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Allison M. Liddell
Washington University School of Medicine in St. Louis

Steven L. Stockham
University of Missouri

Michael A. Scott
University of Missouri

John W. Summer
Centers for Disease Control

Christopher D. Paddock
Centers for Disease Control

See next page for additional authors

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Authors
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Predominance of *Ehrlichia ewingii* in Missouri dogs

A. Allison M. Liddell,† Steven L. Stockham,‡ Michael A. Scott,§ John W. Sumner,Christopher D. Paddock,¶ Monique Gaudreault-Keener,¶ Max Q. Arens,¶ and Gregory A. Storch¶,†,‡,*

Department of Internal Medicine and Pediatrics,† Washington University School of Medicine, and Diagnostic Virology Laboratory, St. Louis Children's Hospital,‡ St. Louis, and Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Missouri–Columbia, Columbia,‡ Missouri, and Viral and Rickettsial Zoonoses Branch, Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia.§

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To investigate the species distribution of *Ehrlichia* present in Missouri dogs, we tested 78 dogs suspected of having acute *ehrlichiosis* and 10 healthy dogs. Blood from each dog was screened with a broad-range 16S rRNA gene PCR assay that detects known pathogenic species of *Ehrlichia* and *Anaplasma*. The species was determined by using species-specific PCR assays and nucleotide sequencing. *Ehrlichia* antibody testing was performed by using an indirect immunofluorescence assay with *Ehrlichia* chaffeensis as the antigenic substrate. The broad-range assay detected *Ehrlichia* or *Anaplasma* DNA in 20 (26%) of the symptomatic dogs and 2 (20%) of the asymptomatic dogs. *E. ewingii* accounted for 20 (91%) and *E. chaffeensis* accounted for 1 (5%) of the positives. *Anaplasma* phagocytophilum DNA was detected in one dog, and the sequences of regions of the 16S rRNA gene and the groESL operon amplified from the blood of this dog matched the published sequences of this organism. Antibodies reactive with *E. chaffeensis* were detected in 14 (67%) of the 21 PCR-positive dogs and in 12 (19%) of the 64 PCR-negative dogs. Combining the results of PCR and serology indicated that 33 (39%) of 85 evaluable dogs had evidence of past or current *ehrlichiosis*. We conclude that *E. ewingii* is the predominant etiologic agent of canine *ehrlichiosis* in the areas of Missouri included in this survey. *E. canis*, a widely recognized agent of canine *ehrlichiosis*, was not detected in any animal. The finding of *E. ewingii* in asymptomatic dogs suggests that dogs could be a reservoir for this *Ehrlichia* species.

*Ehrlichiosis* is an important emerging infection of dogs and humans. The first species recognized, *Ehrlichia canis*, causes monocytic *ehrlichiosis* in dogs. A closely related species, *E. chaffeensis*, was subsequently identified as the cause of an *E. canis*-like disease in dogs. A closely related species, *E. chaffeensis*, has also been detected in *E. canis*-infected dogs (12), coyotes (21), goats (13), and deer (3, 10). A more closely related species, *E. ewingii*, was initially recognized as the cause of granulocytic *ehrlichiosis* in dogs (15) and was recently found to cause asymptomatic carriers of granulocytic *ehrlichiosis* in humans (7). Most cases of human granulocytic *ehrlichiosis* are caused by a species referred to as the agent of human granulocytic *ehrlichiosis* (4). This bacterium has also been detected in dogs (19), deer (5), foxes (20), and rodents (31). The name *Anaplasma* phagocytophilum has recently been proposed to include this bacterium, in addition to the species previously known as *B. phagocytophilum* and *E. equi* (14), and this proposed name is used in the present study.

