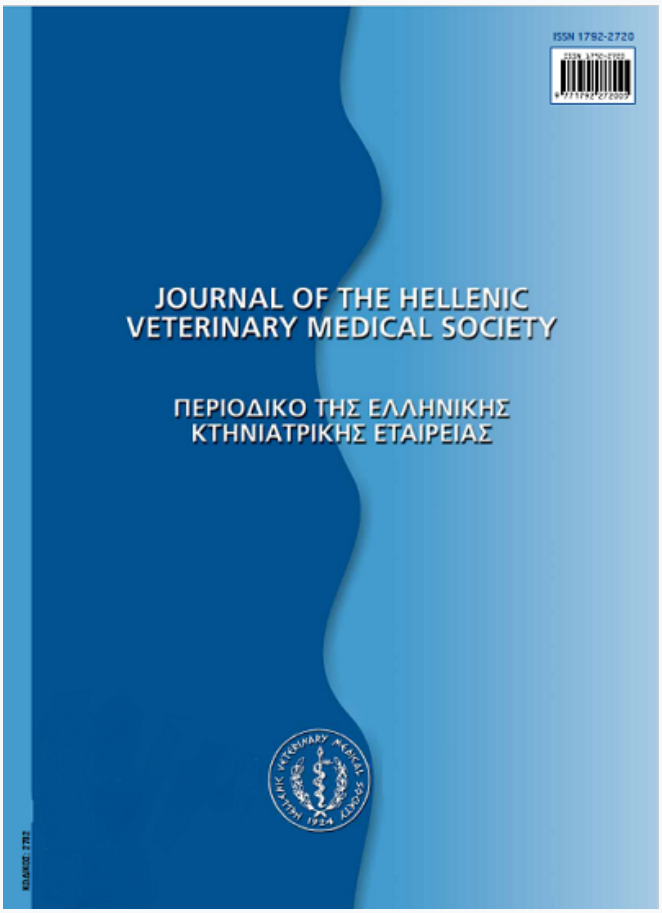
Journal of the Hellenic Veterinary Medical Society

Vol 57, No 3 (2006)



Seroprevalence of Ehrlichia canis infection among privately-owned dogs in northern Bulgaria

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doi: 10.12681/jhvms.15041

ΠΕΡΙΟΔΙΚΟ ΤΗΣ ΕΛΛΗΝΙΚΗΣ ΚΤΗΝΙΑΤΡΙΚΗΣ ΕΤΑΙΡΕΙΑΣ 2006, 57(3): 212-216
JOURNAL OF THE HELLENIC VETERINARY MEDICAL SOCIETY 2006, 57(3): 212-216

Ερευνητική
Original article

Ορολογική διερεύνηση για τη μόλυνση από *Ehrlichia canis* σε δεσποζόμενους σκύλους στη βόρεια Βουλγαρία

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ΠΕΡΙΛΗΨΗ. Σκοπός της μελέτης αυτής ήταν η ανίχνευση αντισωμάτων κατά της *Ehrlichia canis* σε δεσποζόμενους σκύλους που ζουν στη βόρεια Βουλγαρία και συγκεκριμένα στις περιοχές Varna, Silistra, Ruse, Montana, Veliko Tarnovo και Pleven. Συνολικά εξετάστηκαν με τη μέθοδο IFA 120 οροί αίματος που πάρθηκαν από ισάριθμους σκύλους για την ανίχνευση IgG αντισωμάτων κατά της *E. canis*. Σαράντα πέντε σκύλοι (37.5%) βρέθηκαν ορολογικά θετικοί, με τις περιοχές της Varna και της Silistra να παρουσιάζουν τα υψηλότερα ποσοστά (60% και 55%, αντίστοιχα), σε σύγκριση με τις άλλες (Ruse: 30%, Montana: 30%, Veliko Tarnovo: 25%, Pleven: 25%). Τα αποτελέσματα της μελέτης αυτής δείχνουν ότι το ποσοστό έκθεσης των δεσποζόμενων σκύλων που ζουν στη βόρεια Βουλγαρία στην *E. canis* είναι υψηλό.

