Ορολογική διερεύνηση για τη μό­
λυση από Ehrlichia canis σε δε-
σποζόμενους σκύλους στη βόρεια
Βουλγαρία

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ΠΕΡΙΛΗΨΗ. Σκοπός της μελέτης αυτής ήταν η ανίχνευση αντι­
σωμάτων 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 εξε­
tάστηκαν 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.

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,

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.

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% sero-
prevalence.

Epidemiological data, which focused on the sero-
prevalence 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.

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
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), Rousse (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 -20°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 (2x10⁶/cells/ml) infected with Ehrlichia canis (Symbiotics 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 Fluorep (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 (x²) 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
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* (Harms 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 Monroe 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

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

<table>
<thead>
<tr>
<th>Region</th>
<th>Varna</th>
<th>Silistra</th>
<th>Rouse</th>
<th>Montana</th>
<th>V.Tarnovo</th>
<th>Pleven</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of tested dogs</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>120</td>
</tr>
<tr>
<td>Number of positive dogs</td>
<td>12</td>
<td>11</td>
<td>6</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>45</td>
</tr>
<tr>
<td>Seroprevalence (%)</td>
<td>60</td>
<td>55</td>
<td>30</td>
<td>30</td>
<td>25</td>
<td>25</td>
<td>37.5</td>
</tr>
<tr>
<td>Titer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:100</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>1:200</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>1:400</td>
<td>3</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>1:8001</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(2%)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Classes of age</th>
<th>Varna total</th>
<th>Varna positive</th>
<th>Silistra total</th>
<th>Silistra positive</th>
<th>Rouse total</th>
<th>Rouse positive</th>
<th>Montana total</th>
<th>Montana positive</th>
<th>V.Tarnovo total</th>
<th>V.Tarnovo positive</th>
<th>Pleven total</th>
<th>Pleven positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>[young adults]</td>
<td>31</td>
<td>11</td>
<td>4</td>
<td>2</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>9</td>
<td>3</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>[middle aged]</td>
<td>66</td>
<td>28</td>
<td>7</td>
<td>7</td>
<td>12</td>
<td>7</td>
<td>15</td>
<td>4</td>
<td>10</td>
<td>3</td>
<td>9</td>
<td>13</td>
</tr>
<tr>
<td>[elder]</td>
<td>23</td>
<td>6</td>
<td>9</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>65</td>
<td>25</td>
<td>11</td>
<td>6</td>
<td>9</td>
<td>7</td>
<td>12</td>
<td>4</td>
<td>9</td>
<td>3</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>Females</td>
<td>55</td>
<td>20</td>
<td>9</td>
<td>6</td>
<td>11</td>
<td>4</td>
<td>8</td>
<td>2</td>
<td>11</td>
<td>3</td>
<td>8</td>
<td>3</td>
</tr>
</tbody>
</table>
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.

**Acknowledgments**

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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