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CHANGING TIMES – CHALLENGE THE PARADIGMS

8th Symposium of the CVBD® World Forum in St. Petersburg, Russia

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INTRODUCTION TO THE 2013 CVBD® WORLD FORUM SYMPOSIUM

This year's CVBD World Forum Symposium will take place in the same year as Bayer's 150th anniversary. The Bayer Group will be celebrating its 150th anniversary with numerous events around the world in 2013. CEO Dr. Marijn Dekkers stated recently, "Bayer can look back on a long and highly successful history as an inventor company. What started as a small but innovative dyestuffs factory in the Barmen district of Wuppertal, Germany, is now a global enterprise with more than 110,000 employees. In the past 150 years, Bayer inventions have helped to improve people's quality of life. This great tradition is also our commitment to the future – entirely in line with our mission at Bayer: **Science For A Better Life**." Bayer's growth and success is strongly based on science and innovation. As one of a few, if not the only company in the world, the Bayer Group comprises globally leading life sciences divisions covering products in agriculture, human and animal medicine. As innovation is based on science, it is with great pleasure that we at Bayer Animal Health welcome the members of the CVBD World Forum to the 8th Symposium. Even though the history of the CVBD World Forum is only recent in comparison to 150 years of Bayer, we feel that a scientific meeting of this kind, conducted annually for the last 7 years, is an outstanding contribution to science in the field of vector-borne diseases. Just as Bayer combines science from the different life sciences, we in the CVBD World Forum bring together the different disciplines, all eager to exchange and share knowledge. We in the CVBD World Forum live and breathe the '**One Health Concept**' and as R. Virchow once said, "... between animal and human medicine there are no dividing lines – nor should there be".

The CVBD World Forum can already look back on an exciting history. It all started in 2006 when we met for the first time in Billesley, UK, for the very **First Edition** of CVBD World Forum. Italy then embraced us in a **World of Information** and we started to build up a **World of Knowledge** of CVBDs. Germany provided us with the **Chances** to meet again for the third time, at home with Bayer in its Monheim head office. It was an opportunity for us to share our passion for the fascinating world of parasites and pathogens. Spain drove us onwards and upwards – no delays for **Investigation** here! And where, if not in the USA, could we consolidate a **Global View** of CVBDs? Incidentally, we will always remember Eyjafjallajökull, and how the volcano eruption strengthened the CVBD World Forum, as most of us were stranded in the Big Apple at the time. France demonstrated to us once again that, as the world – together with CVBDs – continues to evolve at an unchecked speed, the CVBD World Forum is setting out along its own distinct path of **Evolution**. There is simply no chance of stopping us now. **Biodiversity is challenging One Health**, which was the theme of last year's meeting in Berlin, Germany.

This year's theme: **Changing times – challenge the paradigms!**
We cordially welcome you for the eighth time at the CVBD World Forum Symposium. We invite you to dive into the paradigmatic, extensive, and still undiscovered world of CVBDs.

The Russian Federation (Российская Федерация) is a fitting venue which will allow us to fully embrace the changing times, offering us an opportunity to challenge what one may consider to be untouchable paradigms. For the 2013 symposium, with the professional and invaluable support of the scientific board, it has been possible to design a meeting for fruitful cross-experience discussions allowing an interdisciplinary learning effect. We hope that this meeting, as in previous meetings, will offer a special opportunity for the scientific community to share the latest research findings in the field of vector-borne diseases.

It is with great pleasure that Bayer Animal Health welcomes the CVBD World Forum members and guests to the 8th CVBD Symposium. We hope that you enjoy the scientific presentations as well as the lively discussion, all with a view to further advancing our understanding, the prevention and treatment of vector-borne diseases. In this great combination of 150 years of Bayer and "Science for a Better Life", we welcome you all to St. Petersburg in Russia and hope you will enjoy the 8th Symposium of the CVBD World Forum!

On behalf of Bayer Animal Health

Dr. Norbert Mencke
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Dr. María de Lourdes Mottier
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CONTENT

1 SITUATION OF CANINE VECTOR-BORNE DISEASES (CVBDs) AND THEIR VECTORS AROUND THE WORLD

8th CVBD WORLD FORUM Symposium

Canine tick-borne diseases: situation in Russia

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ABSTRACT

Russia is the largest areal of arthropod-borne diseases in the world. Ixodid ticks are widespread across all the territory of the country (Figure 1).

Ixodes persulcatus and *Ixodes ricinus* are the main vectors of *Borrelia burgdorferi* s.l. and *Anaplasma phagocytophilum*. The prevalence of *B. burgdorferi* s.l. and *A. phagocytophilum* in ticks in different parts of Russia varies from 10 to 50 % and from 1 to 5 % accordingly.[1–4] *Borrelia afzelii* and *Borrelia garinii* dominate in ticks in Russia. *B. burgdorferi* s.s. was found in *I. ricinus* only and the prevalence is 1 – 5 %.

Figure 1. **Tick areas on Russian map.**

Dermacentor marginatus, *Dermacentor reticulatus* and *Rhipicephalus sanguineus* are the main vectors of *Babesia* spp. The prevalence of *Babesia* in ticks is 1 – 5 %.[5]

R. sanguineus – the main vector of *Ehrlichia canis* – occupies most of southern regions of Russia and has been registered in Moscow since 2008. The prevalence of *E. canis* in ticks in Russia has not been studied.

The spread of babesiosis (*Babesia canis* and *Babesia vogeli* only) coincides with the spread of the disease vectors (Figure 1). *B. canis* was determined in dog blood samples in Moscow, Rostov, and Novosibirsk.[5] Almost all the dogs with *B. canis* (≈ 99 %) have had acute forms of the disease: hemolytic anemia, systemic inflammatory response syndrome, and acute renal failure. Chronic forms of babesiosis caused by *B. canis* were observed in < 1 % of the cases.

B. vogeli was determined in dog blood samples in the southern regions of Russia, causing acute, chronic, and subclinical forms of babesiosis. Vaccinations against babesiosis are very rare.

Dog vaccinations against borreliosis have never been used in Russia (and ever certified here). In contrast with western and Central Europe, where most of the dogs receive acaricide and insecticide treatment, in Russia there are large dog populations that have never been treated against ticks and mosquitoes. This provides ability to obtain significant results for such "natural" populations.

The seroprevalences of *B. burgdorferi* s.l., *A. phagocytophilum*, and *E. canis* in dogs were studied in the European part of Russia only. We studied anaplasmosis, borreliosis, and ehrlichiosis in dogs in 6 cities (St. Petersburg, Moscow, Voronege, Rostov-on-Don, N.-Novgorod, Kazan). IDEXX 4Dx SNAP kits were used to determine antibodies against *B. burgdorferi* s.l., *A. phagocytophilum*, and *E. canis* in dog blood samples.

Two groups of dogs were studied: 1) Population of the dogs that had never been treated against ticks and mosquitoes. Moreover the territory occupied by this population has never been treated too; 2) Vet clinics canine patients that had been treated against vector-borne diseases (VBD) vectors, but had tick bite history and have shown clinical signs of VBDs.

Non-treated dogs (Group 1) had higher seroprevalences to *B. burgdorferi*, *A. phagocytophilum*, and *E. canis* (**Table 1**). Almost all dogs with infections had no clinical signs. Only 3 free-living mix-infected dogs (*A. phagocytophilum* + *B. burgdorferi* s. l., *A. phagocytophilum* + *E. canis* + filarial infection, and *D. immitis* + *A. phagocytophilum*) showed no specific clinical signs like conjunctivitis, tachypnea, cough, and lameness.

Table 1. Seroprevalence to *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, and *Ehrlichia canis* among dogs in different groups

Dog group	Number of samples	Number *A. phagocytophilum*-positive (%)	Number *B. burgdorferi*-positive (%)	Number *E. canis*-positive (%)
1) Non-treated dogs	82	28 (34)	2 (2.4)	2 (2.4)
2) Dogs having treatment	440	5 (1.1)	1 (0.2)	0 (0)

The treated dogs (Group 2) had lower seroprevalence to *B. burgdorferi, A. phagocytophilum*, and *E. canis*. But all the dogs with anaplasmosis showed specific clinical signs – fever, anemia, splenitis, hepatitis, renal failure. Two dogs from Group 2 died in a few days. Dogs with borreliosis had no clinical signs.

What is better in the Russian case? To remain most part of the dog population without acaricide treatment but still having natural immunity to tick-borne diseases? Or to expand acaricide treatment coverage, assuming probability of severe illness in some cases? Or something different, like vaccination with a vector protein? These questions should be addressed in future researches.

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Seropositivity rates for agents of canine vector-borne diseases in Spain: a multicenter study

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ABSTRACT

BACKGROUND: Controlling canine vector-borne diseases (CVBD) is a major concern, since some of these diseases are serious zoonoses. This study was designed to determine seropositivity rates in Spain for agents causing the following five CVBD: leishmaniosis (*Leishmania infantum*: Li), heartworm (*Dirofilaria immitis*: Di), ehrlichiosis (*Ehrlichia canis*: Ec), anaplasmosis (*Anaplasma phagocytophilum*/*Anaplasma platys*: An), and Lyme disease (*Borrelia burgdorferi*: Bb).

METHODS: Anti-An, -Bb, and -Ec antibodies and the Di antigen were determined using the 4DX SNAPW Test (IDEXX Laboratories) and anti-*L. infantum* (Li) antibodies using the *Leishmania* SNAPW Test (IDEXX Laboratories) in blood and/or serum samples.

RESULTS: Among 1,100 dogs examined, overall seropositivity rates were: Li (15.7 %), Ec (5 %), An (3.1 %), Di (1.25 %), and Bb (0.4 %). While seropositivity towards Bb and Di was similar in all geographic regions, rates were significantly higher in the East of Spain (8.3 %) for An, significantly higher in the North (20 %) for Ec, and significantly higher in the Southeast (46.6 %) and South (27.4 %), and significantly lower in the North (0 %) for Li. No statistical associations were observed between sex and the CVBD analyzed ($p \geq 0.05$), while the following associations with other variables were detected: a higher seropositivity to Ec (40 %) and Bb (6.7 %) in dogs under one year of age compared with adults ($p < 0.05$); and a higher seropositivity to An and Li in dogs that lived outdoors versus indoors ($p = 0.01$; $p < 0.001$, respectively). Seropositivity rates of 2.1 %, 0 %, 1.7 %, 0.5 %, and 4.2 % were recorded for An, Bb, Ec, Di, and Li, respectively, in dogs with no clinical signs ($n = 556$) versus 3.8 %, 0.6 %, 7.5 %, 1.8 %, and 25.9 % for those with signs ($n = 507$) suggestive of a CVBD.

CONCLUSION: The data obtained indicate a risk for dogs in Spain of acquiring any of the five CVBD examined. Veterinarians in the different regions should include these diseases in their differential diagnoses and recommend the use of repellents and other prophylactic measures to prevent disease transmission by arthropod vectors. Public health authorities also need to become more involved in the problem, since some of the CVBD examined here also affect humans.

KEYWORDS: Leishmaniosis, Heartworm, Ehrlichiosis, Anaplasmosis, Lyme disease, Dog

BACKGROUND

The term canine vector-borne diseases (CVBD) includes a wide variety of diseases of infectious or parasitic aetiology whose agents are transmitted by ectoparasites such as ticks, fleas, lice, mosquitoes, and sandflies.[1] Controlling these infectious agents is important because some are responsible for serious diseases in humans (e.g., *Anaplasma phagocytophilum*, *Bartonella* spp., *Borrelia burgdorferi*, *Leishmania infantum*, *Thelazia callipaeda*, etc.).[2] However, their control can be extremely complex since they show a wide geographical distribution, and clinical signs in infected dogs can vary significantly.[3,4]

In addition, there is evidence to suggest that changing factors linked to climate, and the environment could determine the expansion of the current geographical distribution ranges of these diseases and their arthropod vectors.[5–9] The transport of infected dogs from endemic areas has also been attributed an important role in the spread of CVBD to the North of Europe.[10,11] Owing to an increase in this transport due to new habits such as travelling with dogs or adopting animals from other countries, the epidemiological status of these diseases in Europe, and especially across the Iberian Peninsula including Portugal, has changed considerably.[12–14]

CVBD may show no specific clinical signs or clinicopathological abnormalities, or alternatively may present a varied clinical picture making the diagnosis of a CVBD extremely complex. Animals with subclinical infection have been described to show an increased risk of disease transmission.[15,16]

Anaplasma platys and *A. phagocytophilum* (An) are the aetiological agents of anaplasmosis, which affects a wide range of vertebrate hosts (rodents, dogs, humans). *A. phagocytophilum* is transmitted by ticks of the genus *Ixodes* and *A. platys* by the tick *Rhipicephalus sanguineus*. Both pathogens infect dogs, in which the clinical picture ranges from subclinical disease to acute illness.[17] *A. phagocytophilum* can also infect humans causing febrile syndrome.[18] The reported seroprevalence of *Anaplasma* spp. in Spain has ranged from 5 to 19 % for Galicia, Catalonia, Balearic Islands, and Castilla-León.[19–21]

Lyme disease is an infectious disease caused by spirochetes belonging to the *Borrelia burgdorferi* (Bb) sensu lato complex, transmitted by ticks of the genus *Ixodes*. Lyme disease shows a worldwide distribution, although its incidence is increasing in North America and Europe because of its association with this vector.[22–24] Bb affects a wide range of hosts, mainly humans and dogs. In humans, Lyme disease can produce chronic weakness with non-specific clinical signs (fever, muscle and joint pain). Though few dogs show clinical signs, most are subclinical reservoirs[25,26] and can be used as sentinels for this infection. In Spain, dogs seropositive for *B. burgdorferi* have been detected in Galicia (6.3 %),[20] Mallorca (1.3 %),[19] and Castilla - León (2.1 to 21 %).[27–29]

Ehrlichia canis (Ec), an intracellular Gram-negative bacterium that infects monocytes, is the causative agent of canine monocytic ehrlichiosis, and is transmitted by the tick *R. sanguineus*.[30] The disease is characterized by three stages of varying severity. The acute stage produces clinical signs such as apathy, depression, anorexia, dyspnoea, fever, lymphadenopathy, splenomegaly, petechiae and echymotic haemorrhage in the skin and the mucous membranes, epistaxis, and vomiting. Laboratory abnormalities are usually thrombocytopenia, leucopenia, and mild to moderate normocytic, normochromic and non-regenerative anaemia. The second stage is subclinical with clinicopathological abnormalities such as thrombocytopenia, anaemia, or hyperproteinemia. The third or chronic stage is characterized by a very complex clinical picture: haemorrhage, weakness, apathy, sustained weight loss, fever, lymphadenopathy, splenomegaly, and peripheral oedema in the hind limbs

and scrotum, and a wide variety of clinicopathological abnormalities.[31,32] In Spain, the seroprevalence of Ec in dogs ranges from 3.1 to 19 %, with cited rates of 3.1–6.5 % for Galicia, Madrid, and Zaragoza[20,33,34] and higher rates for Mediterranean regions (Catalonia, Valencia, Baleares),[19,35,36] and Castilla-León (12–20%).[35]

Dirofilaria immitis (Di) is a filarial worm transmitted by mosquitoes (Culicidae) to carnivores and other hosts. Since the vector is not very host-specific, many mammals can become infected including humans.[37] *D. immitis* is a cosmopolitan parasite, mainly found in southern European countries including Spain, where it is endemic in the regions Valencia, Balearic Islands, Andalucia, Aragon, and the Canary Islands with prevalences of 6.3–67.02 %.[38–42] Lower prevalences have been reported for other regions, although recently cases have been detected in two northern provinces: La Rioja (12 %) and La Coruña (4.2 %).[43,44] *D. immitis*, also known as heartworm, mainly affects dogs but has also been detected in cats.[45] In dogs, the course of disease is chronic due to changes in the pulmonary arteries and lung parenchyma.[46] In humans, the parasite cannot complete its whole life cycle, yet produces a serious infection in which parasitic granulomas can be observed in the lung parenchyma.[47]

Canine leishmaniosis (CanL), a zoonotic disease endemic in southern Europe caused by the protozoan *Leishmania infantum* (Li), is transmitted to humans and animals by blood-sucking phlebotomine sandflies.[48,49] Until recently, CanL was considered to be limited to the Mediterranean Basin, with an estimated seroprevalence in Spain ranging from 3 % in the North[50] to 34.6 % for Malaga province on the South coast.[51] Northern Spain was considered a non-endemic area, but CanL and its sandfly vectors have been detected in the Northeast and Northwest where the disease was previously unknown.[50,52–54] At our latitude, dogs are considered the main reservoir for human infection.[55,56] Clinical CanL shows a wide spectrum of clinical signs and severity because of the many pathogenic mechanisms involved and the particular immune response produced in the host.[57] The main clinical signs of CanL are one or more of the following: weight loss, lethargy, muscular atrophy, anaemia, lymphadenomegaly, splenomegaly, epistaxis, diarrhoea, renal disorders, ocular lesions, polyarthritis, onycogryphosis, and skin lesions.[58–60] In endemic areas, a high proportion of clinically healthy dogs are able to transmit the infection causing a serious public health problem.[61] In Europe, human infection with *L. infantum* is observed mainly in children and immunocompromised adults, but a recent outbreak (2010) in Southwest Madrid (Spain) indicates the epidemiology of this disease is complex and subject to constant change.[62,63]

Information emerging from Spain on some of these vector-borne diseases has been limited. Most studies have addressed canine leishmaniosis in the Mediterranean Basin and a few reports have dealt with ehrlichiosis, dirofilariosis, *Borrelia* and *Anaplasma* infection in specific areas of the country.

The present study was designed to establish seropositivity rates and epidemiological associations for these five CVBD by determining antibodies against *Anaplasma* spp. (An), *Borrelia burgdorferi* (Bb), *Ehrlichia canis* (Ec), and *Leishmania infantum* (Li), and the *Dirofilaria immitis* (Di) antigen in dogs from different Spanish regions.