Most studies of the prevalence of infection with *Ehrlichia* spp. in dogs have been based on serologic methods that do not provide identification of the species that elicits production of anti-*Ehrlichia* antibodies in the host animal. Four studies have used molecular techniques and/or cell culture methods to identify the *Ehrlichia* species infecting dogs. In these studies, carried out in North Carolina (6, 22), Virginia (11), and Oklahoma (25), 24 dogs were infected with *E. chaffeensis*, 21 were infected with *E. canis*, 19 were infected with *E. ewingii*, 10 were infected with *E. platys*, and 1 was infected with *A. phagocytophilum*. A recent study described 15 dogs with *E. ewingii* infection, proven by PCR (18).

In our laboratory at Washington University Medical Center in St. Louis, Missouri, we have detected nearly 200 cases of *Ehrlichia* infection in recent years by using PCR; 89% of these cases were caused by *E. chaffeensis* and 11% were caused by *E. ewingii*. To learn more about possible relationships between humans and canine *ehrlichiosis*, we studied the occurrence and species distribution of *Ehrlichia* in pet dogs in Missouri. The focus of the study was on ill dogs with clinical manifestations suggestive of *ehrlichiosis*, but we also studied a small number of asymptomatic dogs.

**Materials and Methods**

Canine subjects and blood samples. Participating *Missouri* veterinarians were recruited by the staff at the *University of Missouri* College of Veterinary Medicine. Participating veterinarians were asked to submit blood samples from dogs that they suspected of having *ehrlichiosis* based on a distributed list of clinical manifestations of granulocytic or monocytic *ehrlichiosis*. Clinical manifestations included fever, evidence of a unipolar endocarditis, polyarthritis, splenomegaly, uveitis, seizures, hemoptysis, and eosinophilia. *Ehrlichia* is.

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presence of a coinfection in a peripheral blood smear, and presence of ticks on the dog. EDTA-anticoagulated whole blood and serum specimens were collected from each dog for laboratory testing. For each dog with suspected ehrlichiosis included in the study, veterinarians were also asked to submit whole-blood and serum specimen from another dog under their care at the same time that was not ill, e.g., dogs being seen for routine immunizations or dogs being boarded under the supervision of the veterinarian. Thirty-five veterinarians submitted samples from 88 dogs from March 2000 through January 2001; the same samples were mailed to the Veterinary Laboratory at St. Louis Children’s Hospital. The veterinarians also provided clinical and epidemiologic data for each dog by using a standardized data collection form. The first day of observed illness was known for 23 dogs. For these 23, samples were obtained after a median interval of 4 days (range, 0 to 31).

PCR testing. Leukocyte lysates were prepared from whole-blood specimens as described previously (7). A broad-range Ehrlichia PCR was performed with primers (ECA and HE3) that bind to conserved euts of the 16S rRNA gene that are conserved among all pathogens in Ehrlichia and A. phagocytophilum. The Ehrlichia species was determined by additional reactions with sets of primers specific for E. chaffeensis (HE1 and HE3) (2), E. ewingii (GW1 and HE3) (33), and E. canis (31). Same samples positive with the broad-range primers were also tested with primers EHR 521 and EHR 747 that amplify A. phagocytophilum, as well as other Anaplasm a spp. (27). Same samples positive with EHR 521 and EHR 747 were also tested with primers GEF9F and GEF2 that amplify a portion of the 16S rRNA gene of A. phagocytophilum, as well as the closely related white-tailed deer agent (23), and also with a nested assay that specifically amplies a 256 bp segment of the A. phagocytophilum groEL gene (28).

Serology. Canine serum specimens were tested for IgM and IgG antibodies reactive with E. chaffeensis by using an indirect immunofluorescent-antibody assay (IFA), as described previously (9). Fluorescein isothiocyanate-labeled goat anti-dog IgG (specific) conjugate was used at a dilution of 1:50. Serum IgG was assessed at a 1:52 dilution; species-specific reactions at this dilution were compared to the endpoint. A monkey IgM was expressed as the greatest reciprocal dilution for which specific reactivity was observed. Dogs were considered seropositive if the IFA titer was >64.

