haematology and biochemistry results of canine ehrlichiosis before (day 0) and after treatment (day 30) for both gorups was compared in Table 1. Among relevant parameters of aforementioned groups, no statistically significant differences

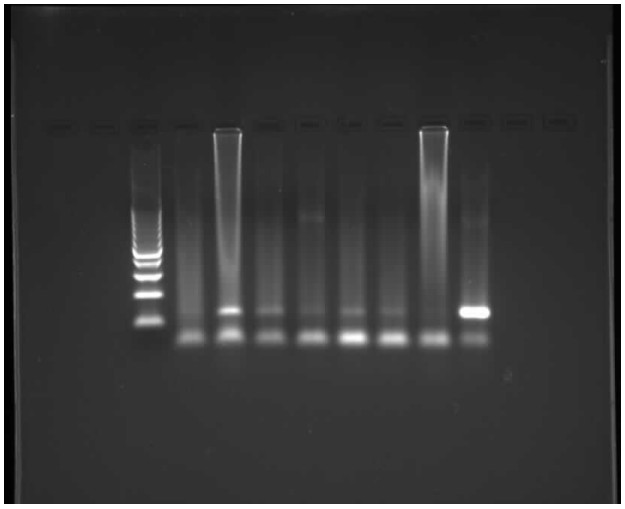

Figure 1. PCR analysis of nested PCR with primers EHR-OUT1, EHR-OUT2, and EHRL3-IP2 for the small 16 S rRNA gene from the *Ehrlichia* genus-specific primers 1: Molecular size markers 2: Negative control 3-8: Samples 9: Positive control (From tick). The PCR products was electrophoresed on 1.5% agarose gel and stained by ethidium bromide.

were detected ($P > 0.05$). Following treatment in both groups the clinical signs were dinished, body temperature of the dogs returned to physiological levels in both groups. Lymphadenopathy was regressed in 1 week, and 1 month later the clinical examination was repeated in which all dogs in combined treated group showed complete clinical recovery, whereas lymphadenopathy was stil evident in some of the dogs in chloroquine group (data not shown).

DISCUSSION

Ehrlichial organisms are susceptible to tetracycline derivatives, and doxycycline [2] may be effective for clearing parasitemia in acute Erlichia canis infection. Clinical recovery may be observed within a few days, however treatment should be commenced for 3 weeks, because some cases may remain carriers even if short treatment protocole is applied [9,11,14]. Most dogs infected with CME usually recover from the acute and subclinical phases when treated with doxycycline or other tetracyclines [9,11]. Some dogs enter the chronic phase of the disease for which the prognosis is grave [18]. Eventhough doxycycline treatment is traditionally used in dogs infected with *E. canis* remain infected [14,34].

Thus relevant information is required to determine effective therapy protocole for CME. Several factors may have influence on the efficacy of threapy applications in infected cases such as the disease phase and schedule of antibiotic administration [23].

The results of the present study confirmed the efficacy of doxycycline+chloroquine combination for amelioration of clinical signs during active phase of CME disease, however we did not performed PCR analysis after treatment, therefore we can not speculate about the potential clearence of *E. canis* from peripheral blood. In the doxycycline+chloroquine group all of the parameters measured returned to normal levels after initiation of this protocole. To the present authors' knowledge this is the first report of attempted doxycycline+chloroquine combination therapy regimen for CME, and the latter result suggests that this protocole could be more efficacious for CME management.

Chloroquine was reported as a chemotherapeutic agent for treatment and prevention of malaria in human being [1,30]. Besides it is the gold standard therapy protocole against malaria because of effectively usage, reasonable price and safety [1,30]. Among relevant antimalarials, chloroquine is one of the choice with the longest half-lives (approximately 60 days), providing a chemoprophylactic effect during the drug elimination phase [21,25]. Besides chloroquine also exposes the parasites to an extended time period although after which it falls down below the therapeutical concentration [25]. In addition chloroquine has been used as a treatment protocole for anti-inflammatory conditions [35,36] .

Published data indicate that serum levels of proinflammatory cytokines involving tumor necrosis factor (TNF)-α [8], interferon-gamma (IFN-gamma) [27], interleukin (IL)-1β and IL-8b [29] are changed in *E. canis* infection. In the latter study *E. canis* induced chronic expression of selected proinflammatory cytokines in dogs experimentally infected. High levels of (IL)-1β and IL-8 observed in that study was suggested in association with clinical signs [29]. In another study evaluating the expression of cytokines, TNF-α has been found to play a role in the pathogenesis of acute canine ehrlichiosis and treatment with doxycycline reduced the systemic effects of the latter cytokine, by reducing or eliminating parasitemia load [8].

Chloroquine has been widely used for its anti-inflammatory effects and treatment of various disorders [20,35]. The latter drug lowered some of the proinflammatory cytokines, in human cases with systemic lupus erythematosus, due to its anti-inflammatory effects [35]. Chloroquine also may be of beneficial in a variety of bacterial and fungal pathogens, both by direct and indirect mechanisms [33]. Therefore it may have the potential of reducing infection for microbiological agents [33]. Due to its evidenced and proofed efficacy against malaria and above mentioned potential effects against various agents, the present authors decided to use chloroquine in combination with doxycycline against *E. canis* infection. Although we did not measured the levels of proinflammatory cytokines in dogs enrolled in the present study chloroquine might have helped to lower the levels of those cytokines within within its anti-inflammatory effects and therefore may hastened clinical recovery in *E. canis* infection, as reported previously [8].

Interestingly, although not statistically significant, BUN levels after therapy was decreased in contrast to initial values in dogs treated with double combination (doxycycline/chloroquine). This may be partly explained within the chloroquine therapy possibly inducing a marked decrease of circulating im-

munocomplexes [24], that might probably be involved in immune-complex glomerulonephritis [2] due to *Ehrlichia* infection. The established efficacy of chloroquine for treatment of autoimmune diseases besides its effectiveness for blocking antigen presentation [5] may all contribute its usefullness, as it is suggested in the present study.

Although several studies regarding canine monocytic ehrlichiosis have been documented, the clinical study presented herein is, to the best of our knowledge, the first study reporting doxycycline/chloroquine combination against canine monocytic ehrlichiosis in Aydin province, Turkey. Detailed clinical trials invoving double combination (doxycycline/chloroquine) must be analysed in a larger sampled dog population with monocytic ehrlichiosis.

SOURCES AND MANUFACTURERS

[1]Monodoks capsule, 100 mg., Deva, Turkey.
[2] Kutlu tablet, 250 mg., Abdi brahim, Turkey.
[3]Roche Diagnostics, Mannheim, Germany.
[4]Promega, Madison, Wisconsin, USA.

***Declaration of interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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