METHODS

Bioclimatic characteristics of the study area

The survey was carried out in seven different ecoepidemiological regions of Spain. The Iberian Peninsula shows two main regions of flora and vegetation, the Mediterranean and Eurosiberian regions. This last region covers the North of Spain, where climate and vegetation are typically

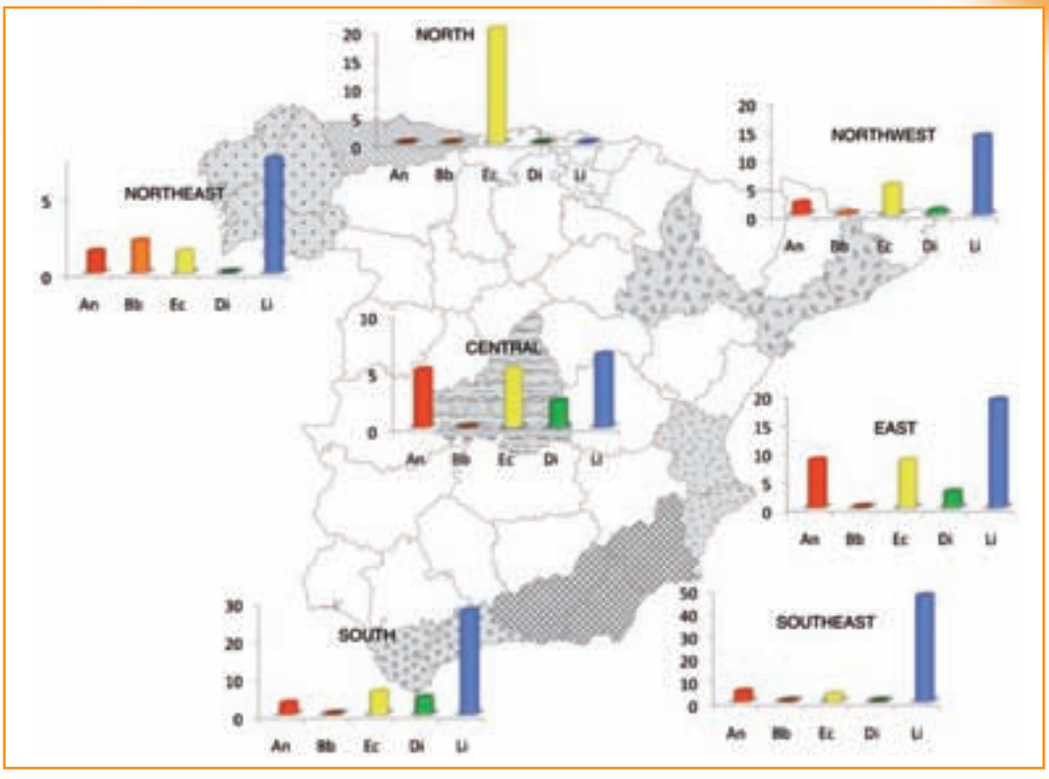

Figure 1. **CVBD seropositivity recorded for seven Spanish geographic regions.**

oceanic, with warm summers and cool winters and rainfall evenly distributed all year round. The rest of the peninsula falls within the Mediterranean region. Here, summers are dry and hot, and most rainfall occurs in autumn and spring. Moreover, coastal areas show a milder, more humid climate, and mountain ranges have a shielding effect from an oceanic influence determining a climate of extremely cold winters and very hot summers.

Veterinary clinics and dogs

The study was carried out in accordance with the International Guiding Principles for Biomedical Research Involving Animals, issued by the Council for the International Organizations of Medical Sciences. The owners of the dogs enrolled were previously informed about the study protocol.

The dogs examined were 1,100 owned dogs attending 57 veterinary clinics in central (187 dogs; 7 clinics), eastern (90 dogs; 5 clinics), southern (75 dogs; 5 clinics), southeastern (105 dogs; 5 clinics), northern (15 dogs; 1 clinic), northeastern (465 dogs; 22 clinics), and northwestern (163 dogs; 11 clinics) Spain (see Figure 1).

Dogs were subjected to the same protocol to compile a brief clinical record based on a questionnaire and physical examination. Blood samples were collected from all dogs. The data collected were correlated with age, sex, abode (indoors, outdoors, or mixed), and the presence of clinical signs compatible with any CVBD. The dogs were 6 months to 18 years of age; 593 were male (489 entire, 104 neutered) and 507 female (405 entire, 102 neutered).

Serologic testing

Anti-An, -Bb, and -Ec antibodies and the Di antigen were determined using the 4DX SNAPW Test (IDEXX Laboratories) and anti-*L. infantum* (Li) antibodies using the *Leishmania* SNAPW Test (IDEXX Laboratories) in blood and/or serum samples.

Statistical analysis

Seropositivity rates were compared according to age, sex, abode, and the presence of clinical signs. Associations between CVBD-agent seropositivity and the remaining variables were assessed using the chi-squared test. All statistical tests were performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Significance was set at $p \leq 0.05$.

RESULTS

Overall seropositivity rates for the five CVBD agents were: Li (15.7 %), Ec (5 %), An (3.1 %), Di (1.25 %), and Bb (0.4 %). Rates obtained by geographic region are shown in **Table 1**. Seropositivity to the Bb antibody and Di antigen was similar in the seven regions. Seropositivity to An was significantly higher (8.3 %) in the East than the remaining regions (0–5 %), the rate for Ec was significantly higher in the North (20 %), and that for Li was significantly higher in the South (46.6 %) and Southeast (27.4 %). In the North of Spain, no dog tested was Li seropositive (**Table 1**).

No associations were observed between sex and any CVBD ($p \geq 0.05$). Seropositivity towards Ec (40 %) and Bb (6.7 %) was higher in dogs under one year of age compared to adults ($p < 0.05$), while no differences in the rates recorded for An, Di, or Li were detected between the two age groups.

Seropositivity to An and Li was significantly higher for dogs that lived outdoors compared to indoors. No link was detected between the seropositivity rate observed for Bb, Ec, and Di, and place of abode.

When stratified by the presence or absence of clinical signs, percentages of An, Bb, Ec, Di, and Li seropositive dogs were 2.1 %, 0 %, 1.7 %, 0.5 %, and 4.2 % for the subset of dogs with no clinical signs ($n = 556$), and 3.8 %, 0.6 %, 7.5 %, 1.8 %, and 25.9 % for those with clinical signs compatible with CVBD, respectively ($n = 507$) (**Table 2**). The main clinical signs described by the veterinarians were: apathy, anorexia, anaemia, lymphadenomegaly, digestive disorders, skin lesions characterized by alopecia, seborrhoeic dermatitis, erythema, scaling, and hyperkeratosis, as well as ulcerative lesions, and onychogryposis.

In addition, seropositivity rates for Li and Ec were correlated with the presence of clinical signs in the dogs examined. No correlation was detected, however, between seropositivity for Bb, An, and Di and the presence of clinical signs.

DISCUSSION

This study is the most complete survey of CVBD-agent seropositivity conducted in Spain. Most prior studies have been limited to a single region.[21] Overall, 37.1 % of the dogs were seropositive for at least one of the five CVBD agents examined. The highest seropositivity rate detected was that of Li (15.7 %) and the lowest of Bb (0.4 %), the seropositivity rate for each of these CVBD pathogens varying according to the geographic region. Despite differences detected in seropositivity to each CVBD-agent among each of the seven regions, the travel history of each dog was not included in the questionnaire, such that we cannot rule out the possibility that dogs were infected outside their home region.[14]

The present study examines several epidemiological variables to assess possible associations with CVBD-agent seropositivity. No link was detected between sex and each CVBD ($p \geq 0.05$), although seropositivity rates for Ec (40 %) and Bb (6.7 %) were higher in dogs under one year of age compared with adults ($p < 0.05$). These data suggest a need for further studies designed to determine the effects of age on CVBD-agent seropositivity since we only examined 17 young dogs.

Table 1. Seropositivity for the CVBD studied by geographic region

Geographic region		CVBD Positive/total (%)				
		An	**Bb**	**Ec**	**Di**	**Li**
Central	(n = 187)	9/180 (5)	0/176 (0)	9/176 (5.1)	4/176 (2.3)	10/157 (6.4)
Northeast	(n = 465)	9/451 (2)	1/451 (0.2)	23/451 (5.1)	3/451 (0.7)	60/434 (13.8)
East	(n = 90)	6/72 (8.3)*	0/73 (0)	6/73 (8.2)	2/73 (2.7)	13/69 (18.8)
Southeast	(n = 105)	2/45 (4.4)	0/30 (0)	1/33 (3.0)	0/31 (0)	41/88 (46.6)**
South	(n = 75)	2/68 (2.9)	0/68 (0)	4/68 (5.9)	3/68 (4.4)	20/73 (27.4)**
Northwest	(n = 163)	2/143 (1.4)	3/143 (2.1)	2/143 (1.4)	0/142 (0)	12/159 (7.5)
North	(n = 15)	0/15 (0)	0/15 (0)	3/15 (20.0)*	0/15 (0)	0/15 (0)**
Total	**(n = 1,100)**	**30/976 (3.1)**	**4/956 (0.4)**	**48/959 (5.0)**	**12/957 (1.25)**	**156/995 (15.7)**
p value		**p = 0.04**	**p = 0.07**	**p = 0.04**	**p = 0.06**	**p < 0.001**

* p < 0.05 ** p < 0.001
Abbreviations: An, *Anaplasma phagocytophilum*/*Anaplasma platys*; Bb, *Borrelia burgdorferi*; Ec, *Ehrlichia canis*; Di, *Dirofilaria immitis*; Li, *Leishmania infantum*

Table 2. Seropositivity for CVBD according to the epidemiological variables analyzed

Epidemiological variable		CVBD Positive/total (%)				
		An	**Bb**	**Ec**	**Di**	**Li**
Age (years)	< 1	2/17 (11.8)	1/15 (6.7)**	6/15 (40)**	0/15 (0)	0/14 (0)
	1–3	12/369 (3.2)	1/361 (0.3)	7/362 (1.9)	2/361 (0.5)	60/370 (16.2)
	3–7	7/269 (2.6)	0/261 (0)	12/261 (4.6)	2/262 (0.8)	50/270 (18.5)
	> 7	7/307 (2.3)	2/305 (0.6)	23/307 (7.5)	6/305 (2.0)	44/327 (13.5)
	Unknown	2/14 (14.3)	0/14 (0)	0/14 (0)	2/14 (14.3)	2/14 (14.3)
p value		**p = 0.14**	**p = 0.001**	**p < 0.001**	**p = 0.3**	**p = 0.14**
Sex	Male	21/531 (4.0)	3/526 (0.6)	23/528 (4.4)	9/527 (1.7)	94/546 (17.2)
	Female	9/445 (2.0)	1/430 (0.2)	25/431 (5.8)	3/430 (0.7)	62/449 (13.8)
p value		**p = 0.08**	**p = 0.16**	**p = 0.4**	**p = 0.14**	**p = 0.16**
Clinical signs	Asymptomatic	9/433 (2.1)	0/416 (0)	7/417 (1.7)	2/416 (0.5)	19/449 (4.2)
	Symptomatic	19/506 (3.8)	3/503 (0.6)	38/505 (7.5)**	9/504 (1.8)	133/513 (25.9)**
	Unknown	2/37 (5.4)	1/37 (2.7)	3/37 (8.1)	1/37 (2.7)	4/33 (12.1)
p value		**p = 0.13**	**p = 0.11**	**p < 0.001**	**p = 0.07**	**p < 0.001**
Place of abode	Indoor	6/360 (1.7)	0/346 (0)	14/346 (4.0)	2/346 (0.6)	26/354 (7.3)
	Outdoor	18/357 (5.0)*	1/355 (0.3)	22/355 (6.2)	5/355 (1.4)	86/362 (23.8)**
	Mixed	5/256 (2.0)	3/252 (1.2)	12/255 (4.7)	5/253 (2.0)	43/276 (15.6)
	Unknown	1/3 (5.2)	0/3 (0)	0/3 (0)	0/3 (0)	1/3 (5.2)
p value		**p = 0.01**	**p = 0.07**	**p = 0.23**	**p = 0.28**	**p < 0.001**

* p < 0.05 ** p < 0.001
Abbreviations: An, *Anaplasma phagocytophilum*/*Anaplasma platys*; Bb, *Borrelia burgdorferi*; Ec, *Ehrlichia canis*; Di, *Dirofilaria immitis*; Li, *Leishmania infantum*

No significant correlation was detected in our study between the presence of clinical signs in a dog and its positivity for Bb, An, or Di. In contrast, Li or Ec positivity was correlated with the presence of clinical signs in the dogs examined ($p < 0.05$). This finding is consistent with reports indicating that clinical signs are commonly observed in dogs infected with *L. infantum* and *E. canis*.[14,16,64,65]

CVBD have been correlated with the presence of vectors such that prevalences should be higher in dogs living outdoors due to their greater cumulative exposure to the agents these vectors transmit. However, we detected no association between positivity for Bb, Ec, and Di, and place of abode, though dogs living outside showed a higher rate of An and Li. Seropositivity towards An was significantly higher in dogs living outdoors, in agreement with data obtained in dog shelters in Northwest (45.3 %) and Central (19 %) Spain.[20,21] It would be interesting to collect information on whether the dogs were protected with an ectoparasiticidal agent since some of these insecticides are able to prevent CVBD.[66–70] Macrocyclic lactones have also been found to be effective against canine heartworm.[46]

In Europe, *A. phagocytophilum* is transmitted by the tick *Ixodes ricinus*, whose distribution range is limited to areas of high humidity and cold temperatures, while *A. platys* could be transmitted by *Rhipicephalus sanguineus*, widely distributed across the Iberian Peninsula. Our results revealed a high seropositivity for this agent in eastern (8.3 %) and central (5 %) Spain, and a lower seropositivity in the North (0–2 %). In prior studies conducted on dogs attending veterinary clinics in the Northwest and East of Spain, similar rates of 5 % and 11.5 %, respectively, have been reported.[19,20] Despite the good sensitivity and specificity of the test used to detect antibodies against *Anaplasma* (99.1 % and 100 %, respectively), serological cross-reactivity between *A. phagocytophilum* and *A. platys* has been described in experimentally infected dogs.[71] Thus, PCR is needed to identify the *Anaplasma* species. Our results could therefore indicate exposure to the *Anaplasma* genus with no information provided at the species level. So far, *A. phagocytophilum* has not been isolated in Spanish dogs. It is likely that the antibodies detected in this study were anti-*A. platys* antibodies since we noted a higher seropositivity to An in areas where the presence of *R. sanguineus* is common. It is also true that *I. ricinus* is the common vector of *B. burgdorferi* and *A. phagocytophilum*, yet we found no An/Bb co-infections. However, dogs from some regions could be infected with *A. phagocytophilum* since this agent has been isolated from *Ixodes* ticks[72,73] and has also been detected in sheep, goats, cows, deer, birds,[73–75] and even human beings.[76,77]

Antibodies against Bb were only detected in four dogs, three in the Northwest (2.1 %) and one in the Northeast (0.2 %). The Snap 4DX kit only detects these antibodies during active infection[78] such that this could explain the low seroprevalence recorded. Other serological studies in which the Snap 3DX or 4DX methods were used have provided similar results.[19,21] Our data indicate significantly higher seropositivity for Bb in the younger dogs (< 1 year), though this finding requires confirmation since our study only included 17 young dogs.

The bacterium Ec is transmitted by *R. sanguineus*. This tick is the most common tick found in dogs which explains the wide distribution of the disease.[79] *R. sanguineus* has been detected across Spain, though we observed a significantly higher seropositivity to Ec in the North, where 6 out of 15 dogs were seropositive, while the overall prevalence of Ec was 5 %. The low number of dogs surveyed precludes reliable estimates of the real prevalence of this disease. However, the higher seropositivity rate detected in the North could reflect the fact that *E. canis* infection is not limited to dogs, and that other wild canids (wolves, foxes, coyotes) may serve as reservoirs of infection.[80] Effectively, in northern Spain (Asturias), wild canids could live in close contact with domestic canids. There is also evidence that the prevalence of Ec is higher in rural areas or among stray dogs[20]

than in urban areas.[81] Other authors have reported a similar Ec seroprevalence for different areas of the country in household dogs (3.13–16.7%)[19,20] along with a higher seroprevalence (54.7 %) in stray dogs from the Northwest of Spain.[20]

D. immitis antigen was detected in 1.25 % of the dogs examined here. Higher rates were recorded for central (2.3 %) and eastern (2.7 %) regions of the country, while Di antigen was not detected in dogs from the North and Northwest. It is known that climate and environmental factors determine the geographical distribution of its vector (Culicidae), allowing it to complete its life cycle and consequently the life cycle of Di.[42,82] In Spain, similar surveys have identified Di in large areas of the country, often in irrigated zones.[42] A high prevalence of Di has been reported in the Canary Islands (19.2–67.02 %),[38,40,41,83] Mediterranean coast (6.3–39 %)[84] and southern regions (8.5–36.7 %).[38,39] At present, while in certain areas (e.g., Gran Canaria) prevalence is decreasing probably due to preventive measures,[41,42] in other areas where Di was considered non-endemic (La Rioja and La Coruña), the first cases of canine dirofilariosis have been detected.[43,44,85] A possible explanation for the lower prevalences detected here is that the ELISA test can give rise to false negatives in dogs with low heartworm burdens or in blood samples from dogs infected only by male worms.[86] Besides, these types of study are difficult to compare due to differences in the diagnostic techniques used (PCR, agglutination test, ELISA, etc.), the size and origin of samples, and the study season.

As expected, seropositivity towards *L. infantum* was the highest of all the CVBD-agents analyzed (15.7 %). Li antibodies were detected in all the geographic regions except the North, and higher rates were recorded for the South (27.4 %) and Southeast (46.6 %). Our Li seropositivity map is similar to those emerging from other Spanish surveys.[65,87,88] Other studies conducted in Spain[50,89] have detected a higher seroprevalence in some northern areas. Such differences between studies could be attributable to the different population analyzed, the sampling season, and the diagnostic technique used.

Among the dogs tested seropositive for Li, 85.2 % (133/156) showed clinical signs of leishmaniosis in the physical exam performed by the veterinarians (**Table 2**). This can be explained by the fact that CanL is well known to Spanish practitioners despite the wide variety of clinical signs this disease can show.[53,90] In addition, Li seropositivity was significantly higher in the subset of dogs that lived outdoors compared to those living at home or even both indoors and outdoors. The explanation for this could lie in the increased exposure of dogs that spend more time outdoors to phlebotomines.[65,88,91] Arthropod vector distribution and density cause differences in the regional distribution of CVBD-agent seropositivity. To understand the potential role of the vector, knowledge of its environmental requirements is fundamental. There is indeed a need for research targeted at the prevention, diagnosis, treatment, and prevention of CBVD. Information on the prevalence and geographical distribution of these infections is essential for planning control measures and their surveillance thereafter. This preliminary overview of the current situation in Spain requires further work to complete the prevalence map of agents causing CBVD in this country.