Sequencing. Amplified products from the Ehrlichia broad-range assay performed on DNA extracted from canine whole blood were sequenced at Washington University School of Medicine. The sequencing reaction contained 125 ng of purified amplicon, 12 pmol of primer, 8 μl 2× loading buffer (Applied Biosystems, Inc., Foster City, Calif.), and 2 μl of the PCR DNA polymerase. Extension products were analyzed in an automated DNA sequencer (model 377; Applied Biosystems). The primers used for sequencing of the broad-range PCR product were HE3 (2) and PER1R (7).

Nucleotide sequence accession numbers. The GenBank accession number of the 1,256-bp groEL sequence amplified from a Missouri dog isAY219849.

Statistical methods. Categorical data were compared by using the Chi-square test or the Fisher exact test. A P value of 0.05 was considered statistically significant. Statistical analyses were carried out by using EpiInfo 2000 (Centers for Disease Control and Prevention).

RESULTS

A total of 88 pet dogs were included in the study, including 78 (89%) that were ill and 10 (11%) that were asymptomatic. The dogs included a wide variety of breeds, of which the most common were Labradors and Golden Retrievers (22, including 18 m, 4 f). Fifty-six percent were female, and the mean age was 4.6 years (range, 1 to 13 years). Fever and a monocytosis (i.e., lameness, reluctance to rise or walk, walking with a stiff or stiffened gait, or pain in the swollen joints) were the most frequent clinical findings. Other reported findings included current or recent hemorage, organomegaly, uveitis, and neurologic signs.

Routine laboratory test results were available for only a minority of the dogs and included that 19 had thrombocytopenia (platelet count: 200,000/μl), 20 had anemia (10 m, 10 f), 10 had leukopenia, and 4 had hypoglobulinemia. IgM antibodies to Borrelia burgdorferi were observed on peripheral blood smear from two dogs that were later found to be PCR positive for E. ewingii.

TABLE 1. Results of Ehrlichia PCR testing of Missouri dogs

<table>
<thead>
<tr>
<th>Status</th>
<th>Total no. of dogs</th>
<th>No. (%) of dogs PCR positive for:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ill</td>
<td>78</td>
<td>E. ewingii</td>
</tr>
<tr>
<td>Symptom atic</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Combined</td>
<td>88</td>
<td>20 (20%)</td>
</tr>
</tbody>
</table>

The results of PCR testing of the 88 dogs are shown in Table 1. Ehrlichia or Anaplasma DNA was detected in the blood of 22 (25%) of the 88 dogs, including 20 (26%) of the ill dogs and 2 (20%) of the asymptomatic dogs. Species-specific PCR testing revealed 19 infections with E. ewingii, 1 with E. chaffeensis, and 1 with A. phagocytophilum. One additional dog was determined to be positive by the broad-range assay but negative by the species-specific assays. The species identity of this dog’s infection was determined to be E. ewingii by nucleotide sequencing of a portion of the 16S rRNA gene. The failure of the species-specific assay to yield the species identity was probably related to the fact that the species-specific assays are less sensitive than the broad-range assay for the detection of Ehrlichia DNA (unpublished data). E. canis was not detected in any dog. Two dogs positive for E. ewingii were also positive in the screening assay for A. phagocytophilum (primers EHR 521 and EHR 747) but negative with the confirmatory assays that amplify segments of the 16S rRNA gene of A. phagocytophilum and the groEL operon of A. phagocytophilum. Two dogs were thus considered to be positive only for E. ewingii. Thus, infection with a multiple Ehrlichia species was not detected in any dogs in the present study.