Seroprevalence of *Ehrlichia canis* infection among privately-owned dogs in northern Bulgaria

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ABSTRACT. A serologic survey was carried out to detect the prevalence of *Ehrlichia canis* antibodies among privately-owned dogs in the counties of Varna, Silistra, Ruse, Montana, Veliko Tarnovo and Pleven of northern Bulgaria. A total of 120 serum samples were IFA tested for anti-*Ehrlichia canis* IgG antibodies. A mean seroprevalence rate of 37.5% was recorded with Varna county showing the highest value (60%) followed by Silistra (55%), Ruse (30%), Montana (30%), Veliko Tarnovo (25%) and Pleven (25%) counties. These results clearly show that dogs residing in northern Bulgaria are highly exposed to *E. canis*.

Key words: dog, *Ehrlichia canis*, seroprevalence, northern Bulgaria, IFA test

INTRODUCTION

The genus Ehrlichia, based on the 16SrRNA gene sequence, includes *Ehrlichia canis, Ehrlichia chaffeensis, Ehrlichia ewingii, Ehrlichia ruminantium* and *Ehrlichia muris* (Dumler et al. 2001). *Ehrlichiae* are small, gram-negative, pleomorphic, obligatory intracellular bacteria that primarily infect leukocytes (Ristic et al. 1984, Rikihisa 1991).

Ehrlichia canis is the main aetiological agent of canine monocytic ehrlichiosis (CME), an infectious disease that was first described in dogs from Algeria (Donatien and Lestoquard 1935) and since then it has been identified in tropical and subtropical areas all over the world (Stephenson and Ristic, 1978, Keefe et al. 1982, Brouqui et al. 1991).

In the neighbouring country Greece, CME, due to *E. canis*, is considered the commonest tick-borne infection among the canine population (Kontos and Athanasiou 1998), while in the Mediterranean region of Turkey (Adana), Batmaz et al. (2001) reported a 65% seroprevalence.

Epidemiological data, which focused on the seroprevalence of CME in the southern part of Bulgaria, have recently been published (Tsachev et al. 2006). The objective of this study was to assess the exposure of privately-owned dogs to *Ehrlichia canis* by IFA serology in six counties of northern Bulgaria.

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Ημερομηνία υποβολής: 26.10.2006
Ημερομηνία εγκρίσεως: 10.01.2007

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Submission date: 26.10.2006
Approval date: 10.01.2007

MATERIALS AND METHODS

Study area and animals

The survey was carried out in the northern part of Bulgaria (approximately 42° 52' and 44° 06' N latitude, 22° 50' and 28° 20' W longitude), the climate of which is typically continental.

Veterinarians from six private clinics were requested to collect serum samples from privately-owned dogs admitted for medical-surgical problems or routine health care. In total, 120 dogs of different breeds were sampled and geographically allocated as follows: Varna (n=20), Silistra (20), Rouse (n=20), Montana (20), Veliko Tarnovo (n=20) and Pleven (n=20). Their age ranged from 1 to 12 years (mean ± SD: 4.26 ± 0.43) and further characterized as young adults (n:31), adults (n:66) and middle aged to elderly dogs (n:23). All the animals entered the study were diseased dogs (various internal medicine or surgical problems) with a lifestyle of both indoor and outdoor living. Additionally, all the animals had a history of previous tick exposure. Animal selection was done by random.

Blood sampling

Five ml of peripheral blood were obtained from each of the dogs by applying cephalic venipuncture and vacuum tubes with clot activator (Vacuette®). Blood was allowed to coagulate at room temperature and sera were separated by centrifugation at 5.000 rpm for 10 minutes and kept at -200°C until assayed. All blood samples were collected between February and March 2004.