CONCLUSION

The findings of this study reveal that dogs in Spain are at risk of acquiring any of the five CVBD examined (leishmaniosis, heartworm, ehrlichiosis, anaplasmosis, and Lyme disease). They also indicate that veterinarians across Spain need to include these diseases in their differential diagnosis and recommend the use of repellents along with prophylactic measures to prevent disease transmission by arthropod vectors. In addition, greater involvement on the part of public health authorities is needed given that some of the CVBD detected can be transmitted to humans.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

GM, AS, and XR designed the survey, GM drafted the first version of the manuscript and finalized the manuscript. AM performed the statistical analysis of data, constructed the tables, drafted the first version of the manuscript, and finalized the manuscript. RG prepared the figures and reviewed and finalized the manuscript. All authors read and approved the final version of the manuscript.

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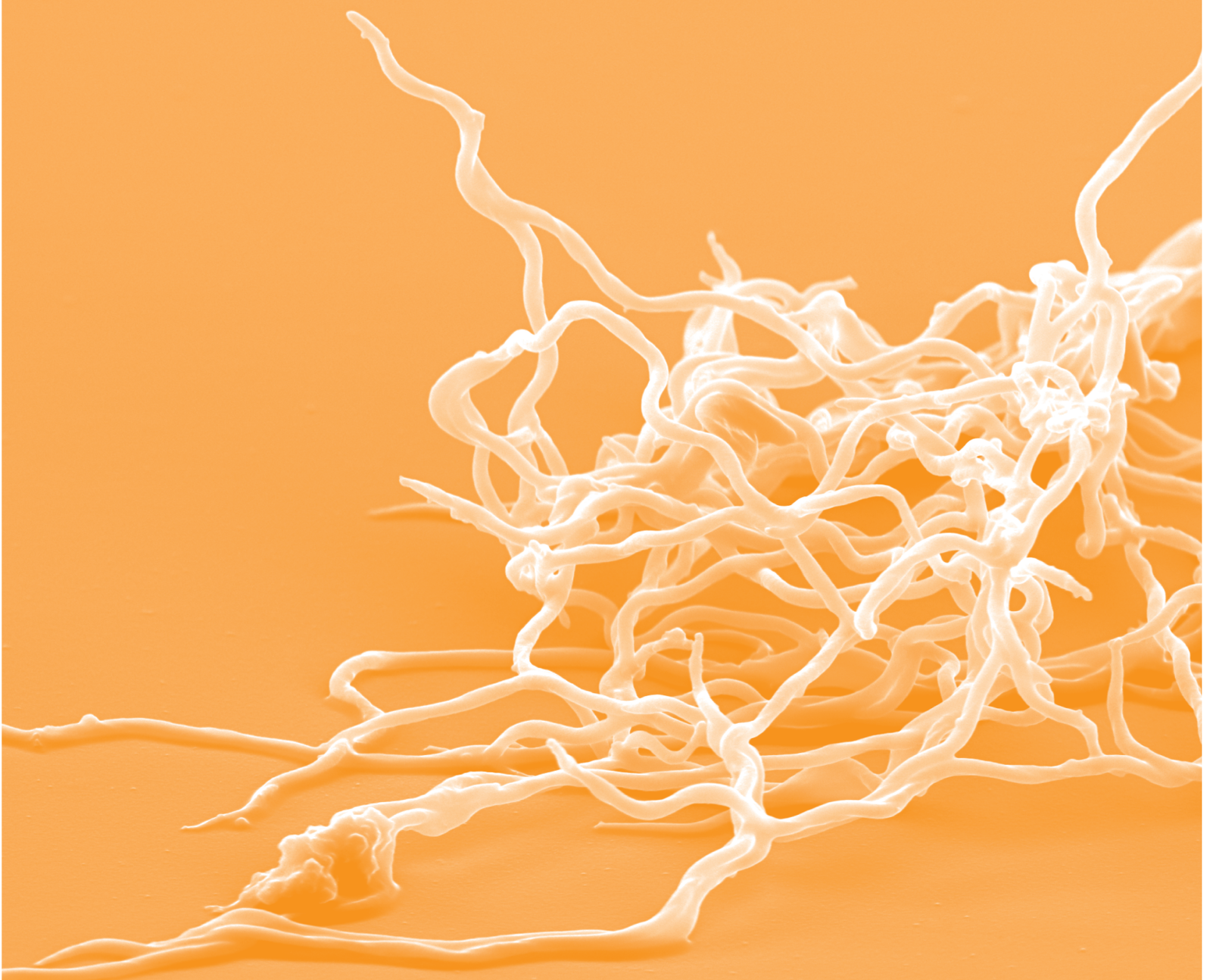

Borrelia burgdorferi

A survey of canine vector-borne diseases in Romania

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INTRODUCTION

Romania is a country in southeastern Europe. Due to its geographical location, it has several different climatic zones and also different landscapes. These different environmental conditions create different ecological zones, which harbor a number of vectors of pathogens for canines and humans. So far, the knowledge of canine vector-borne diseases in Romania is very rudimentary. Due to the geographical location of Romania, it may serve as a transition zone for spreading vector-borne pathogens from southeastern Europe, Asia, and possibly Africa to Europe.

OBJECTIVE

A literature survey was conducted to summarize and review current knowledge of vectors and pathogens of canine vector-borne diseases. In addition, own results of tick-borne pathogens and of clinically manifest diseases in dogs are reported.

RESULTS

VECTORS

Vectors – mosquitoes

There are no current data available on the prevalent mosquito species in Romania. In course of outbreaks of West Nile fever in 1996 in Bucharest, a total of 5,500 mosquitoes were collected in urban epidemic areas.[1] 96 % of collected mosquitoes belonged to the species *Culex pipiens pipiens*. Two other *Culex* species, *Culex annulatus* and *Culex territans,* were identified. One *Aedes* species, *Aedes geniculatus,* and two *Anopheles* species, *Anopheles maculipennis* complex and *Anopheles plumbeus*, could also be identified. In some older publications, at least five different species of the *A. maculipennis* complex were identified, among them a so far non-described species.[2] A comprehensive review of mosquito species in Romania in the mid 1990s lists a total of 55 different species of mosquitoes.[3]

Vectors – sandflies

During the last 35 years, no studies on the presence of sandflies were conducted in Romania. Therefore, no actual data are available on the occurrence of Phlebotominae or from pathogens transmitted by Phlebotominae.

Vectors – fleas

No current studies are conducted on flea species in Romania. It may be assumed that cat and dog fleas are prevalent in the dog and cat populations in Romania. A recent study in dogs from Romania found dogs with infection of *Dipylidium caninum*, a tapeworm in whose transmission cycle fleas of the genus *Ctenocephalides* are involved.[4] Also *Hymenolepis nana*, the dwarf tapeworm, which needs fleas as intermediate hosts for its development is prevalent in children and adults in different Romanian regions.[5]

Vectors – ticks

In a very recent review, a total of 25 different hard tick species of the genera *Ixodes*, *Dermacentor*, *Haemaphysalis*, *Rhipicephalus*, and *Hyalomma* have been described.[6] There seems to be a trend of more prevalent distribution of *Ixodes* species in the northern and western part of Romania, while *Hyalomma* and *Rhipicephalus* seem to be more prevalent in the southern and eastern parts of the country. *Dermacentor* species can be found all over Romania. The described number and species of ticks clearly show the different ecological zones found in Romania.

PATHOGENS

Pathogens – *Dirofilaria*

Recently, human cases of *Dirofilaria repens* and *Dirofilaria immitis* were described in Romania. A recent study resulted in the detection of 1/29 *D. immitis* in dogs from Bucharest.[6] Our own data show that up to 7 % of dogs from an animal clinic in Bucharest yielded positive results by molecular technologies for *Dirofilaria* spp. (Chitimia, unpublished results). A serosurvey resulted in 3 % seropositive dogs against *D. immitis* antigen.[7]

Pathogens – *Hepatozoon canis*

In a recent study in dogs from Bucharest, 1/97 dogs were found positive by molecular technologies for *Hepatozoon canis* (Chitimia, unpublished results).

Pathogens – *Babesia*

In a study, 37 % of dogs from Bucharest were found positive for *Babesia canis*. A further molecular characterization of *B. canis* resulted in 34/37 *Babesia canis canis* and of 2/36 *Babesia canis vogeli* (Chitimia, unpublished results). In a recent publication, 45 % of dogs from Romania were found positive for *B. canis canis*.[8] Also *Babesia occultans* and *Babesia microti* have been detected in ticks from cattle (*Dermacentor marginatus*, *Hyalomma marginatum*, *Ixodes ricinus*).[8] Other *Babesia* spp. (*Babesia gibsoni*, *Babesia felis*) have not been detected so far in dogs from Romania. Serological data show that up to 20 % of dogs in a rural and urban environment in western Romania[9] and between 6 and 42 % in dogs in Bucharest exhibited antibodies against *B. canis*.[10]

Pathogens – *Theileria*

In a recent study, *Theileria equi* could be detected in 1/139 ticks of the species *Hyalomma marginatum* from Romania.[8] Furthermore, *Theileria orientalis/ bufeli/ sergenti* group could be detected in 16 % of *I. ricinus* and in 6 % of *H. marginatum*.[8]

Pathogens – *Acanthocheilonema*

So far, *Acanthocheilonema reconditum* was detected in one out of 109 dogs imported from Romania to a German animal rescue organization.[10] Molecular testing of 29 dogs in an animal clinic in Bucharest did not result in any positive results.

Pathogens – *Leishmania*

In a recent study, no *Leishmania* could be detected in dogs from an animal clinic in Bucharest by using molecular techniques. However, using serological techniques 3/29 dogs showed a positive serological reaction.[10] These data imply that *Leishmania* spp. may occur in parts of Romania and that dogs may play a role in the transmission cycle of the *Leishmania* spp.

Pathogens – *Ehrlichia canis*

E. canis was detected in 1/29 dogs from a Romanian animal clinic in Bucharest using molecular technologies.[10] A serological survey in more than 1,100 dog sera from all over Romania resulted in a seroprevalence rate of 2.1 % in the southeastern part of Romania (Constanta County).[7]

Pathogens – *Neoehrlichia*

There have been no data available so far on the occurrence of *Neoehrlichia mikurensis* in Romania.

Pathogens – *Borrelia* spp.

Borrelia burgdorferi was detected for the first time in Romania only in 2010.[11] The first available data on the prevalence of *B. burgdorferi* s.l. in ticks in the county of Sibiu show that 19.5 % (39/200) of ticks of the species *I. ricinus* were infected with *B. burgdorferi* s.l. The species differentiation in this small study resulted in two species, *Borrelia afzelii* (31/39 positive ticks) and *Borrelia garinii* (8/39 positive ticks). These data are within the range of prevalence rates of other European countries. A serological study in more than 1,100 dogs from different areas of Romania resulted in 0.5 % (6/1,146) seropositive dogs.[7] Especially high seropositivity rates were detected in the Central Romanian counties of Harghita, Valcea, and Arges. It may therefore be assumed that studies in other Romanian counties will result in the detection of more different species of *Borrelia*.

Pathogens – *Anaplasma*

At least two *Anaplasma* species, *Anaplasma phagocytophilum* and *Anaplasma platys,* have been detected so far in Romania. *A. phagocytophilum* was detected in 3 % of dogs imported from Romania to Germany[7] and in *R. sanguineus* ticks.[8] Recently, *A. platys* was described for the first time in a dog in Romania.[12] Serological evidence of *Anaplasma* infection in dogs was found in 6 % (7) and in 17 % (10) in the dog populations studied.

Pathogens – *Bartonella* spp.

There are no data available on the occurrence of *Bartonella* spp. in Romania so far.

Pathogens – *Rickettsia* spp.

So far, no detailed studies on the occurrence of rickettsiae arthropods exist in Romania. Preliminary studies in about one hundred ticks of different species revealed the presence of at least 4 different species of rickettsiae, *Rickettsia raoultii*, *Rickettsia slovaca*, *Rickettsia monacensis,* and *Rickettsia aeschlimannii* (Chitimia, unpublished results). In a recent publication, *Rickettsia helvetica* and *R. monacensis* were detected in *Ixodes* ticks and *R. slovaca* and *R. raoultii* were detected in *Dermacentor* ticks.[8] It may be assumed that due to the climatic and geographic location of Romania several more species are present in ticks and other arthropods in Romania.

Pathogens – tick-borne encephalitis virus

Tick-borne encephalitis is the most important tick-borne virus in Central and eastern Europe. So far, few data are available on the occurrence of tick-borne encephalitis in Romania. In a recent technical report of the European Centers for Disease Control between 2 and 8 human cases were reported annually by the Romanian health system to the ECDC.[13] The most affected county was the county of Salaj, followed by the neighboring counties of Cluj, Bihor, Mures, and Bistrita-Nasaud. To date, no tick-borne encephalitis virus could be isolated from ticks or patients in the region.

Pathogens – West Nile virus

West Nile virus is the most important vector-borne virus in Romania. There is evidence of the presence of the virus since the 1960s. However, the most important epidemics of West Nile virus infections in Romania occurred in 1997, with a total of 322 patients.[14] Again, in 2010 another big outbreak was reported in the parts of Romania.[15] While in 1997 the virus circulation seems to be restricted mainly to the South and Southeast of Romania, in 2010 human cases were diagnosed throughout the country.

CONCLUSIONS

The current knowledge of vector-borne pathogens in Romania is rudimentary. The most prevalent canine vector-borne disease in Romania seems to be babesiosis. Other pathogens seem to be prevalent but in much lower prevalence rates. So far, it is unclear whether and where a risk of leishmaniosis occurs in some areas of Romania. The overall data imply that Romania shows the pathogen fauna of Central Europe as well as the Mediterranean fauna with tick and mosquito species found mainly in southern Europe. The distribution of West Nile virus infections in humans during the last two big epidemics (1997, 2010) shows that there may be changes in the prevalence of vector-borne diseases and especially the example of West Nile virus implies a western and northern spread into the direction of Central Europe.

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A serological and molecular survey of canine vector-borne diseases in hunting dogs from Korea

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ABSTRACT

A serological and molecular survey for vector-borne diseases in hunting dogs from the Republic of Korea (South Korea) was conducted. From a total of 440 dogs from five major provinces of the country, the number of serologically positive dogs for any of *Dirofilaria immitis*, *Anaplasma phagocytophilum*, *Ehrlichia canis*, and *Borrelia burgdorferi* was 93 (40.6 %). The percentages of dogs positive for *D. immitis* (18.4 %) and *A. phagocytophilum* (18.0 %) were similar, whereas seropositive dogs to *E. canis* (5.7 %) and *B. burgdorferi* (1.4 %) were relatively low in number. Blood samples from these dogs were also analyzed by a series of real-time PCR assays for major vector-borne pathogens (*Anaplasma* spp., *Babesia* spp., *Bartonella* spp., *Mycoplasma haemocanis*, '*Candidatus* Mycoplasma haematoparvum', *Ehrlichia* spp., *Hepatozoon* spp., *Leishmania* spp., *Neorickettsia risticii*, and *Rickettsia* spp.). The number of dogs positive for any of the ten pathogens by real-time PCR was 274 (62.3 %) with the highest prevalence observed in Gyeongsang province (20.9 %), followed by Jeolla (15.9 %), Gyeonggi (10.5 %), Chungcheong (8.6 %), and Gangwon (6.4 %) provinces. The number of dogs positive for '*Cand.* M. haematoparvum' was the highest (43.2 %) followed by *M. haemocanis* (38.2 %), *Babesia gibsoni* (5.2 %), *Anaplasma* spp. (2.3 %), and *Hepatozoon canis* (0.2 %). No dogs were positive for *Bartonella*, *Ehrlichia*, *Leishmania*, *Neorickettsia*, or *Rickettsia* by PCR.

Tick infestation, tick prophylaxis, and risk of tick-borne infections in dogs in Northeast Germany: a tick collection study

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INTRODUCTION

Canine ticks are of outstanding importance as vectors of various agents causing diseases in humans and animals. Dogs in particular bear a high risk for tick-borne infections since they are frequently subject to tick infestation.

OBJECTIVE

The objective of this tick collection study was the prospective evaluation of tick infestation, seasonality of tick species, tick prophylaxis, and risk of tick-borne infections in dogs in the Berlin/Brandenburg area.

METHODS

A total of 392 owners of 441 dogs were included in the study between March 2010 and April 2011. Dogs participated in the study between one and 13 months (median 11). Owners were asked to collect ticks from their dogs and to fill out a questionnaire for each tick (including questions regarding tick location on the dog, use of tick repellents, etc.). Ticks were stored by the owners in laboratory tubes containing ethanol. Species, developmental stage, gender, and scutal indices of the ticks were determined. The ticks were analyzed for different infectious agents such as *Babesia* spp., *Rickettsia* spp., *Anaplasmataceae,* and *Borrelia* spp. by PCR and sequencing or high-resolution melting curve analysis.

RESULTS

From 251 dogs, 1–60 ticks (median 4) were sent in by their owners; no ticks were sent in from the remaining 190 dogs due to different reasons (dogs not infested, ticks not collected, no information available).

Dog-specific characteristics such as coat length and dog size were significant factors for infestation with ticks.

An overall number of 1,728 ticks belonging to four different species were collected: *Ixodes ricinus* (46 %), *Dermacentor reticulatus* (45 %), *Ixodes hexagonus* (9 %), *Rhipicephalus sanguineus* (0.1 %). 52 %, 34 %, and 13 % of the dogs were infested by one, two, or three different tick species, respectively, during the course of the study.

Tick infestation was observed throughout the year. The highest number of ticks was found in May/June (peak of activity of *I. ricinus* on dogs) and September/October (peak of activity of *D. reticulatus* on dogs).

Measuring of scutal indices revealed that more than 60 % of the *I. ricinus* and more than 40 % of the *D. reticulatus* ticks were removed only after feeding for more than two days. The head, neck, chest and limbs of the dogs were found to be the most common attachment sites.

Data regarding tick prophylaxis with substances licensed for dogs by the Medicinal Products Act (correctly or incorrectly treated) or non-treated dogs were available for 1,195 ticks. About two thirds of the ticks were collected from dogs that had been treated incorrectly or not treated at all. One third of the ticks were collected from dogs that had been treated correctly according to their owners.

DNA of *Babesia* spp. was detected in 2.5–3 %, DNA of *A. phagocytophilum* in 4–6.5 %, DNA of *Rickettsia* spp. in 44–61 %, DNA of '*Candidatus* Neoehrlichia mikurensis' in 4.3–5.9 %, and DNA of *Borrelia* spp. in 11.2–11.6 % in ticks of the species *I. ricinus/I. hexagonus*, respectively. In *D. reticulatus*, no DNA of *Babesia canis* or other piroplasms was detected, but DNA of *Rickettsia* spp. was detected in more than 39 % of the ticks examined.