Because hum an cases of ehrlichiosis caused by A. phagocytophilum have been rare in Missouri (24), we carried out nucleotide sequencing of portions of the 16S rRNA gene and the groEL heat-shock operon am phly amplified from the blood of the dog that was positive for A. phagocytophilum. Sequencing of the 16S rRNA gene segment was performed by using the sequencing primer PER1R, which provides the sequences of a 126-bp segment that spans the highly variable region. The sequences determined matched the published sequence of A. phagocytophilum (GenBank accession no. U02521) (8). The nucleotide sequence of the segment of the groEL operon amplified by PCR was very similar to or identical to sequences previously determined for A. phagocytophilum.

There were no significant differences between PCR-positive and PCR-negative dogs in gender, proportion fertile, mean age, or the presence of fever or a monocytosis in dogs (Table 2). Differences in tick exposure (ticks currently or bedded or recently removed) were reported in 75% of PCR-positive compared to 46% of PCR-negative dogs (P = 0.05 [chi-square]). As shown in Fig. 1, the mean (81%) of the PCR-positive cases occurred during May through July. Figure 2 shows the distribution of PCR-positive and PCR-negative samples within the state of Missouri. Most specimens were submitted from the southern portion of the state. Positive dogs were located throughout this region, with a cluster of positives in four counties (Jefferson, Washington, St. Francois, and St. Genevieve) located south of St. Louis and a smaller cluster in the southwest portion of the state.

Serum from 85 dogs, including 78 ill and 9 asymptomatic
TABLE 2. Clinical characteristics of Missouri dogs tested for Ehrlichia

<table>
<thead>
<tr>
<th>Characteristic or finding</th>
<th>No. of animals with data available</th>
<th>No. (%) of animals that were:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL (n = 78)</td>
<td>Asymptomatic (n = 10)</td>
</tr>
<tr>
<td></td>
<td>PCR positive (n = 20)</td>
<td>PCR negative (n = 58)</td>
</tr>
<tr>
<td>Male</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>Mean age (yr)</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>Febrile</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>Malignant findings</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>Tick exposure</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Anemia</td>
<td>22</td>
<td></td>
</tr>
</tbody>
</table>

a Except where indicated (i.e., mean age), entries in the table indicate the number of dogs and the percentage of those tested that had the indicated characteristic or finding.

b Defined as lameness, reluctance to move, walking with a stiff or stilted gait, or painful or swollen joints.

defined as lameness, reluctance to move, walking with a stiff or stilted gait, or painful or swollen joints.

DISCUSSION

This study of the Ehrlichia species present in dogs in Missouri revealed several notable results. The first was the finding that more than 90% of dogs with molecular evidence of current Ehrlichia infection were infected with E. ewingii. Although E. ewingii had previously been demonstrated as a cause of ehrlichiosis in Missouri, no study had yet documented its presence by molecular methods. The distribution of Ehrlichia species in Missouri dogs differs dramatically from that in humans with ehrlichiosis acquired in the state. In our laboratory, which receives human specimens from a geographic region similar to the region from which dog samples were provided for the present study, E. chaffeensis has accounted for 89% of the cases, with E. ewingii accounting for the remaining 11%. One possible explanation for this discrepancy may be differences in host pathogenicity; namely, E. chaffeensis may be more pathogenic for humans, and E. ewingii may be more pathogenic for dogs. Additional molecular studies of the prevalence of Ehrlichia in asymptomatic dogs would help clarify these results.

The absence of E. canis in the present study is also noteworthy. One other molecular study of canine ehrlichiosis, performed in Virginia, found only E. chaffeensis and E. ewingii, without any cases of E. canis infection (11). We do not think the absence of E. canis in the present study is the result of the failure of the PCR assay used to detect E. canis, since the PCR primers in the broad-range assay used for initial screening con...
FIG. 2. Geographical distribution of dogs included in the present study by county of residence. The results of Ehrlichia PCR assays are shown as designated in the key.
amplify the DNA of *E. canis*. Previous studies of the causes of canine ehrlichiosis that were based on serology may have failed to make definitive species identification because of serologic cross-reactions among members of the *Ehrlichia*, including *E. chafeensis* and *E. ewingii*. Specifically, it is possible that some cases of *E. ewingii* infection were mistaken attributed to *E. canis* infection. An alternative explanation for the predominance of infections with *E. ewingii* is that participating veterinarians selected dogs for inclusion in the present study who had symptoms such as arthritis that are associated with *E. ewingii* infection.