IFA test

All sera were assayed by the IFA test for the detection of specific to *E. canis* IgG antibodies. The antigen applied was a formalin-inactivated cellular suspension ($2x10^6$/cells/ml) infected with *Ehrlichia canis* (Synbiotics Europe, France) and fixed in 15-well special immunofluorescence slides. After serial dilutions (1:100, 1:200, 1:400, 1:800 and 1:1600) in PBS (pH 7.2), sera (10μl) were added to the slides. Positive (serum provided by Professor Kontos, National School of Public Health, Athens) and negative (physiological saline) control sera were also tested. Slides were incubated at 37°C for 30 min and washed twice in PBS (5 min each time). The monospecific rabbit anti-canine IgG labelled with fluorescein isothiocyanate (Sigma, Germany) was added at a 1/25 dilution in PBS and further incubated for 30 min at 37°C. Following washing as before, slides were air-dried, mounted with Fluoprep (Bio-Merieux, France) and observed under a fluorescence microscope (Olympus, Japan) at 400x magnification. Titers equal to or in excess of 1/100 were considered positive.

Statistical analysis

Biostatistical analysis was performed by the statistical SPSS package, version 12.0 for Windows (SPSS Inc., Chicago, IL, USA). Chi-square (x^2) test was used for the comparison of prevalence rates among studied counties as well as age groups, while Fisher's exact test was used in the comparison of the corresponding rates between sexes.

The level of 5% was considered significant for all the comparisons made.

RESULTS

Seropositive dogs were found in all regions with a seroprevalence varying from 25% to 60% (Tables 1 and 2). The highest prevalence was noticed in Varna (60%) and Silistra (55%), whereas the lowest in both Veliko Tarnovo and Pleven (25%, each). Forty five out of 120 sampled dogs were found seropositive thus rising the mean seroprevalence to 37.5%. Antibody titre distribution was 1:100 in 22 dogs, 1:200 in 15, 1:400 in 7 and 1: 800 in one dog (Table 1).

Comparison of seroprevalence rates between different counties showed no significant differences (P>0.05). Also, the comparison of seroprevalence rates between age groups and between sexes showed no significant differences (P>0.05).

DISCUSSION

The prevalence of *E. canis* is much dependent on the geographical distribution of its arthropod vector *Ripicephalus sanguineus,* which is mainly found in the tropical and subtropical regions of the world, including Bulgaria (Serbezov 2002). The results of this survey are in agreement with a recently published study on canine ehrlichiosis in southern Bulgaria also showing a high seroprevalence rate (30%) among pet dogs (Tsachev et al. 2006). Consequently, in both southern and northern parts of Bulgaria the infection rate to *Ehrlichia canis* among pet dogs is high (30 and 37.5%, respectively).

In serosurveys conducted in Algeria, Israel, Egypt, Mexico, Spain, Turkey and South Africa, it has been shown that the prevalence of *E.canis* infection ranges from 9 to 53% (Donatien and Lestoquard 1935, Baneth et al. 1996, Botros et al. 1995, Rodriguez-Vivas et al. 2005, Saintz et al. 1996, Batmaz et al. 2001, Pretorius and Kelly 1998). These variations have been attributed to certain epidemiological factors, such as abundance and distribution of the vector, animal behavior and the average age of the study population (Serbezov 2002). Additionally, IFA cut off point in some of the above mentioned studies was below 1/100, hence increasing the sensitivity and seropositivity rate in the study. The

Table 1. Serological evaluation of *E.canis* infection from different regions of the Northern Bulgaria

Region	Varna	Silistra	Rouse	Montana	V.Tarnovo	Pleven	Total
Number of tested dogs	20	20	20	20	20	20	120
Number of positive dogs	12	11	6	6	5	5	45
Seroprevalence (%)	60	55	30	30	25	25	37.5
Titer							
1:100	6	4	4	4	2	2	22 (49%)
1:200	2	3	2	2	3	3	15 (33%)
1:400	3	4					7 (16%)
1:8001	1						(2%)