CONCLUSION

In conclusion, this study revealed that infestation with ticks occurred frequently in the majority of the dogs from the Berlin/Brandenburg area. Determination of the scutal index showed that ticks were often removed after feeding for more than two days which is sufficient to infect the host with pathogens such as *Borrelia burgdorferi* or *Anaplasma phagocytophilum*. For the most part, prophylactic measures to prevent infestation (regular examination for ticks, use of ectoparasitic products) were not performed correctly. It is recommended that thorough information be given to dog owners with regard to ectoparasitic prophylaxis; this should become an important feature in veterinary practice as part of the annual health check.

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Flea species infesting dogs in Florida and *Bartonella* spp. prevalence rates

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INTRODUCTION

Of the flea species known to infest domestic and wild canids worldwide, *Ctenocephalides felis* is the most common and has a wide range of mammalian host species. In contrast, *Ctenocephalides canis* is less common and is mostly a parasite of wild and domestic canines.[1] However, in some studies, *Pulex* spp. have been reported to be the most common flea species infesting dogs.[2,3] A number of *Bartonella* spp. including *B. henselae*, *B. vinsonii* subsp. *berkhoffii*, *B. clarridgeiae*, and *B. rochalimae* are known to infect dogs and humans and can be associated with a variety of clinical abnormalities including vasculitis, endocarditis, and myocarditis.[4–12] Few studies have concurrently investigated the prevalence of *Bartonella* in the blood of canids and the fleas taken from the dogs with current infestations.

OBJECTIVES

The objectives of this study were to determine the genera of fleas infesting dogs in an animal shelter in Florida, the distribution of *Bartonella* spp. within the fleas, and the distribution of *Bartonella* spp. within the healthy dogs from which the fleas were collected.

MATERIALS AND METHODS

Fleas, whole blood in EDTA, and serum were collected from 80 healthy dogs upon admittance to shelter in North-Central Florida as part of routine screening for *Dirofilaria immitis* and other vector-borne diseases. The samples were stored at –20 °C until shipped to Colorado State University. Fleas from 43 dogs were examined under dissecting microscope and the genus determined based on key distinguishing features. Fleas determined to be from the *Ctenocephalides* genus were further distinguished as *C. felis* or *C. canis*, whereas *Pulex* were classified simply as *Pulex* spp., given the inherent difficulty in distinguishing *P. irritans* from *P. simulans*. The fleas from the 80 dogs were grouped (< 5 fleas per pool) by genera when known, total DNA was extracted from each flea group and the blood of the corresponding dog, and *Bartonella* spp. DNA was amplified using a previously reported genus-specific, conventional PCR assay that amplifies the 16S-23S ribosomal rRNA intergenic region of *Bartonella* spp.[13] Positive amplicons were sequenced (Macromolecular Resources, Colorado State University) to confirm the *Bartonella* species; the species with the highest %-homology is reported. Amplicons for which sequencing revealed homology to *B. vinsonii* subsp. *berkhoffii* were shipped to North Carolina State University for specific genotyping as described previously.[14]

RESULTS

The flea genera were determined for 43 dogs; 28 dogs (65.1 %) were infested with *C. felis* alone, 8 dogs (18.6 %) were infested with *Pulex* spp. alone, and 7 dogs (16.3 %) had mixed infections. *Bartonella*

spp. DNA (*B. vinsonii* subsp. *berkhoffii* or *B. rochalimae* DNA) was amplified from blood of 14 of 80 dogs (17.5 %). *Bartonella* spp. DNA (*B. vinsonii* subsp. *berkhoffii*, *B. rochalimae*, or *B. clarridgeiae*) was amplified from fleas taken from 9 of 80 dogs (11.3 %). Of the *B. vinsonii* (BV) DNA samples from blood that were successfully genotyped, five were BV genotype II and four were BV genotype I. Of the BV DNA samples from flea groups that were successfully genotyped, three were BV genotype II and two were BV genotype I. The *Bartonella* spp. amplified from dog blood did not always match the *Bartonella* spp. DNA amplified from the fleas collected from the dog.

DISCUSSION

This study confirms that dogs in this area of North-Central Florida can be infested simultaneously with both *C. felis* and *Pulex* spp., and that mixed infection can be relatively common. To our knowledge, this is the first study to amplify DNA of *B. vinsonii* subsp. *berhoffii* from *Pulex* spp. collected from domestic dogs. Since *Pulex* spp. also feed on humans, this genus may be a source for zoonotic transfer of *B. vinsonii* subsp. *berhoffii* to people. These findings provide evidence to support the hypothesis that dogs may be a reservoir host for this pathogen. Experimental transmission studies should be performed to explore this hypothesis further.

B. rochalimae DNA was amplified from blood of 7.5 % of the dogs described here, and two of the dogs were infested with *Pulex* spp. that were also positive for this putative human zoonotic agent. The role dogs play in the potential zoonotic transmission of *B. rochalimae* to humans and the role this agent plays in clinical disease syndromes in dogs should be explored.

B. henselae DNA was not amplified from the blood of any dog or flea group in this study. In contrast, *B. henselae* DNA was amplified from the blood of 68.4 % of the cats and five of nine *C. felis* groups in the same animal shelter in a previous study.[15] *B. henselae* is only present in low levels in blood of dogs and so the failure to detect the organism in the dogs described here may merely reflect the sensitivity of the screening PCR assay used. Failure to detect *B. henselae* in the *C. felis* flea groups described herein suggests that *C. felis* from cats in the shelter were not shared with the dogs.

B. clarridgeiae has been associated with clinical illness in some dogs and the agent is commonly amplified from the blood of cats and their fleas.[8,15] While *B. clarridgeiae* DNA was amplified from *C. felis* and *Pulex* spp. groups, none of the dogs in the studies described here were PCR-positive. As discussed for *B. henselae*, this may merely reflect the sensitivity of the assay used and levels of bacteremia induced by *B. clarridgeiae* in dogs.

Overall, the results of this study support the recommendation that flea control should be provided to all dogs to potentially lessen the risk of transmission amongst dogs and humans.

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Ctenocephalides felis

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Seroepidemiology of canine leishmaniosis in Évora (southern Portugal): 20-year trends

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ABSTRACT

BACKGROUND: Canine leishmaniosis (CanL) is an endemic zoonosis in the southern regions of Europe. This paper reports the trend in CanL seroprevalence in the municipality of Évora (southern Portugal), where the disease is endemic, over a period of 20 years. The work comprises three different studies that were conducted in the years of 1990 (n = 3,614), 1999 (n = 3,563), and 2010 (n = 1,485 dogs). Blood samples were collected during the anti-rabies vaccination campaigns. Anti-*Leishmania* antibodies were detected with the direct agglutination test (DAT).

FINDINGS: The total percentages of DAT seropositive dogs were 3.9 % (in 1990), 9.4 % (in 1999), and 5.6 % (in 2010). The overall seroprevalence was significantly higher in 1999 compared to 1990, but in 2010 a significant decrease was found in comparison with 1999. However, compared to 1990 the overall seroprevalence was still significantly higher in 2010. From 1990 to 2010, seroprevalence has switched from significantly lower to higher in the rural areas. Relatively few dogs showed clinical signs of overt disease (0.8 % to 2.0 %) with lymphadenopathy, onychogryphosis, and skin involvement as most frequently observed. Gender-associated differences in seroprevalence were not found, and most commonly seropositive dogs were working or stray animals. The mean age of seropositive dogs was significantly higher than seronegative dogs in all three sampling rounds.

CONCLUSIONS: A high proportion of dogs, which are apparently healthy, yet seropositive, may remain an important factor in limiting the outcome of zoonotic leishmaniosis control efforts.

KEYWORDS: Canine leishmaniosis, Direct agglutination test, Évora, Portugal, Seroprevalence

FINDINGS

Leishmaniosis, a disease caused by protozoan parasites of the genus *Leishmania*, is one of the major communicable diseases in the world and one of the most important vector-borne parasitic diseases after malaria.[1] Dogs are the main domestic reservoir for human infection where zoonotic

leishmaniosis is caused by *Leishmania infantum*.[2] Canine leishmaniosis (CanL) is a systemic chronic disease and clinical manifestations usually include lymphadenopathy, dermatitis, alopecia, cutaneous ulcerations, onychogryphosis, lameness, anorexia, weight loss, cachexia, ocular lesions, epistaxis, anaemia, diarrhoea, and renal failure.[3] Even when they receive treatment, severely affected dogs do not often survive the disease. Nevertheless, a significant proportion of infected animals remain asymptomatic,[4] but these asymptomatic infected dogs may act as carriers of *L. infantum* and are capable of transmitting the parasite to the vector, the phlebotomine sandflies.[5] CanL is endemic in all countries of the Mediterranean Basin, including the European countries Portugal, Spain, France, Italy, Greece, Croatia, Albania, Malta, and Cyprus. Emerging trends in the seroprevalence of CanL in many traditional leishmaniosis foci are being reported.[4,6–8] Possibly due to global warming, CanL is currently also being reported in foci outside the classical area of the disease in the Mediterranean countries.[9–11] For example, cases of autochthonous CanL have recently been reported from Hungary[12] and Germany.[13]

In Portugal, there are four main CanL foci: the province of Trás-os-Montes e Alto Douro (in the North), the region of Lisbon (in the West), the province of Algarve (in the South), and the municipality of Évora (in the southern region of Alentejo). Previous studies conducted in Trás-os-Montes e Alto Douro have demonstrated that there is a considerable prevalence of CanL in northern Portugal with approximately 20 % of dogs being seropositive.[14,15] In the context of emerging disease, the present study describes trends in seroprevalence of CanL over two decades in the municipality of Évora in southern Portugal (38° 34′ 17′′ N, 07° 54′ 31′′ W). Évora comprises 19 different administrative sections (parishes) of which eight are considered to be urban and the other rural, covering an area of 1,307.04 km^2 with an average altitude of 300 m above sea level. The climate and vegetation are typically Mediterranean, with dry hot summers (32 – 35 °C) and maximum rainfall in spring and autumn, and mild winters with temperatures rarely going below 5 °C. The sandfly season runs from May to October: *Phlebotomus sergenti* is the most abundant species, followed by *Phlebotomus perniciosus*.[16] The known species of *Leishmania* is *L. infantum* MON-1.[17]

This study was approved by the University of Évora ethics committee for research in health sciences and well-being as complying with the Portuguese legislation for the protection of animals (Law no. 92/1995, from September the 12th). Dogs were surveyed during the yearly anti-rabies vaccination campaigns in 1990 (n = 3,614), 1999 (n = 3,563), and 2010 (n = 1,485). All animals were clinically examined by veterinary practitioners and considered as either apparently healthy or clinically suspect, when either none or at least one clinical sign or lesion compatible with CanL was noted. Blood samples were collected from the cephalic vein and spotted into the middle of a filter paper allowed to air-dry and stored at −20 °C until testing with the direct agglutination test (DAT) for titration of anti-*Leishmania* antibodies.[18,19] The samples analyzed in 1990 and 1999 were tested by using a liquid DAT antigen prepared from *Leishmania donovani* promastigotes (strain 1-S) with a cut-off titer of 1:320.[16] The samples collected in 2010 were analysed with DAT based on freeze-dried antigen, with a cut-off titer of 1:400.[18,19] Chi-square test compared proportions of positivity (no. of dogs found seropositive divided by the no. of dogs tested) related to the categorical dependent variables location, gender, and ability. The exact binomial test was used to calculate confidence intervals (CI) for proportions, with a 95 % confidence level. Differences between the ages of dogs were compared with the Mann–Whitney U test. Analyses were done with StatLib or SPSS 11.5 software for Windows. A probability (p) value < 0.05 was regarded as statistically significant.[20] The serology data obtained in 1990 have been previously reported,[16] but again presented in the current paper to allow a comparison over a time period of 20 years. The total numbers of DAT seropositive dogs were 141 (3.9 %) in 1990, 335 (9.4 %) in 1999, and 84 (5.6 %) in 2010. The overall seroprevalence was significantly higher in 1999 (9.4 %) compared to 1990 (3.9 %), but in 2010 a

significant decrease in seroprevalence (5.6 %) was found compared to 1999 (Table 1). Nevertheless, the overall seroprevalence was significantly higher in 2010 compared to 1990. From 1990 to 1999 the total increase in seroprevalence was simultaneous with significant increases in both the urban and rural areas. On the other hand, from 1999 to 2010, the decrease in seroprevalence was simultaneous with a significant decrease only in the urban area (Table 1). At last, when comparing the years 1990 and 2010, the increase in seroprevalence was simultaneous with a significant increase only in the rural area. A questionnaire conducted in Portugal in the year 2006 revealed that dog owners in urban areas had a significantly higher knowledge on CanL compared to the rural ones.[21] No data are available on the prophylactic measures applied to dogs from Évora, but it might be possible that preventatives,[22] especially insecticides with a repellent effect, have increasingly been used on dogs from the urban area.

Table 1. Percentages of seropositive dogs found in urban or rural settings in Évora municipality

Study area	Year 1990		Year 1999		Year 2010	
	% seropositive (No. dogs tested)	95 % CI (%)	% seropositive (No. dogs tested)	95 % CI (%)	% seropositive (No. dogs tested)	95 % CI (%)
Urban	4.9[a] (1,524)	3.9–6.1	↑ 10.1 (1,661)	8.7–11.7	↓ 3.3[b] (675)	2.1–4.9
Rural	3.2[a] (2,090)	2.5–4.0	↑ 8.8 (1,902)	7.5–10.10	7.6[b] (820)	5.8–9.6
Total	**3.9 (3,614)**	**3.3–4.6**	**↑ 9.4 (3,563)**	**8.5–10.4**	**↓ 5.6 (1,495)**	**4.5–6.9**

a, b: statistically significant difference between areas; ↑↓: statistically significant difference to the previous year, for the same area

Out of the 141 seropositive dogs in 1990, 113 (80.1 %) were apparently healthy and 28 (19.9 %) were considered clinically suspect (0.8 % of the total study population in 1990). Similar observations were made in 1999 and 2010, when 263 healthy dogs (78.5 %) and 72 (21.5 %) suspect dogs (2.0 % of the total study population) were DAT-positive (out of the total of 335 animals) and 71 (80.1 %) healthy and 13 (9.9 %) suspect dogs (0.9 % of the total study population) were seropositive (out of 84), respectively. From 1990 to 1999, there was a significant increase in the percentages of suspect dogs among the total populations; and from 1999 to 2010, there was a significant decrease. However, differences between the proportions of clinically suspect dogs among the seropositive ones were not statistically significant when comparing the years 1990–1999 or 1999–2010. The percentage of clinically suspect dogs among the total populations seems to have followed that of the seropositivity. Among every 5.0 (year 1990), 4.7 (year 1999), or 6.5 (year 2010) seropositive dogs, there was one animal clinically suspect of CanL. Lymphadenopathy (69.2–89.3 %), followed by onychogryphosis (53.2–59.7 %) and skin involvement (38.9–46.4 %), were the most frequently observed clinical manifestations in the present study and are among those most commonly found.[3,4] The number of dogs with overt disease is thus relatively low, but at a comparable level found in other studies in the Iberian Peninsula.[4,14]

Gender-associated differences between male and female seroprevalences were not found in this study (Table 2) and are in line with previous observations,[4,14,23] but in contrast to some other studies in which a higher prevalence was observed in males.[24] With respect to ability or occupation of the dogs, it was noted that the highest levels of seropositivity were found among stray dogs, but the majority of seropositive dogs in absolute numbers were mainly working animals, including guard and hunting dogs (Table 2). An association between age and seroprevalence was also observed (Table 3) in all three rounds of sample collection and confirmed previous findings that dogs of older age are at a higher risk of being seropositive.[4,8,15]

Table 2. Percentages of seropositive dogs in relation to gender and ability

	Year 1990 % seropositive (No. dogs tested)	Year 1999 % seropositive (No. dogs tested)	Year 2010 % seropositive (No. dogs tested)
Gender			
Female	3.4 (1,926)	↑ 9.3 (1,837)	6.2 (796)
Male	4.5 (1,688)	↑ 9.5 (1,726	5.0 (699)
Ability			
Guard	3.6 (1,483)	↑ 7.9* (1,702)	↓ 3.7* (854)
Hunting	3.8 (992)	↑ 13.7* (774)	11.0* (227)
Pet	4.7 (656)	5.9* (612)	3.7 (246)
Shepherd	3.4 (351)	↑ 12.1 (298)	↓ 5.0 (119)
Stray	11.5 (61)	17.9* (117)	↑ 41.4* (29)
ND	0.0 (71)	3.3 (60)	0.0 (20)
Total	**3.9 (3,614)**	**↑ 9.4 (3,563)**	**↓ 5.6 (1,495)**

DAT: direct agglutination test; SD: standard deviation;
a, b, c: statistical significant difference between seropositive and seronegative;
↑: statistical significant difference to the previous year;
*: age was not determined for one dog

The findings of the presented surveys reveal that a considerable number of dogs from Évora are seropositive for CanL. A peak in seropositivity was observed in 1999 and there is a decline in the number of positive cases in 2010. It should, however, be noted that the total number of dogs sampled in 2010 was much lower (< 50 %) compared to previous sample rounds. This could be due to the fact that people, perhaps influenced by the current economic crisis, are becoming reluctant to have their dogs anti-rabies-vaccinated and tested, and may dispose of animals with ill health (in 2010 only 13 animals with clinical signs of CanL were found in the present study). This may bias, i.e., underestimate, the true seroprevalence in the region. The reported seroprevalences are in a comparable range to those reported in other studies from Portugal.[25,26] Also, in other countries in the Mediterranean Basin, a rather wide range of seroprevalence is being reported.[4,6,8]

Table 3. Relationship between age of dogs and seropositivity

DAT	Year 1990		Year 1999		Year 2010	
	Mean age (± SD) – years	No. dogs	Mean age (± SD) – years	No. dogs	Mean age (± SD) – years	No. dogs
Urban	7.04 (± 2.71)[a]	141	6.96 (± 2.98)[b]	335	6.57 (± 3.37)[c]	84
Rural	4.71 (± 2.40)[a]	3,473	↑ 5.26 (± 2.76)[b]	3,227	5.34 (± 2.81)[c]	1,411
Total	**4.80 (± 2.46)**	**3,614**	**↑ 5.42 (± 2.82)**	**3,562***	**5.41 (± 2.86)**	**1,495**

DAT: direct agglutination test; SD: standard deviation; a, b, c: statistical significant difference between seropositive and seronegative;
↑: statistical significant difference to the previous year; *: age was not determined for one dog

CONCLUSIONS

In conclusion, there is a high proportion of dogs that are seropositive for CanL in the municipality of Évora in Portugal but that appear as clinically healthy. If in analogy to human infections[27] where asymptomatic cases are thought to be also in the majority, with an estimated ratio of > 100 asymptomatic individuals per each clinical case, the number of infected dogs could be enormous. Indeed, it is difficult to provide an actual number of infected dogs, but it has been estimated based on seroprevalence studies that 2.5 million dogs from Italy, France, Spain, and Portugal are infected.[2] It remains a concern that so many animals are possibly infected with *L. infantum* and that they may

transmit the parasite either to other dogs or humans.[3,28] Therefore, control efforts should remain focussed on canines, but also the human population in these regions should be better monitored, too.