The detection of a dog infected with *A. phagocytophilum* or a closely related species was surprising. *W.* have not detected *A. phagocytophilum* in our extensive experience with hum an ehrlichiosis in M issouri. It is possible that the organism does exist at low levels in M issouri and simply escapes detection as a hum an pathogen. It is also possible that the agent detected was a species related to but not identical to *A. phagocytophilum* , although we think this is unlikely because of the very close similarity of the groESL sequence determined in the present study to any different *A. phagocytophilum* sequences determined in the laboratory of one of the authors (J.W.S.). Because complete travel histories were not available, we cannot exclude the possibility that this dog was infected out of the state.

Serologic testing for antibodies reactive with *E. chafeensis* revealed that 31% of dogs had serologic evidence of past or present infection with *Ehrlichia*. Combining the results of PCR and serologic testing, 39% of all dogs tested had evidence of past or present infection with *Ehrlichia*, indicating frequent exposure of M issouri dogs residing in the survey regions of M issouri to this group of bacteria. D iscrepancies between the results of serologic and molecular tests observed for some animals were not unexpected. The 12 dogs that were seropositive but PCR negative probably had past *Ehrlichia* infection. The fact that these cases were evenly distributed throughout the year supports this explanation. It is also possible that some of these dogs had recent infection but were PCR negative because of antibiotic treatment. However, only seven dogs in the study were known to have received antibiotics with activity against *Ehrlichia*, and only one was PCR positive and IFA negative, which indicates that antibiotic therapy was not the explanation for this finding. Finally, the sensitivity of PCR as a method for detecting acute canine ehrlichiosis has not been determined, and it is possible that PCR was falsely negative in some of these dogs.

Several explanations are possible for the seven dogs that were PCR positive but seronegative. Some of these dogs may have been sampled very early in the course of their infection before an antibody response had occurred. Unfortunately, the interval between the day of onset of symptoms and the day when the blood sample was obtained was not available for all dogs. A nother possible explanation may have been failure to make an antibody response to acute *Ehrlichia* infection in some of these dogs. Convalescent-phase samples were not available to test this hypothesis. It is also possible that in some dogs, the *E. chaffeensis* antigen used in the IFA may have failed to detect antibodies produced in response to infection with *E. ewingii*. This possibility is supported by the observation of inconsistent seroreactivity with *E. canis* antigen in serum from dogs found to be positive for *E. ewingii* DNA by PCR (16, 18).

There were no differences among the dogs with or without confirmed ehrlichiosis by sex, age, breed, or fertility status. The larger overall representation of retrievers in the study sample may be explained by the popularity of these breeds as pets, but data on breed prevalence for the state were not available. Expected early summer peaks in both total suspected tick-borne illnesses and in actual PCR-positive cases of ehrlichiosis were noted. Prior studies have noted higher incidence, mortality rate, and chronicity among dogs than in other regions. This finding raises the possibility that chronic canine *Ehrlichia* infection could be a source for subsequent infections with *Ehrlichia* in humans residing in the same area. It is probably more likely that dogs and humans share similar exposures to infecting ticks, suggesting that cases of canine ehrlichiosis may serve as sentinels for human cases, as described for other tick-borne infections, including Rocky Mountain spotted fever (26). *M* oat cases of suspected canine ehrlichiosis do not currently undergo testing to reveal the etiologic agent. If confirmatory testing becomes more widely adopted, results could assist human public health officials in identifying environments were the risk of acquiring human ehrlichiosis is high.

ACKNOWLEDGMENTS

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