Table 2. Seropositive dogs to *E.canis* according to their age and sex in each county in northern Bulgaria

Overall population			County											
			Varna		Silistra		Rouse		Montana		V. Tarnovo		Pleven	
	total	positive	total	positive	total	positive	total	positive	total	positive	total	positive	total	positive
Classes of age														
[young adults]	31	11	4	2	5	4	3	2	9	3	7	0	3	0
[middle aged]	66	28	7	7	12	7	15	4	10	3	9	3	13	4
[elder]	23	6	9	3	3	0	2	0	1	0	4	2	4	1
Sex														
Males	65	25	11	6	9	7	12	4	9	3	12	2	12	3
Females	55	20	9	6	11	4	8	2	11	3	8	3	8	2

results of this study did not reveal any correlation between the age of the animals and seropositivity rate. This finding is in agreement with some reports (Botros et al. 1995, Baneth et al. 1996, Harrus et al. 1997), but not with others (Batmaz et al. 2001). As a result, *E.canis* seems to affect equally young adults, middle aged and elder dogs. Although neither the age nor the sex seems to be related to the infection, lifestyle should be considered critical, since outdoor living facilitates tick infestation. Some of these dogs were probably suffering from acute CME, subsequently recovered, but remained seropositive at the time of testing, while others would be considered subclinical carriers (Stephenson and Ristic 1978, Keefe et al. 1982). However, the seropositivity in some dogs may be the result of cross-reactivity to other *Ehrlichia* species (Mylonakis et al. 2003). *E. chaffeensis,* which is the causative agent of human monocytic ehrlichiosis (HME), was first described in the USA in 1987 (Anderson et al. 1991). Subsequent studies have demonstrated that human infections also occur in Europe (Morais et al. 1991) and Asia (Uhaa et al. 1992).

In the USA, dogs are potential reservoirs of *E. chaffeensis* organism and are susceptible to both natural and experimental infections (Davoust 1994). It has been shown that sera from dogs infected with *E.canis* or *E.chaffeensis* are highly cross-reactive (Dawson et al. 1996). Cross-reactivity can occur with some other members of *Ehrlichia* and *Neorickettsia* genera, some of which can infect dogs (Dumler et al. 2001). These species include *Anaplasma phagocytophilum* (Harrus et al. 1997), *Neorickettsia risticii* (Saintz et al. 1996), *Neorickettsia helminthoeca* (Rodriguez-Vivas et al. 2005), *Ehrlichia ewingii* (Anderson et al. 1992) and *Ehrlichia ruminantium* (Leib and Monrroe 1997). However, IFA for *E. canis* is the most commonly used technique to monitor canine ehrlichiosis infections (Waner et al. 2001, Belanger et al. 2002). Furthermore, it has been shown that the majority of the *E.ewingii* and *A.phagocytophilum* molecular test positives are negative by *E.canis* IFA (Sirigireddy and Ganta 2005). Western Blot and real time reverse transcriptase PCR have been

quite useful in the validation of IFA specificity for *E.canis* (Suksawat et al. 2001, Sirigireddy and Ganta 2005). Accordingly, although cross-reactivity cannot be excluded, our results could be considered quite reliable regarding the seroprevalence of *E.canis* among privately-owned dogs in northern Bulgaria.

In conclusion, these results indicate a high exposure to *E. canis* of dogs residing in northern Bulgaria that necessitates the raise of suspicion among clinicians, dog owners and travelers who visit this part of the country for this potentially fatal infectious disease of dogs.

Acknowlegments

The authors would like to gratefully thank the staff of Athens Animal Hospital, Athens, Greece for their technical assistance and collaboration and Mr Zavras Dimitrios for statistical analysis. ❑

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