ABBREVIATIONS

CanL: Canine leishmaniosis; DAT: Direct Agglutination Test

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

HDFHS performed DAT (2010) and data analysis and wrote the manuscript. LC performed data analysis, tabulation and revision of the manuscript. SJSS organized the collection of canine samples and data and performed DAT analysis (1990 and 1999). All authors read and approved the final manuscript.

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A cross-sectional study on *Leishmania infantum* infection by serology and non-invasive PCR in sick and clinically healthy dogs from Cyprus

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BACKGROUND

Limited studies have been performed in Cyprus on canine *Leishmania infantum* infection. The study aims were: 1) to evaluate non-invasive diagnostic methods in the screening of apparently healthy dogs living in an endemic region as well as confirming leishmaniosis in sick dogs; 2) to evaluate two assays targeting the kDNA and ITS loci for real-time PCR; 3) to investigate the rate of infection based on PCR and serological tests in dogs living in Cyprus.

METHODS

L. infantum serology by ELISA and real-time PCR of blood, conjunctival and oral swab samples were performed in sick (n = 19) and apparently healthy (n = 120) dogs. Cytological evaluation of lymph node aspirates was also carried out in sick dogs.

RESULTS

Seropositivity in the apparently healthy and sick dog group was 17.9 % (20/117) and 100 % (18/18), respectively. The overall rate of infection by PCR was 34.17 % (41/120) (calculated by ITS or kDNA PCR-positive on any tissue sample) in apparently healthy group. For the sick dogs, the kDNA PCR-positivity rates in blood, conjunctival and oral swabs were: 74 % (14/19), 67 % (10/15), and 15 % (2/13), respectively. The ITS1 PCR rates in blood of apparently healthy dogs and sick dogs was 14 % (17/119) and 73.7 % (14/19), respectively. The presence of amastigotes by cytology was only detected in one sick dog (5.6 %). For the apparently healthy group, the positive kDNA PCR percentages in blood, conjunctival and oral swabs were: 23 % (28/120), 11.7 % (14/120), and 0 % (0/114), respectively. The kDNA PCR was significantly more sensitive than the ITS1 PCR ($p < 0.0001$) in the detection of leishmanial DNA in the blood of the apparently healthy dogs, with 23 % of the dogs positive by kDNA PCR versus 14 % by the ITS PCR. Finally, based on the apparently healthy dogs, the overall rate of infection (calculated by seropositive and/or ITS or kDNA PCR-positive dogs in any one tissue) was 45.83 % (55/120).

CONCLUSIONS

This study demonstrated a high prevalence of *L. infantum* infection in dogs in Cyprus. The similar positive PCR rates found in blood and conjunctival swabs suggest the use of conjunctival swabs as a non-invasive alternative technique for the detection of *Leishmania* infection. In contrast, oral swab PCR proved not to be a sensitive tool for detection of this infection. kDNA real-time PCR was more sensitive than ITS PCR.

Hemotropic *Mycoplasma* species infection in people and animals

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ABSTRACT

Hemotropic *Mycoplasma* spp. (hemoplasmas) are obligate epierythrocytic bacteria that infect numerous animal species, including Homo sapiens. Using PCR targeting the 16S rRNA and the RNaseP genes, we describe the molecular characterization of known and several novel hemotropic *Mycoplasma* found in the blood of a cohort of human (n = 703) and non-human primates (n = 52), white-tailed deer (n = 73), pet dogs (n = 506), and white-nose bats (n = 68).

The PCR prevalence of hemotropic *Mycoplasma* varied substantially among each group: humans (0.4 to 4.5 %, depending on the risk of arthropod and animal exposure) as compared to cymologous monkeys (84.6 %); dogs (0.14 %); white-tailed deer (89 %); and white-nose bats (47%). With the advent of molecular techniques, the mechanisms of intra- and interspecies transmission and pathogenic potential of hemotropic *Mycoplasmas* as a cause of human and animal disease requires additional study.

A survey of canine ectoparasites contributing to vector-borne diseases in India

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BACKGROUND

Ectoparasites of domestic dogs can cause dermatitis, anaemia, or act as vectors of pathogenic agents, resulting in disease not only in dogs but in some cases also in humans. This study aimed to determine the prevalence, burdens, and associated risk factors for species of ticks, fleas, lice, and biting flies found on shelter and stray dogs in four cities representing unique climatic niches in India.

RESULTS

Of 525 dogs examined, 417 (86.5 %) were infested with ectoparasites. A total of 1,496 ectoparasites were collected from 419 dogs, consisting of 832 ticks, 403 fleas, 202 biting flies, and 60 lice. The most common flea species isolated from dogs was *Ctenocephalides felis* (95.8 %), followed by *Ctenocephalides canis* (4 %), and *Pulex irritans* (0.2 %). Molecular characterization of ticks found *Rhipicephalus sanguineus* to be the most abundant species, followed by *Rhipicephalus microplus*, *Haemaphysalis longicornis*, *Rhipicephalus haemaphysaloides*, *Rhipicephalus* sp. voucher USNTC, and *Haemaphysalis punctata*. *Hippobosca longipennis* was observed on more than half the dogs sampled in Ladakh. Multivariate analyses revealed a significant correlation between location and ectoparasite infestation.

CONCLUSIONS

Ectoparasites are endemic among stray and shelter dogs in India. Almost all ectoparasite genera reported in this study act as a vector for at least one vector-borne pathogen of dogs and/or humans.

New insights into the taxonomy of *Rhipicephalus sanguineus*: implications for pathogen transmission?

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ABSTRACT

Rhipicephalus sanguineus is an ixodid tick originally described in 1806 by Latreille, based on specimens collected somewhere in Europe. However, morphological, cross-breeding, and molecular studies carried out over the past century have gathered irrefutable evidence supporting the existence of a cryptic species complex under the name *R. sanguineus*, whose number of sibling species around the world has yet to be ascertained. This fact is of medical and veterinary concern, considering that ticks currently identified as *R. sanguineus* have been regarded as proven or putative vectors of several pathogenic microorganisms infecting dogs and humans as well. However, differences in the occurrence and prevalence of some of these microorganisms (e.g., *Ehrlichia canis* and *Hepatozoon canis*) further support the being of a cryptic species complex and suggest that the vector competence of tick species belonging to this group may vary widely. The implications of current research on taxonomy and vector role of *R. sanguineus* ticks are discussed in this review.

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>>> Capelli *et al.*, *Parasites & Vectors* 2013; **6**:60 – http://www.parasitesandvectors.com/content/6/1/60

Risk of canine and human exposure to *Dirofilaria immitis*-infected mosquitoes in endemic areas of Italy

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ABSTRACT

BACKGROUND: The occurrence of infections by *Dirofilaria immitis* in canine and human populations depends on several factors linked to both the definitive and intermediate hosts. Little data are available on the risk of human and dog exposure to *D. immitis* in endemic areas. Data collected on dog- and human-bait traps in endemic areas of northeastern Italy were used to estimate the likelihood of a receptive host coming into contact with an infected vector.

METHODS: From 1997 to 1999, mosquitoes were collected from three sampling sites of northeastern Italy on *D. immitis* microfilaraemic dogs and on human baits. The bite/night/host rates were determined based on the number of feeding and probing mosquitoes on dogs and humans, respectively. The survival/mortality rates of different species of mosquitoes following the blood meal, and the rate of natural *Dirofilaria* infection in unfed specimens were estimated. The risk of exposure of dogs and humans to infected mosquito species was determined by combining the bite/night/host and the mosquito infection rates.

RESULTS: A total of 1,165 mosquitoes were collected on human (n = 815) and dog (n = 350) baits with varying species composition (i.e., *Culex pipiens*, 87.3 % and *Ochlerotatus caspius*, 11.6 %). Overall, dogs were more attractive to *C. pipiens* than humans (feeding rate 70.2 % vs. probing rate 25.9 %). The highest bite/night/host rate was 84.0 for dogs and 26.5 for humans. *C. pipiens* displayed a mortality rate of 76.3 % within 13 days and *O. caspius* of 100 % within two days following the infective blood meal. In addition, *D. immitis* DNA was detected in unfed *C. pipiens* (infection rate of 0.26–2.07 %). The infection rate adjusted for mosquito mortality was 0.38 %. Based on data collected, the contact between an infected mosquito and a host can occur as often as every four nights for *D. immitis*-infected mosquitoes in dogs and within two weeks for humans.

CONCLUSIONS: *C. pipiens* was confirmed as the most efficient natural vector of *D. immitis* in the studied area. In endemic areas, the risk of transmission can be very high for dogs and relevant for humans. Despite the increased awareness of veterinarians and owners on canine dirofilarioses, dogs from rural areas still maintain the natural life cycle of *Dirofilaria* spp., therefore acting as a source of infection to humans through vector bites.

KEYWORDS: *Dirofilaria immitis*, Mosquito vectors, Dogs, Humans, Risk of exposure

BACKGROUND

Dirofilaria immitis (Filarioidea, Onchocercidae) is responsible for cardiopulmonary dirofilariosis. This filarioid is transmitted by many species of mosquito vectors (genus *Culex*, *Aedes*, *Ochlerotatus,* and *Anopheles*), in which it develops into the infective third stage within different time frames, which depend upon several factors.[1–4] Among vector-borne helminths, *Dirofilaria* spp. have been recognized as emerging zoonotic agents, currently spreading throughout Europe.[5,6] The risk of canine and human infection by *D. immitis* is linked to a combination of several factors related with both the definitive and the intermediate hosts. For example, the rates of infection in the intermediate host depend on vector densities, host-seeking activity/feeding preference, and vector competence.[4,7,8] Mosquito species of the genus *Culex* and *Ochlerotatus* have been reported as major vectors of *Dirofilaria* in Italy and other European countries.[3,9,10] Current data on the vectors of *Dirofilaria* spp. derive from laboratory experiments, occasional findings in naturally infested insects, or from fieldworks using dogs and / or human-bait traps.[11] In these studies, the rate of mosquito infection has been estimated by insect dissection or biomolecular methods. Nevertheless, the risk of dogs and humans to be exposed to *D. immitis*-infected vectors in endemic areas has never been investigated.

In the present study, data on risk for dog and human exposure to *D. immitis*-infected mosquitoes in endemic areas of Italy have been examined and discussed.

METHODS

Sampling area and mosquito collections

From 1997 to 1999, nocturnal mosquito collections were carried out in lowland areas of north-eastern Italy, endemic for dirofilariosis.[5,6,10] Dog- and man-attracted mosquitoes were collected in three peri-urban sites i.e., Rodeano (site A; province of Udine, Friuli Venezia Giulia region; 46°06'43"N – 13°00'13"E, 130 m above sea level [a.s.l.]), Piove di Sacco (site B; province of Padua; Veneto region; 45°17'49"N – 12°02'06"E, 5 m a.s.l.), and Sarzano (site C; province of Rovigo; Veneto region; 45°04'50"N – 11°49'38"E, 5 m a.s.l.). The mosquito collection started in each site when a minimum temperature of > 15 °C was recorded, and terminated at the end of September of each year. Microfilaraemic dogs naturally infected by *D. immitis* (i.e., a 7-year-old male pure-breed dog (Bobtail) in site A; a 5-year-old male cross-breed dog (medium size) in site B; and a 6-year-old male cross-breed dog (small size) in site C) were used as bait in each sampling site. Average values of microfilaraemia, expressed as number of microfilariae per milliliter (mf/ml), were calculated by ten counts serially performed on 10 µl of blood samples collected from each dog at the beginning and at the end of the study.

Mosquitoes were also collected while landing on two persons. The same persons were employed in site B and C, whereas one of the two persons was replaced in site A. One dog- and one man-bait trap were employed simultaneously in the three sites for 17 sampling nights, i.e., for six nights in site A (August 5 – September 15, 1997), six nights in site B (July 29 – September 23, 1998), and five nights in site C (June 23 – September 15, 1999). The traps consisted of a cylindrical structure of wood (2.3 m of diameter x 2.0 m in height for dog and 2.0 m x 1.5 m for humans) covered by a net, five cm above the ground to allow mosquitoes to enter the trap[12,13] (Figure 1). The risk of mosquitoes escaping from the trap was considered negligible, due to both

Figure 1. **Dog-bait trap (site C).**

the insect host-seeking behaviour (in which mosquitoes rarely fly downwards when leaving an enclosed space), and to the fact that engorged females do not move after the blood meal, resting on the net.[14] In each site, the human and dog baits were located at a distance of at least ten meters to avoid interference between mosquito attractants.

Mosquito collections were performed using a paper cup aspirator[15] from 8:00 p.m. until 6:00 a.m. In dog-bait traps, engorged insects resting on the net were collected every two hours. Unfed mosquitoes were left in the traps until the last sampling (6:00 a.m). Mosquitoes were collected by using the above procedures in the human-bait traps, with two persons acting as bait and collectors, simultaneously. Only mosquitoes attempting to probe on humans were collected.

Laboratory procedures

The mosquitoes were identified according to morphological keys.[16] The unfed mosquitoes collected in dog-bait traps and all the mosquitoes collected in human-bait traps (probing and resting on the net) were pooled (minimum 1 – maximum 12 specimens) according to species, date, and site of collection.

The numbers of human-probing mosquitoes and of dog-fed specimens were used to evaluate the bite/night/host rates. Alive dog-fed insects were kept under standard insectary conditions (25–27 °C, ~90 % relative humidity) for 13 days, and observed daily in order to estimate the mortality rate after the microfilaraemic blood meal.[17]

The DNA was extracted separately from pools of the insect abdomens and thorax-heads, respectively, in order to detect potentially infective specimens.[18]

The pooled samples were analyzed by PCR amplification with *D. immitis*- and *D. repens*-specific pair of primers (R1-R2, and I1-I2, respectively) as previously described.[19] All sequences generated were compared to sequences available in GenBank using standard BLAST searches.

Ethical statement

The procedures for sampling mosquitoes attracted to humans and dogs followed a standard protocol.[12] At the time of the study, in absence of any Ethical for Animal Experimentation Committee at the University of Padua, the study was performed according to the legislative decree no. 116 (27 January 1992), implementing the Council directive no. 86/609/EEC on the protection of animals used for experimental purposes. All humans involved in the field study were staff employed by the Faculty of Veterinary Medicine of Padua and provided their informed consent to all components of the study. Since in human-bait traps two persons collected mosquitoes while probing and not following feeding, the infection risk with any pathogen was considered negligible. The dogs used in the study were naturally infected by *D. immitis* and were usually kept outdoors during the night. Dogs were neither anesthetized nor forced under the traps, which were placed at their usual sleeping site. The dog owners declared their unwillingness to treat them against dirofilariosis. However, a free treatment was offered to dogs at the end of the study.

Statistical analyses

The differences among the proportions of mosquitoes that fed on dogs or landed on humans were tested by the chi-square test or the Fisher exact test when appropriate.[20] The mean numbers of different mosquito species captured on dogs and humans were compared by ANOVA, after $\log_e (x+1)$ transformation of the data (software SPSS, version 13.0 for windows).

The rate of infection in mosquitoes was adjusted for pooled samples, calculating the Estimated Rate of Infection (ERI) using the following formula:[21]

ERI = 1-(1-n/N)1/k

where n is the number of positive pools, N the number of examined pools, and k the average number of specimens in each pool.[22]

Before calculating ERI for *D. immitis*, the positive pools detected from the pooled mosquito abdomens (infected and not still infective) was adjusted with the mean mortality rate found in reared *C. pipiens*.

The exposure risk, i.e., the risk of contact with an infected mosquito species, was calculated by combining the bite/night/dog and the bite/night/human rates at each sampling with the ERI as follows:

Days to contact a *Dirofilaria* spp.-infected mosquito = 100/(bite/night/host)/ERI

The ERI calculated in this study was used for site C, while the ERI estimated in a separate study performed in the same area in 2010[11] was used for site B. No data on mosquito ERI are available for site A. The risk of exposure was expressed as "risk in days" (i.e., the minimum number of nights of exposure necessary to come in contact with an infected mosquito under the specific epidemiological conditions of each site at each data time/point). Therefore, the shorter the time, the higher the risk of contact.

RESULTS

The dogs trapped in site A, B, and C displayed a microfilaraemia of 30–35,000, 100–110,000, and 110–125,000 mf/ml at the beginning and at the end of the study, respectively.

Of the 1,165 mosquitoes captured on host baits (i.e., 350 on dogs and 815 on humans), the most represented species was *Culex pipiens* (n = 1017; 87.3 %), followed by *Ochlerotatus caspius* (n = 135; 11.6 %), *Culex modestus* (n = 5; 0.4 %), *Coquillettidia richiardii* (n = 4; 0.3 %), and *Aedes vexans* [*Aedimorphus vexans*] (n = 4; 0.3 %). The latter two species were captured on humans only (Table 1).

Table 1. Total number and species of mosquitoes collected using dog- and human-bait traps and number and proportion (%) of fed (dog) or landed (man) mosquitoes

	Dog (n = 3)			Man (n = 6)[a]		
	Total no. mosquitoes	Fed	%	Total no. mosquitoes	Landed	%
Culex pipiens	329	231	70.2*	688	178	25.9*
Ochlerotatus caspius	20	18	90.0	115	92	80.0
Coquillettidia richiardii	0	0	–	4	4	100.0
Culex modestus	1	1	100.0	4	4	100.0
Aedes vexans	0	0	–	4	3	75.0
Total	**350**	**254**	**72.6***	**815**	**281**	**34.5***

a: Two persons per trap. Statistically significant differences are marked (*) ($p < 0.01$)

Although the mean number of mosquitoes per dog/human which entered the traps did not differ significantly, the rate of *C. pipiens* that fed on dogs was more than double that of mosquitoes probing on humans (i.e., 70.2 % vs. 25.9 %; p < 0.01) (Table 1). The same tendency (p = 0.053) was observed for *O. caspius* (i.e., 90.0 % vs. 68.9 %) in site C (Table 2).

The attractiveness of *C. pipiens* to dogs and humans varied significantly within the same host species (Table 2). Specifically, the feeding rate of mosquitoes feeding on dogs at site B was higher than that recorded on the other two dogs (i.e., 97.5 % vs. 38.6 % at site A and 46.4 % at site C). In addition, the two persons at site A were more attractive (landing rate of 44.4 %) than the ones at sites B and C (i.e., landing rate of 16.8 % and 21.2 %, respectively) (Table 2).

Table 2. *Culex pipiens* and *Ochlerotatus caspius* collected using dog- and human-bait traps and number and proportion (%) of fed (dog) or landed (man) mosquitoes according to site and night of collection (*)

Site	Date dd/mm/yy	*Culex pipiens pipiens*				*Ochlerotatus caspius*			
		Dog		Man§		Dog		Man§	
		fed/tot	%	landed/tot	%	fed/tot	%	landed/tot	%
Site A (Udine)	05/08/97	2/12	16.7	0/11.5	0.0	0	–	0	–
	13/08/97	10/15	66.7 [a]	26.5/45.5	58.2 [a]	0	–	0	–
	20/08/97	1/9	11.1	3.5/8	43.8	0	–	0	–
	17/09/97	3/6	50.0	1.5/3	50.0	0	–	0	–
	22/09/97	1/8	12.5	4/10	40.0	0	–	0	–
	25/09/97	5/7	71.4 [b]	2/7.5	26.7 [b]	0	–	0	–
	Total	**22/57**	**38.6 [A]**	**38/85.5**	**44.4 [CD]**	**0**	**–**	**0**	**–**
Site B (Padova)	29/07/98	84/84	100.0 [c]	9.5/68.5	13.9 [c]	0	–	2/2	100.0
	10/08/98	64/67	95.5 [d]	2/4.5	44.4 [d]	0	–	3.5/3.5	100.0
	20/08/98	7/7	100.0	1/1	100.0	0	–	0/0.5	0.0
	26/08/98	3/3	100.0	0/0	0.0	0	–	0	–
	23/09/98	0/1	0.0	0/0.5	0.0	0	–	0	–
	Total	**158/162**	**97.5 [eAB]**	**12.5/74.5**	**16.8 [eC]**	**0**	**–**	**5.5/6**	**91.7**
Site B (Padova)	23/06/99	20/36	55.6 [f]	4.5/75.5	6.0 [f]	6/7	85.7	0.5/2.5	20.0
	29/06/99	6/8	75.0	10.5/24.5	42.9	0	–	4.5/5.5	81.8
	14/07/99	2/35	5.7	1/7.5	13.3	6/6	100.0	10/17	58.8
	27/07/99	16/19	84.2 [g]	21.5/69	31.2 [g]	6/7	85.7	17.5/21.5	81.4
	19/08/99	7/10	70.0	1.5/7.5	20.0	0	–	3/5	60.0
	15/09/99	0/2	0.0	0/0	–	0	–	0	–
	Total	**51/110**	**46.4 [iB]**	**39/184**	**21.2 [iD]**	**18/20**	**90**	**35.5/51.5**	**68.9**

* Equal letters correspond to significant difference, p < 0.01 (uppercase = comparison within the same host species at the three sites, vertical; lowercase = comparison among dog/humans at the same site, horizontal)

§ Landed and total number of mosquitoes collected per person

The overall mortality rate of fed *C. pipiens* 13 days following collection was 76.3 %. In particular, of the 148 mosquitoes, 95 (64.2 %) died spontaneously within one week (Table 3). Mortality rates varied among sites with the highest rate (i.e., 91.3 %) recorded in mosquitoes which fed on the dog

from site C (i.e., the animal harbouring the largest number of microfilariae) (Table 3). However, a variation of mf density throughout the study period cannot be excluded.

O. caspius specimens, collected only at site C, died within two days (data not shown).

Out of the 915 unfed mosquitoes processed by PCR, eight pools of *C. pipiens* from site C were positive for *D. immitis*, one in thorax/head pools and seven in abdomen pools, corresponding to an estimated rate of infection (ERI) of 0.26 % (95 % CI 0.01–1.2) and of 2.07 % (95 % CI 1.03–4.11), respectively. After adjustment for mortality, the overall ERI for site C was 0.38 %; this value was

Table 3. Mortality in laboratory-reared *Culex pipiens* (n = 148) within 13 days following the blood meal, according to microfilaraemic dogs and site of sampling (mf = microfilariae)

Site	mf/ml[a] (x 1,000)	Days of mosquito breeding														Total no. dead	Overall mortality rate
		0	1	2	3	4	5	6	7	8	9	10	11	12	13		
Site A	30–35	18	4	6		2				1			1			14	77.8 %
Site B	100–110	84	3	6	7	6	10	5	5	5	1	2	2	2	3	57	67.8 %
Site C	110–125	46		27	6	4	1	2	1						1	42	91.3 %
Total		**148**	**7**	**39**	**13**	**12**	**11**	**7**	**6**	**6**	**1**	**2**	**3**	**2**	**4**	**113**	**76.3 %**

a: Microfilaraemic values detected at the beginning and at the end of each mosquito collection

used to estimate the risk of exposure to a *D. immitis*-infective *C. pipiens* at the same site. Conversely, the risk of exposure to *D. immitis* at site B was calculated using the ERI recorded in a study performed 10 years later in the same area.[11] For site A, no data on mosquito *Dirofilaria* spp. rate of infection are available.

Based on this data and according to biting rates and ERI recorded at different sites (Table 4), the risk for dogs and humans to be exposed to *D. immitis*-infected *C. pipiens* could range from a minimum of four to a maximum of 300 days and a minimum of 12 to a maximum of 901 days, respectively (Table 4).

Table 4. Potential risk of exposure (days) to *Dirofilaria immitis*-infected *Culex pipiens* based on fed (dog) or landed (man) mosquitoes per night, according to date and site)

Site	Date dd/mm/yy	Bites/Night/Host		ERI *	Days §	
		Dog	Man	%	Dog	Man§
Site B (Padova)	29/07/98	84	9.5	0.278	4	38
	10/08/98	64	2	0.111	14	450
	20/08/98	7	1	0.111	129	901
	26/08/98	3	0	0.111	300	–
	23/09/98	0	0	0.421	–	–
Site C (Rovigo)	23/06/99	20	4.5	0.38	13	58
	29/06/99	6	10.5	0.38	44	25
	14/07/99	2	1	0.38	132	263
	27/07/99	16	21.5	0.38	16	12
	19/08/99	7	1.5	0.38	38	175
	15/09/99	0	0	0.38	–	–

* ERI = estimated rate of infection. For site C, ERI was calculated in this study and adjusted for *C. pipiens* mean mortality; for site B, ERI is from ref. 11

§ Minimum number of days to come in contact with a *D. immitis*-infected mosquito

DISCUSSION

The results of this study contribute to current understanding of the epidemiology of *Dirofilaria* spp. in an endemic area of northeastern Italy. Based on the dog/human attractiveness, on the survival after feeding on dogs with high microfilariae concentrations and on the overall rate of positive mosquitoes, *C. pipiens* was the most efficient natural vector of *D. immitis* in the studied area, where highly microfilaraemic dogs are expected.[7,23] Although the small num-

ber of *O. caspius* collected does not allow any definitive conclusion to be drawn, the high mortality rate of this species following a blood meal on a highly microfilaraemic dog indicates that *O. caspius* might be an effective vector in non-endemic areas. Indeed, the survival of different vectors was linked to *Dirofilaria* microfilariae concentration in the mosquito species.[24]

The results also indicate that in northeastern Italy, where *C. pipiens* is most prevalent,[25] the risk of exposure to *Dirofilaria* spp.-infected vectors can be very high for dogs and relevant for humans.

Indeed, based on our calculations, during the seasons characterized by higher biting pressure, the contact between an infected mosquito and a host may occur as often as every four nights for *D. immitis* in dogs, whereas the possibility for a human to come into contact with an infected mosquito occurs within two weeks of exposure. The higher risk of *Dirofilaria* spp. transmission was recorded in late July and August, in accordance with previous reports.[23]

In contrast, in a study in which more than 40,000 culicids collected from May to October 2010 in the areas of site B and C were screened for *D. immitis* and *D. repens*,[11] the rates of *C. pipiens* infection did not vary significantly through the season, indicating that over spring a certain number of dogs may act as a source of infection to suitable vectors. This finding indicates that the mosquito abundance is one of the key factors in the epidemiology of dirofilariosis. Interestingly, the rate of *C. pipiens* infection with *D. immitis* estimated in this study in 1999 (0.26 %–0.38 %, site C) was very similar to that calculated more than ten years later (0.21–0.33 %).[11] This finding shows that, in spite of the availability of several chemoprophylactic treatments used for the prevention of canine dirofilarioses in endemic areas, prevalence of microfilaraemic dogs has not decreased significantly in rural areas.

However, since the most conservative approach was used to infer the risk of host exposure to *D. immitis* (i.e., assuming that mosquitoes harboring microfilariae in the abdomen would have suffered the same rate of mortality of *C. pipiens* fed on high microfilaraemic dogs, 76.3 %), is likely that in certain sites, the risk of exposure of dogs and humans is higher than indicated.

In addition, due to the introduction of *Aedes albopictus* (*Stegomya albopicta*) in Italy as well in other European countries,[26] the risk of *Dirofilaria* transmission is higher throughout the day time, as a consequence of the fact that this species is diurnal and it acts as natural vector of both filariae in Italy.[9,27] Consequently, categories of dogs and humans previously considered not at risk for dirofilariosis (e.g., animals kept indoors at night or children playing in private and public gardens) should be included in the population at risk of exposure.

Without any doubts, host attractiveness plays a key role in the determination of the population at risk of exposure. Indeed, mosquito-host preferences have been well documented, both at species and at individual level.[28] Accordingly, in the present investigation, *C. pipiens* fed preferentially on dogs (70 %) than on humans (26 %), and was differently attracted by the three dogs used in the study, independently from their sizes. Besides the individual host attractiveness to mosquitoes, the microfilaraemic status of the dogs may enhance the host preference of *C. pipiens.* Accordingly, *C. pipiens* displayed a higher feeding rate on a microfilaraemic dog, compared to a dog under preventative treatment (i.e., 47 % vs. 6.7–12.9 %).[13] Similarly, the mosquito host attractiveness is also enhanced in human patients infected by *Plasmodium* spp.[29,30] indicating that alterations of the physiological status (disease conditions) and consequently of physiological parameters may ultimately change the animal cue attractants (fever, sweat and breathing rhythm, and odour) to competent vectors. Although these factors might have an impact on the spread of the infection, they remain purely speculative for dirofilarioses.

CONCLUSIONS

The quantification of the most important entomological parameters (i.e., mosquito-host preferences, biting rate, mortality induced by the pathogen, rate of infection), which affect the risk of transmission of a vector-borne disease to a susceptible host population, is pivotal for the estimation of the dog/human exposure to infected bites.

Data herein presented on the *Dirofilaria* spp. transmission from arthropod to vertebrate host, and vice versa, are of importance for setting prediction models of dirofilariosis in animals and human beings in a given geographical area. The similarity in the mosquito infection rates recorded over a period of ten years suggests that, in rural areas, dogs maintain the natural cycle of *Dirofilaria* spp., which enhances the risk of transmission to humans. Therefore, strategies to minimize the contact between animal/humans and vectors are strongly recommended.

COMPETING INTERESTS

Authors declare that they have not competing interests.

AUTHORS' CONTRIBUTIONS

AFR and MP conceived the study and performed the field collections, AFR, GCap and DO wrote the manuscript, GCan performed the biomolecular analyses, RC, GS and SC participated to the field and laboratory analyses, GCap performed statistical analyses, all the authors read and approved the final version of the manuscript.

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Distribution and habitat characterization of the recently introduced invasive mosquito *Aedes koreicus* (*Hulecoeteomyia koreica*), a new potential vector and pest in northeastern Italy

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BACKGROUND

Italy is characterized by a climate and an environment that offer many favorable habitats to different species of mosquitoes. Many invasive mosquitoes belonging to *Aedes* genus may have the opportunity to establish after their accidental introduction.[1] The Asian tiger mosquito, *Aedes albopictus* (Skuse) (*Stegomyia albopicta*), represents the best example of spreading and colonization of an invasive species in Italy and Europe.[2] *Aedes* species are proven or potential vectors of important arboviruses,[3,4] and their establishment in new areas pose a threat for human and animal health. A new species of exotic mosquito has been added in 2011 to culicid fauna of Italy: *Aedes koreicus*[5] (*Hulecoeteomyia koreica*).[6] This finding, after a report of the same species in Belgium in 2008,[7] represents the first colonization of this mosquito outside its native area (Asia and East Russia). *Ae. koreicus* is reported to transmit Japanese encephalitis and *Dirofilaria immitis*, but its potential vector competence for other arboviroses and its biology/ecology are largely unknown. The aims of this study were to define the current distribution of this mosquito and to characterize the habitat suitable for its development in northeastern Italy.

METHODS

Starting from the village of the first finding, the monitoring included the whole Belluno province and the neighboring provinces of Vicenza, Treviso, and Trento. Possible larval breeding sites were monitored from May to October 2011 and from March to November 2012. Larvae and adults were collected using a standard larval dipping and BG-Sentinel™ traps, respectively. Adults and larvae obtained from the hatching of eggs collected by standard ovitraps were also examined. The end of *Ae. koreicus* activity was defined when there were no more adults, larvae, or positive ovitraps.

Larvae and adults were identified following taxonomic keys.[5,8–11] For specimens found in a new territory, a molecular confirmation was carried out using the primers and protocol suggested by Cameron *et al.*.[12] The chi-square test was used to compare the *Ae. koreicus* prevalence according to breeding sites.

RESULTS

Ae. koreicus was detected in 37 municipalities out of 94 monitored (39.7%) in an range of altitude from 173 to 1,250 m.a.s.l. The mosquito colonized 40.2% of places and 37.3% of larval habitats checked. Garden centers were the places more positive (66.7%) and catch basins were the larval habitats preferred by *Ae. koreicus* (48.5%), followed by artificial water containers (41.8%) (Figure 1). The ovitraps proved attractive for adult females seeking for oviposition (15/21; 71.4%) (Table 1).

Table 1. Larval breeding sites monitored in 2011–2012 in northeastern Italy for the presence of *Aedes koreicus* and significant differences*

Breeding sites	Positive/Monitored
Vases	16/86 (18.6%)^{ABC}
Water containers	28/67 (41.8%)^{Ad}
Catch basins	16/33 (48.5%)^{B}
Puddles	3/4 (75%)
Tires	4/9 (44.4%)
Basin of fountains	5/7 (71.4%)
Ovitraps	15/21 (71.4%)^{Cd}
Dunghill	0/1 (0.0%)
Tree holes	0/1 (0.0%)
Total	**84/225 (37.3%)**

* Significant differences are marked with the same letter (uppercase = $p < 0.01$; lowercase = $p < 0.05$).

Figure 1. Various typical artificial larval breeding sites of *Aedes koreicus* and *Aedes albopictus*.

Out of 96 breeding sites positive for larvae, *Ae. koreicus* represented the only species in 68 cases (70.8%), whereas the rest of the sites was associated with *Culex pipiens* (17, 15%), *Ae. albopictus* (13, 11.5%), and *Culex hortensis* (6, 5.3%). *Ae. koreicus* was prevalent in places between 400–600 m.a.s.l. (71.4%), well represented at 800–1,000 m.a.s.l. (28.1%) and was found in two sites above 1,000 m.a.s.l. *Ae. albopictus* was prevalent in places under 200 m.a.s.l. (61.9%) ($p < 0.01$).

CONCLUSION

The new species *Ae. koreicus* is clearly established in the area and is now overlapping with other vector species and colonizing areas over 800 m a.s.l., not yet or sporadically reached by the tiger mosquito. The larvae of *Ae. koreicus* develop in the same breeding sites of *Ae. albopictus*, mainly in artificial container in urban areas. Although larvae may coexist with other mosquito species, the majority of breeding sites were colonized by *Ae. koreicus* only, suggesting the existence of competition strategies. Considering the extent of the area colonized, the species likely entered Italy three/four years ago, i.e., at least two years before his discovery. The current scenario seems to represent the third phases of colonization characterized by exponential growth and fast dispersion.[13] The data herein collected are essential to assess the risk of colonization of other parts of Italy and Europe and the risk of spreading of pathogens potentially transmitted. New implication for monitoring and control of mosquitoes must be considered.

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2 NEWS ON FELINE VECTOR-BORNE DISEASES (FVBDs) FROM AROUND THE WORLD

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>>> Vilhena *et al., Parasites & Vectors* 2013; 6:99 – http://www.parasitesandvectors.com/content/6/1/99

Feline vector-borne agents in the North and Center of Portugal

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ABSTRACT

BACKGROUND: In recent years, several clinical cases and epidemiological studies of feline vector-borne diseases (FVBD) have been reported worldwide. Nonetheless, information on FVBD agents and their prevalence in Portugal is scarce.

METHODS: Three hundred and twenty domestic cats presented to 30 veterinary medical centers in the North and Center regions of Portugal were randomly sampled. Blood was assayed by real-time polymerase chain reaction (PCR) for genera *Anaplasma/Ehrlichia*, genus *Babesia*, *Hepatozoon canis*, *Hepatozoon felis*, *Leishmania infantum*, and genus *Rickettsia*. *Babesia*-positive samples were further tested for *Babesia canis* and *Babesia vogeli*.

RESULTS: Eighty (25.0%) out of the 320 cats were positive to at least one vector-borne agent, including seven (2.2%) cats co-infected with two agents. Two cats (0.6%) were infected with *Anaplasma/Ehrlichia* spp., four (1.3%) with *B. canis*, 26 (8.1%) with *B. vogeli*, 50 (15.6%) with *H. felis*, one (0.3%) with *L. infantum* and four (1.3%) with *Rickettsia* spp. No cat tested positive to *H. canis*. One cat (0.3%) was co-infected with *B. canis* and *B. vogeli*, three (0.9%) with *B. vogeli* and *H. felis*, one (0.3%) with *H. felis* and *L. infantum*, and two (0.6%) with *H. felis* and *Rickettsia* spp.

CONCLUSIONS: A considerable prevalence of infection with vector-borne agents among the domestic feline population of the North and Center of Portugal has been revealed by the present study. Additionally, this is the first detection of *B. vogeli* in cats from Europe and of *H. felis* in cats from Portugal.

KEYWORDS: Cats, Feline vector-borne diseases, PCR, Portugal

8th CVBD WORLD FORUM Symposium

BACKGROUND

Vector-borne diseases compromise a variety of infectious illnesses caused by several agents, including viruses, bacteria, protozoa, and helminthes, which are transmitted by ticks, fleas, mosquitoes and phlebotomine sandflies.[1,2] Many of these agents are emerging or re-emerging pathogens[3] and some of them are of zoonotic concern.[4,5] The frequency of some vector-borne diseases is increasing in Europe, partially due to climatic alterations that have a direct impact on the abundance, geographical distribution, and vectorial capacity of arthropod vectors, but also due to the increased mobility of human beings and animals, which further promote the circulation and exchange of vectors and infectious agents.[1,3,6,7]

Although several vector-borne agents cause morbidity and mortality in the domestic feline population,[8] the importance of some of them as a cause of disease has not yet been clearly determined.[9] This lack of knowledge, associated with the unawareness of the distribution and ecology of feline vector-borne diseases (FVBD) of zoonotic concern, has impaired the implementation of effective control measures to prevent infection of cats, other animals, and human beings.[1]

The diagnosis of infectious diseases in cats may be challenging, as some infections can occur in healthy cats,[10] and whenever present, clinical signs are frequently non-specific.[1,11] Most of the agents are often present in low numbers in peripheral blood, are difficult to cultivate *in vitro*, elicited specific antibody responses may be inconsistent,[11] and serological cross-reactivity can exist between some organisms.[12] Many problems of serology are circumvented by the use of the polymerase chain reaction (PCR).[12,13] In addition to accurate detection of infectious agents in animals, human beings and arthropod vectors, DNA-based techniques allow species characterization of different pathogens, which can be important for treatment and prognosis.[6] Compared with the conventional method, real-time PCR can have a higher sensitivity in some diseases[14] and is a useful tool both for diagnosis and treatment monitoring.[13]

Previous clinical case reports[15] and serological or molecular surveys[16–19] have described infection with different vector-borne organisms in cats from Portugal. Nonetheless, information about agents of FVBD and their prevalence in Portugal is scarce. The aims of the present study were to identify the presence and prevalence of vector-borne agents from genera *Anaplasma*, *Babesia*, *Ehrlichia*, *Hepatozoon*, *Leishmania*, and *Rickettsia* in cats from the North and Center regions of Portugal, by means of real-time PCR, and to identify risk factors associated with infection.

METHODS

Cats and samples

320 domestic cats from the North (n = 140) and Center (n = 180) of Portugal were randomly sampled in 30 veterinary medical centers, without inclusion/exclusion criteria or pre-established minima/maxima. The number of cats sampled per center ranged from 1 to 58. This study was ethically approved by the board of the University of Trás-os-Montes e Alto Douro veterinary teaching hospital as complying with the Portuguese legislation for the protection of animals (Law no. 92/1995, from September the 12th).

Whole blood samples were obtained by jugular or cephalic venipuncture into EDTA tubes and stored at −20 °C until DNA extraction. Whenever available, medical and geographic data of each cat were collected, including gender, age, breed, living conditions, clinical status, and feline immunodeficiency virus (FIV)/feline leukaemia virus (FeLV) infection status (**Table 1**). Practitioners classified the cats as clinically suspect, if they had clinical signs compatible with a FVBD, or non-suspect, when they were apparently healthy or had clinical signs not compatible with an infectious disease.

Table 1. Prevalence of infection with vector-borne pathogens in cats from the North and Center of Portugal, as determined by PCR

Independent variable/ category	No. of cats tested (%)	No. of positive cats (%)						
		Anaplasma/ Ehrlichia	*B. canis*	*B. vogeli*	*H. felis*	*L. infantum*	*Rickettsia*	≥1 agent(s)
Gender	**320**							
Female	142 (44.4)	2 (1.4)	2 (1.4)	14 (9.9)	19 (13.4)	1 (0.7)	0 (0.0)	36 (25.4)
Male	178 (55.6)	0 (0.0)	2 (1.1)	12 (6.7)	31 (17.4)	0 (0.0)	4 (2.2)	44 (24.7)
Age (years)	**312**							
0.4–1.5	90 (28.8)	0 (0.0)	2 (2.2)	11 (12.2)[a]	13 (14.4)	0 (0.0)	0 (0.0)	23 (25.6)
2.0–6	157 (50.3)	1 (0.6)	2 (1.3)	13 (8.3)	25 (15.9)	1 (0.6)	3 (1.9)	42 (26.8)
7–20	65 (20.8)	1 (0.6)	0 (0.0)	1 (1.5)[a]	11 (16.9)	0 (0.0)	1 (0.6)	14 (21.5)
Breed	**320**							
DSH	274 (85.6)	2 (0.7)	4 (1.5)	19 (6.9)	43 (15.7)	1 (0.4)	3 (1.1)	66 (24.1)
Pure breed	46 (14.4)	0 (0.0)	0 (0.0)	7 (15.2)	7 (15.2)	0 (0.0)	1 (2.2)	14 (30.4)
Housing	**316**							
Totally indoors	124 (39.2)	0 (0.0)	0 (0.0)	7 (5.6)	20 (16.1)	0 (0.0)	2 (1.6)	26 (21.0)
Outdoors access	192 (60.8)	2 (1.0)	4 (2.1)	19 (9.9)	30 (15.6)	1 (0.5)	2 (1.0)	54 (28.1)
FeLV	**117**							
Negative	107 (91.5)	0 (0.0)	1 (0.9)	9 (8.4)	15 (14.0)	0 (0.0)	2 (1.9)	24 (22.4)
Positive	10 (8.5)	0 (0.0)	0 (0.0)	1 (10.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (10.0)
FIV	**117**							
Negative	97 (82.9)	0 (0.0)	1 (1.0)	10 (10.3)	13 (13.4)	0 (0.0)	2 (2.1)	23 (23.7)
Positive	20 (17.1)	0 (0.0)	0 (0.0)	0 (0.0)	2 (10.0)	0 (0.0)	0 (0.0)	2 (10.0)
Clinical status	**300**							
Non-suspect	132 (44.0)	2 (1.5)	2 (1.5)	16 (12.1)	17 (12.9)	1 (0.8)	1 (0.8)	34 (25.8)
Suspect	168 (56.0)	0 (0.0)	2 (1.2)	10 (6.0)	33 (19.6)	0 (0.0)	3 (1.8)	46 (27.4)
Total	**320 (100)**	**2 (0.6)**	**4 (1.3)**	**26 (8.1)**	**50 (15.6)**	**1 (0.3)**	**4 (1.3)**	**4 (1.3)**

[a]p = 0.031; *B. canis*: *Babesia canis*; *B. vogeli*: *Babesia vogeli*; DSH: domestic short-haired; FeLV: feline leukaemia virus; FIV: feline immunodeficiency virus; *H. felis*: *Hepatozoon felis*; *L. infantum*: *Leishmania infantum*; PCR: polymerase chain reaction

Compatible physical signs and clinicopathological abnormalities comprised anorexia, weight loss, gastrointestinal alterations, anemia, thrombocytopenia, leukocytosis or leukopenia, jaundice, and dermatological or ocular manifestations without any other attributable aetiology.

DNA extraction, PCR amplification, and sequencing

DNA was obtained from 0.5 ml of peripheral blood, as previously described.[14]

Real-time quantitative (q) PCR for *Leishmania infantum* was carried out according to the method described by Francino *et al.*[14] The targets of primers (Table 2) and TaqMan-MGB probes were conserved regions of the kinetoplastic minicircle of *L. infantum*.

Table 2. Primer sequences of the tested vector-borne pathogens (genera or species)

Agents	Amplified region	Primer forward (5'–3')	Primer reverse (5'–3')	Final [primer] (µM)
Anaplasma/Ehrlichia spp.	16S rRNA	GGGTGAGTAATGCRTAGGAATCTACCTAGTA	GGATTATACAGTATTACCCAYCATTTCTARTG	0.5
Babesia spp.	18S rRNA	GTGGCTTTTCCGATTCGTCG	TTCCTTTAAGTGATAAGGTTCACAAAACTT	0.3
Babesia canis	18S rRNA	CGGTTTGACCATTTGGTTGGTTA	CCATGCTGAAGTATTCAAGACAAAAGT	0.3
Babesia vogeli	18S rRNA	CATTCGTTTGGCTTTTTCGAG	CCATGCTGAAGTATTCAAGACAAAAGT	0.3
Hepatozoon felis	18S rRNA	CTTACCGTGGCAGTGACGGT	TGTTATTTCTTGTCACTACCTCTCTTATGC	0.3
Leishmania infantum	kinetoplast DNA	AACTTTTCTGGTCCTCCGGGTAG	ACCCCCAGTTTCCCGCC	0.9
Rickettsia spp.	16S rRNA	AGCCTGATCCAGCAATACCGA	CGGGGCTTTTTCTGCAAGTAA	0.3

For the other agents, samples were submitted to different qPCR for genera *Anaplasma/Ehrlichia*, genus *Babesia*, *Hepatozoon canis*, *Hepatozoon felis*, and genus *Rickettsia*. *Babesia*-positive samples were further tested for *Babesia canis* and *Babesia vogeli*. All primers were designed by The Molecular Genetics Veterinary Service, Universitat Autònoma de Barcelona (Table 2). Specificity of each primer was: (i) tested in silico, to avoid cross-amplification with other FVBD agents, using sequence information available in GenBank and RDP II databases; and (ii) also validated by the amplification of the positive control for which the PCR had been designed and by the absence of amplification in samples positive to other pathogens; and (iii) by DNA sequencing of some positive samples to confirm agents *B. canis* (n = 1), *B. vogeli* (n = 3), and *H. felis* (n = 4).

For all agents, qPCR amplification was carried out in a final volume of 20 µl using FastStart Universal SYBR Green Master (Roche), 4 µl of diluted DNA, and a final primer concentration depending on the amplified pathogen (Table 2). Thermal cycling profile was 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Specificity assessment of qPCR was performed by adding a dissociation curve analysis at the end of the run. The internal reference for cat genomic DNA was the eukaryotic 18S RNA Pre-Developed TaqMan Assay Reagents (Applied Byosystems), which ensured proper qPCR amplification of each sample and that negative results corresponded to true negative samples rather than to a problem with DNA loading, sample degradation or PCR inhibition. Positive qPCR controls were obtained from clinical samples previously amplified and sequenced to confirm the pathogen. Water was used as a negative control. The product of the real-time PCR was sequenced with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (AB, Live Technologies) using the same primers. Sequences obtained were compared with GenBank database (www.ncbi.nlm.nih.gov/BLAST).

Statistical analysis

Prevalences of infection relative to the independent variables (i.e., gender, age, breed, housing conditions, FeLV/FIV infections, and clinical status) were compared by chi-square or Fisher's exact tests. A p value < 0.05 was considered as statistically significant. Analyses were performed with SPSSW 11.5 software for Windows (SPSS Inc).

RESULTS

Table 1 displays data on the prevalence of infection with vector-borne agents among the 320 cats assessed in this study. Cats (n = 312) were aged from 5 months to 20 years (mean: 4.32 years ± 3.88 standard deviation). Pure breed cats were mainly Persians and Siamese. Absolute numbers and proportions (positive/tested cats) of single and co-infections are shown in Table 3.

Table 3. Prevalence of single and co-infections withvector-borne pathogens in 320 cats from North and Center Portugal, as determined by PCR

Agent(s)	No. of positive cats	%
Single infections	73	22.8
Anaplasma spp./*Ehrlichia* spp.	2	0.6
B. canis	3	0.9
B. vogeli	22	6.9
H. felis	44	13.8
L. infantum	0	0.0
Rickettsia spp.	2	0.6
Co-infections	7	2.2
B. canis + *B. vogeli*	1	0.3
B. vogeli + *H. felis*	3	0.9
H. felis + *L. infantum*	1	0.3
H. felis + *Rickettsia* spp.	2	0.6
Total	**80**	**25.0**

B. canis: *Babesia canis*; *B. vogeli*: *Babesia vogeli*; *H. felis*: *Hepatozoon felis*; *L. infantum*: *Leishmania infantum*; PCR: polymerase chain reaction

80 cats (25.0 %) were qPCR-positive to at least one of the tested agents, including seven (2.2 %) cats co-infected with two agents. Two cats (0.6 %) were infected with *Anaplasma*/*Ehrlichia* spp., four (1.3 %) with *B. canis*, 26 (8.1 %) with *B. vogeli*, 50 (15.6 %) with *H. felis*, one (0.3 %) with *L. infantum*, and four (1.3 %) with *Rickettsia* spp. No cat tested positive to *H. canis*. One cat (0.3 %) was co-infected with *B. canis* and *B. vogeli*, three (0.9 %) with *B. vogeli* and *H. felis*, one (0.3 %) with *H. felis* and *L. infantum*, and two (0.6 %) with *H. felis* and *Rickettsia* spp.

Sequencing confirmed *B. canis* in the one cat (100 % relatedness to GenBank HQ662634.1), *B. vogeli* in the three cats (100 % relatedness to GenBank JX871885.1), and *H. felis* in the four cats (100 % relatedness to GenBank JQ867388.1) whose PCR products were sequenced. Because of the small quantities of amplified bacterial DNA in the genus-specific PCR, additional species-specific PCR assays or DNA sequencing were not performed for those samples positive to genera *Anaplasma*/*Ehrlichia* and *Rickettsia*.

A statistically significant difference (p = 0.031) was found between the prevalence of infection with *B. vogeli* in cats aged 5 – 18 months (0.42 – 1.5 years) and in cats aged 7 – 20 years old (12.2 % versus 1.5 %, respectively) (Table 1). No other significant differences were found for the prevalence of infection with each vector-borne agent, at least one agent (≥ 1 agent[s]) or co-infections (2 agents), regarding all the independent variables.

DISCUSSION

The present study represents the most comprehensive investigation on FVBD performed in Portugal, in terms of the number of tested cats and extension of the covered geographical area, and reveals a considerable prevalence of infection in domestic cats from the North and Center regions of Portugal. *Anaplasma*/*Ehrlichia* spp., *B. canis*, *B. vogeli*, *H. felis*, *L. infantum*, and *Rickettsia* spp. were detected among the assayed feline population.

Several ehrlichial and rickettsial infections are shared by man and companion animals.[20] In the present study, *Anaplasma*/*Ehrlichia* spp. and *Rickettsia* spp. DNA were detected in 0.6 % and 1.3 %

of the cats, respectively. In cats from southern Portugal, seroprevalences (by immunofluorescence antibody tests) were 13.5 % for *Anaplasma phagocytophilum* and 18.9 % for *Rickettsia conorii*,[17] and 26.3 % for *R. conorii*/*Rickettsia felis*.[19] Furthermore, Breu *et al.*[19] also reported feline infection by *Ehrlichia canis* in Portugal. A national serological study on canine vector-borne diseases in Portugal detected a significantly higher seroprevalence of antibodies to *Anaplasma* spp. and *E. canis* in dogs from southern Portugal, when compared to dogs from the northern and central regions.[7]

The present study represents the first report on the prevalence of *Babesia* spp. in cats from Portugal. A higher prevalence of *Babesia* spp. was found in Portuguese cats (9.4 %), in comparison with that detected in cats from Barcelona, Spain, (0/100) by Tabar *et al.*[12] Interestingly, it can also be presumed that the most prevalent piroplasm in the Portuguese feline population is *B. vogeli*, instead of *B. canis*, which was the piroplasm most frequently detected in dogs with babesiosis from the North of Portugal.[21] So far, feline infection with *B. vogeli* has only been described in cats from Trinidad, Trinidad and Tobago,[22] and Bangkok, Thailand.[23] To the best of our knowledge, this is the first time that infection with *B. vogeli* has been detected in cats from Europe.

Feline co-infections with other erythrocytic pathogens such as *Mycoplasma* spp., *Cytauxzoon felis*, or other species of *Babesia* may be possible.[24,25] In the present study, only one cat was found co-infected with *B. canis* and *B. vogeli* out of the 29 cats infected with *Babesia* spp. Infection with *B. canis* and/or the *Babesia microti*-like piroplasm (syn. *Theileria annae*) was previously described in three cats from Portugal,[26] but no information is available on the geographical origin of those cats. The *B. microti*-like piroplasm has also recently been found in dogs from northern Portugal affected by babesiosis.[27] In the present study, other species of the genera *Babesia* and *Theileria* were not assessed. Nevertheless, as the entire results positive to the genus *Babesia* had an assigned species (i.e., *B. vogeli*, *B. canis*, or both), although not impossible, a co-infection with the *B. microti*-like piroplasm seems unlikely.

Age has been described as a predisposing factor for feline infection with *Babesia* spp., with younger cats (less than 3 years old) more predisposed to infection in endemic areas,[24,28] and older cats more susceptible to the disease following relocation to an endemic area or in conjunction with concurrent disease, immunosuppression, or severe trauma.[24] In the present study, juvenile cats (≤ 1.5 year) had a significantly higher prevalence of *B. vogeli* infection in comparison with geriatric cats (≥ 7 years), probably because of the less mature immune status of young cats.

Infection with *Hepatozoon* spp. is frequently reported in dogs[29] but not in cats. Furthermore, the *Hepatozoon* species that infect cats have not been definitely characterized.[30,31] Some authors have suggested that *H. canis* is the agent responsible for feline infection, but a new, yet unnamed species of *Hepatozoon* genetically distinct from *H. canis* was recently detected in cats from southern and northeastern Spain.[30,32] Infection with *H. felis* was firstly described by Tabar *et al.* in cats from Barcelona.[12]

This is also the first report of feline infection with *H. felis* in Portugal. Molecularly confirmed infection with *H. canis* in cats from southern Portugal[19] and in a dog from northern Portugal[21] had already been described. The detected 15.6 % prevalence of infection with *H. felis* in the present study is similar to that of *Hepatozoon* spp. (16 %) described by Ortuño *et al.*[30] in stray cats from Barcelona ($p = 0.960$), but higher than the 0.6 % of *Hepatozoon* spp. found in Spanish domestic cats from a non-identified geographical background[32] and the 4.0 % of *H. felis* in domestic cats from Barcelona[12] ($p < 0.001$ and $p = 0.002$, respectively). Moreover, the present study sustains the fact that *Hepatozoon* infection is widespread in the feline population of the Iberian Peninsula. Baneth

et al.[33] in a study from Israel, detected that most infected cats were young domestic short-haired males and that there was an overrepresentation of cats with retroviral disease. In the present study, no statistically significant association was found between infection with *H. felis* and independent variables including clinical status and FIV/FeLV infection (**Table 1**).

Leishmaniasis is an endemic zoonosis prevalent in the Mediterranean Basin.[34,35] The increase in the number of infections and disease cases reported in recent years, together with the results described in different prevalence studies, suggest that cats can act as a secondary reservoir host instead of an accidental one in areas where *Leishmania* spp. are endemic.[36–38] Several surveys of *Leishmania* spp. infection in cats have been performed in different countries by different techniques, with prevalences ranging between 0 % and 68 %.[9,16,18,39–43] In the present study, detected prevalence (0.3 %) might have been different if the qPCR was carried out with another tissue sample, such as bone marrow, spleen, or liver. Results are lower than the 2.8 % seroprevalence (by the direct agglutination test and an enzyme-linked immunosorbent assay) found in cats from northern Portugal[18] and also lower than the 20.3 % obtained in blood samples from cats of Greater Lisbon (southern-central Portugal) by PCR[16] ($p = 0.01$ and $p < 0.001$, respectively). For the appropriate agents under assessment in the present study, comparative differences in the prevalence values can be related to different detection techniques (serology versus molecular analysis) as well as to a different geographical origin of cats (North and Center versus South). The latter may determine differences in climatic conditions, arthropod vector survival, and agent transmission rate.

Associations between housing conditions and prevalence of infection were not found among cats for any one of the agents. Conversely, in a comparable study on canine vector-borne diseases, also in Portugal, a significantly higher sero-positivity to at least one agent (i.e., *Dirofilaria immitis*, *E. canis*, *Borrelia burgdorferi* sensu lato, *Anaplasma* spp. and/or *L. infantum*) has been found in clinically suspect dogs with an outdoor or mixed lifestyle.[7]

CONCLUSIONS

In the North and Center regions of Portugal, a high prevalence of infection with *B. vogeli* and *H. felis*, and a relatively low prevalence of infection with *Anaplasma*/*Ehrlichia* spp., *B. canis*, *L. infantum*, and *Rickettsia* spp. were found in cats. Further studies on these and other vector-borne agents are needed to better understand their epidemiological and clinical importance. It is also necessary to call on veterinarians and owners to adopt effective control measures, including chemoprophylaxis against the ectoparasite vectors, in order to prevent infection of cats with agents of FVBD and their potential transmission to other domestic and wild animals as well as to human beings.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

HV collected samples and clinical data, performed data analysis and drafted the manuscript; VLM-D performed DNA extraction and molecular analyses; LC co-supervised the study, performed data analysis, and revised the manuscript; LV collected samples and clinical data; LA and OF designed the primers, supervised molecular analyses, and provided conceptual advice; JP and ACS-F planned and supervised the study, coordinated sample collection, and reviewed the manuscript. All authors read and approved the final manuscript.

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Babesia lengau associated with cerebral and haemolytic babesiosis in two domestic cats

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ABSTRACT

BACKGROUND: Although reported sporadically from various countries, feline babesiosis appears to be a significant clinical entity only in South Africa, where *Babesia felis* is usually incriminated as the causative agent. *Babesia lengau*, recently described from asymptomatic cheetahs, has now possibly been incriminated as the causative agent in two severe clinical cases in domestic cats.

FINDINGS: Both cats were euthanized in extremis. While typical feline babesiosis in South Africa is an afebrile disease with a chronic manifestation, there was acute onset of severe clinical signs in both cats and their body temperatures were above the normal range when they were presented for treatment. Haemolytic anaemia was confirmed in one case. To our knowledge, this is the first report of cerebral babesiosis in cats. On reverse line blot 18S rDNA PCR products obtained from both cats showed positive hybridization profiles with the *B. lengau* species-specific probe. The two partial parasite 18S rRNA gene sequences obtained, showed high sequence similarity (99.9 %) to *B. lengau*. In a representative tree constructed by the neighbor-joining method using the two-parameter model of Kimura, the two obtained partial 18S rDNA sequences and that of *B. lengau* formed a monophyletic group with *Babesia conradae* and sequences previously isolated from humans and wildlife in the western USA.

CONCLUSION: All clinical cases of feline babesiosis in South Africa are not necessarily caused by *Babesia felis*. Other piroplasms, e.g., *B. lengau*, may be incriminated in clinical cases, especially those occurring outside the known endemic area.

KEYWORDS: *Babesia lengau*, Cerebral babesiosis, Feline babesiosis, Haemolytic anaemia

FINDINGS

Babesiosis of domestic cats has been reported sporadically from various countries, including France,[1] Germany,[2] India,[3] Israel,[4] Poland,[5] Portugal,[6] Thailand,[7] and Zimbabwe,[8] but appears to be a significant clinical entity only in South Africa where it occurs primarily along the eastern and southern seaboard (KwaZulu-Natal, Eastern and Western Cape Provinces), as well as in isolated foci along the eastern escarpment of Mpumalanga and Limpopo Provinces[9–11] (**Figure 1**). This would suggest that the vector(s) only occur(s) in these areas, but the vector(s) remain(s) unknown. Clinical cases occurring inland, e.g., in Gauteng Province, usually concern pets taken along when the owners visited the

coast.[9,12] Feline babesiosis has been well documented in South Africa. Apart from case reports,[13] there have been detailed studies on signalment, clinical manifestation, and pathology[9,14,15] as well as treatment.[12,16] Lethargy, anorexia, and anaemia generally occur, while icterus is only occasionally seen.[14] Elevated body temperature is not a feature of this disease.[14] In clinical cases, parasitaemia is usually high and may exceed 50 %.[14]

Figure 1. Map of South Africa, showing the localities of the two cases. The feline babesiosis endemic area extends along the eastern and southern coast (KwaZulu-Natal, Eastern and Western Cape Provinces), as well as along the eastern escarpment in Mpumalanga and Limpopo Provinces in the northeastern part of the country. Case 1 (Rustenburg): Open circle. Case 2 (Wellington): Black circle.

Babesia felis, the piroplasm usually incriminated as causing feline babesiosis in South Africa, was initially described from a Sudanese wild cat (*Felis ocreata*, syn. *F. sylvestris*).[17] Fourteen domestic cats inoculated with blood from the wild cat became persistently infected, with parasitaemias < 1 %, but did not develop any clinical signs.[17] When feline babesiosis was first described in South Africa a few years later, the parasite was considered to be morphologically similar to *B. felis*, but due to its pathogenicity in domestic cats it was called *Nuttallia felis* var. *domestica*.[13] It remains unresolved whether the pathogenic parasite in South Africa is indeed conspecific with the one described from Sudan.[17]

Although the famous Kenyan lioness "Elsa" was reported to have died of babesiosis,[18] *Babesia felis* sensu stricto has not been incriminated in causing disease in felids other than domestic cats. It has been reported from 18 out of 97 clinically normal captive cheetahs (*Acinonyx jubatus*) in South Africa and 3 out of 40 free-ranging cheetahs in Namibia, as well as from a single lion (*Panthera leo*) and serval (*Leptailurus serval*) in South Africa.[19] *Babesia leo*, commonly found in lions in South Africa, has also been reported from a clinically healthy domestic cat.[19,20]

Recently, *Babesia lengau* was described from clinically healthy cheetahs.[21] In this retrospective study, we report two severe clinical cases of feline babesiosis in domestic cats associated with *B. lengau* infection. As far as we could ascertain, this is the first report of cerebral babesiosis in a domestic cat. In both instances, which occurred before the formal description of *B. lengau*, we did not have access to the clinical cases but interpreted necropsy reports. Both cats had been euthanized in extremis. One case was submitted for necropsy and nucleic acid-based diagnostics to the Faculty of Veterinary Science, Onderstepoort, South Africa. Blood specimens from the second case were submitted to confirm babesiosis. The two cases occurred in different parts of South Africa (North West Province and Western Cape Province, the latter within the known endemic area of feline babesiosis) and there was no connection between them. The results showed the presence of a *Babesia* parasite very close or similar to *B. lengau*, recently described from asymptomatic cheetahs.

Case 1 (Rustenburg, North West Province; June 2004)
An entire, 2-year-old Siamese tomcat, which had been eating and playing in the morning, was presented at a veterinary practice in a collapsed state that evening. He had a temperature of 40.3 °C with pale-icteric mucous membranes. Blood smears revealed intraerythrocytic protozoan parasites morphologically resembling *Babesia* species, as well as erythrocytes phagocytosed by leukocytes. The cat was euthanized and the frozen carcass was submitted for necropsy to the Pathology Section, Faculty of Veterinary Science, University of Pretoria.

Macroscopic findings were as follows: The overall body condition of the animal was very good. External examination revealed moderate flea infestation and marked icteric mucous membranes. No ticks were found on the body of the animal. On evisceration, pronounced icterus was observed, associated with watery blood (indicative of severe anaemia) on blood vessel incision. Anaemia was initially masked by severe icterus. Mild hydrothorax and hydropericardium as well as acute, centrilobular hepatosis were observed. Moderate diffuse splenomegaly due to red pulp hyperplasia and mild, diffuse pulmonary congestion and oedema occurred. On cytological examination, a splenic impression smear showed numerous free-lying groups / aggregates of spherically shaped piroplasm-like protozoan organisms among leukocytes and lytic erythrocytes. Occasional intact erythrocytes contained the same organism resembling a *Babesia* or *Theileria* species. Freeze artefacts may have influenced parasite morphology.

The histopathological conclusion was that this animal had suffered from severe prehepatic icterus and anaemia, which appeared to have resulted from a combination of intra- and extravascular haemolysis. Freeze artefacts had a negative impact on examination and interpretation.

Case 2 (Wellington, Western Cape Province; July 2007)
An 18-month-old domestic short-haired cat was presented to a private veterinarian with a complaint of weakness. It was in good condition, had a temperature of 40.2 °C and showed anisocoria. There was mild lameness of the left hind limb, which appeared to be more of extensor rigidity. There was severe anaemia and icterus, and the cat was covered in large numbers of ticks. A blood smear revealed large numbers of white blood cells, as a result of lymphocytosis, but no blood parasites were observed by the attending veterinarian. The smear also showed evidence of a non-regenerative anaemia. Treatment consisted of intravenous fluids (Ringer's lactate) and injections of 0.3 ml dexamethasone, 0.4 ml amoxycillin and clavulanic acid (Synulox), and 0.4 ml Vitamin B Complex. The cat did show some appetite but was force-fed. During the evening nervous signs such as paddling, vocalisation, and clonic spasms of the neck developed and the cat was euthanized by intravenous administration of an overdose of barbiturates.

Figure 2. The brain of the cat (Case 2): The entire brain shows pronounced congestion with clear red-pink discoloration of the grey matter, as well as multifocal petechiae (Photograph: Tertius Gous).

A necropsy was performed by a private diagnostic pathologist. The carcass was in fairly good condition. There were marked anaemia and icterus. The entire brain showed very pronounced congestion with clear red-pink discoloration of the grey matter, as well as multifocal petechiae (Figure 2). There was severe congestion and oedema of the lungs. The pericardium contained a moderate amount of serosanguineous fluid. Moderate congestion was noted in the spleen, mesenteric lymph nodes, and pancreas.

Examination of blood smears by the pathologist revealed a mild parasitaemia of piroplasm trophozoites in erythrocytes, but brain smears showed massive numbers of piroplasms in and outside of erythrocytes (Figure 3). The brain capillaries were packed with heavily parasitized erythrocytes that resulted in sludging. The lesions and parasites in the brain were confirmed histopathologically.

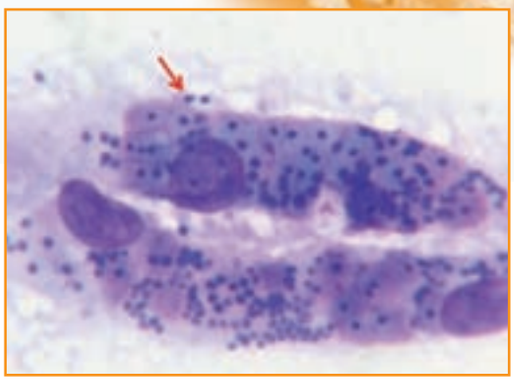

Figure 3. **Brain smear (Diff Quick stain) (Case 2): Cerebral capillary sludging of red blood cells that are heavily parasitized by a large *Babesia* (x 1,000) The dark, purple-bluish spots seen on the photograph represent the parasite in the erythrocyte (Photograph: Tertius Gous).**

Confirmation of the presence of parasite DNA using partial 18S rRNA gene sequencing and phylogenetic analysis

DNA was extracted from 0.2 g pooled spleen and lymph node from Case 1 (BF226) and from 0.2 g brain tissue from Case 2 (BF463), using the commercially available QIAampW DNA Mini Kit (Qiagen, Whitehead Scientific, South Africa), according to the manufacturer's instructions. The V4 hypervariable region of the parasite 18S rRNA gene was amplified and screened using the reverse line blot (RLB) hybridization assay.[22,23] PCR products were subsequently sequenced (Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa) using primers RLB-F and RLB-R.[23]

The obtained sequence data were assembled and edited using GAP 4 of the Staden package (Version 1.6.0 for Windows).[24] A search for homologous sequences was performed using BLASTn (www.ncbi.nlm.nih.gov) and sequences of closely related *Babesia* species were aligned with the obtained parasite sequences using ClustalX (Version 1.81 for Windows). The alignment was manually truncated to the size of the smallest sequence (405 bp). A phylogenetic tree was constructed using the neighbor-joining method[25] in combination with the boot-strap method (1,000 replicates/tree).[26] The consensus tree was edited using MEGA v4.0.2.[27]

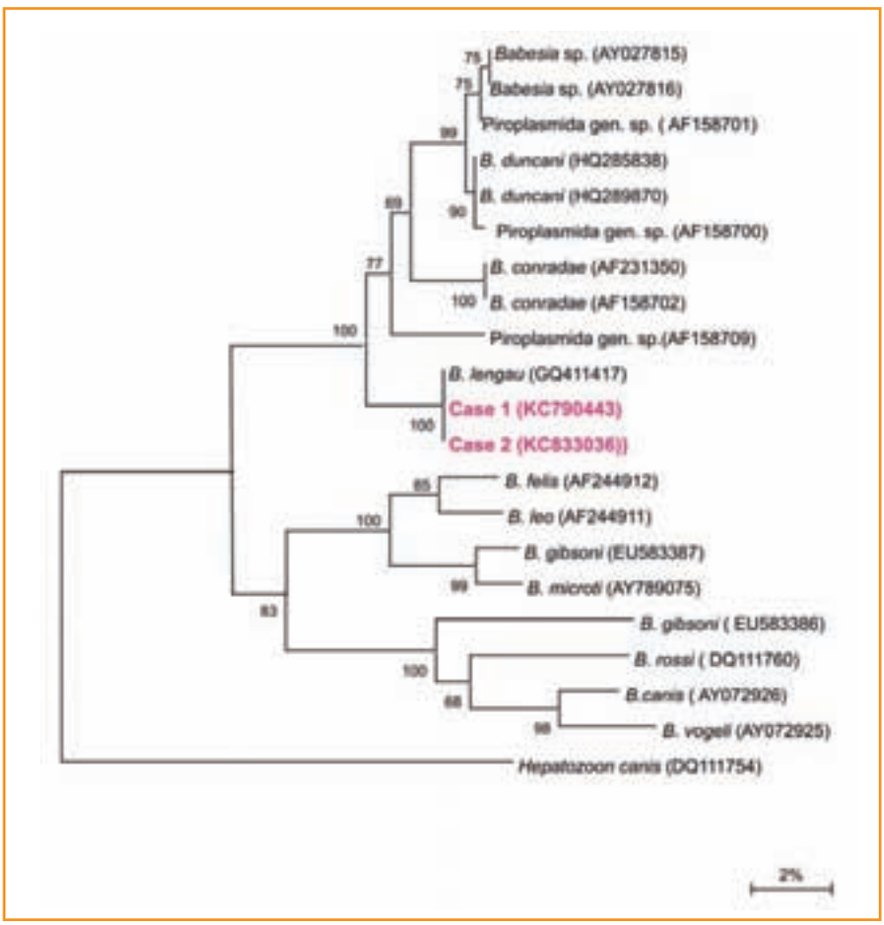

Figure 4. **Results of the neighbor-joining analysis of the 18S rRNA gene of the two piroplasms: The phylogenetic relationship of the piroplasms involved in Cases 1 and 2 with other *Babesia* and *Theileria* species is shown** (The scale bar represents the % nucleotide differences).

Reverse line blot results from Case 1 (BF226) and Case 2 (BF463) showed positive hybridization profiles with the *Babesia lengau* species-specific probe.[21] The sequence data revealed that the two 18S rDNA sequences (KC790443 and KC833036) obtained (405 bp) were identical. A BLASTn search showed a high similarity (99.9 %) with *B. lengau* (accession nrs. GQ411405 to GQ411417);[21] it differed by one deletion

from *B. lengau*. A representative tree constructed by the neighbor-joining method using the two-parameter model of Kimura[28] is shown in Figure 4. Case 1 (BF226), Case 2 (BF463), and *B. lengau* (GQ411417) formed a monophyletic group with *B. conradae* (AF158702, AF231350), *Babesia duncani* (HQ285838, HQ289870), and other sequences previously identified from humans and wildlife in the western USA (AY027815, AY027816, AF158700, AF158701, AF158709) (Figure 4).

DISCUSSION

Both cases reported here differed in some respects from the usual clinical signs associated with feline babesiosis in South Africa.[14] Feline babesiosis is usually afebrile, while the body temperature of both cases was above the normal range when they were presented for treatment. Haemolytic anaemia, seen in Case 1, is not usually associated with typical feline babesiosis.[14] A *B. lengau*-like organism has recently been incriminated in causing a haemolytic disease in sheep in Greece.[29]

Until recently, all small piroplasms of domestic cats that morphologically resembled *B. felis* were assumed to belong to that species. It has now been demonstrated that various small *Babesia* spp. can infect domestic cats.[19] Microscopic identification of piroplasms on blood smears is routinely done by practitioners to diagnose babesiosis in cats and other domestic animals. Confirming species identity, however, often requires molecular diagnostic tools. The reverse line blot hybridization assay and phylogenetic analysis are also more sensitive than blood smear examination in demonstrating the presence of *Babesia* spp. in subclinically infected animals.

The macroscopic and histopathological findings of both cases reported here suggested severe illness due to infection by a *Babesia* parasite, as described for dogs.[30] Molecular diagnostic methods confirmed the presence of *Babesia* parasites in blood and brain smears. Phylogenetic analysis revealed a high similarity to *B. lengau*.

Babesia lengau was described in asymptomatic cheetahs[21] and to date no evidence existed to suggest that *B. lengau* causes clinical disease in that host. The only other piroplasm *B. lengau* genotypically relates to closely is a canine parasite, *B. conradae*, which was described from dogs in California, USA, and is associated with haemolytic anaemia in the host.[31,32] *B. lengau* and *B. conradae* form part of the previously described "western clade" of piroplasms, which also includes *B. duncani* and piroplasms isolated from both humans and wildlife from the western USA. It clustered separately from the *Babesia* sensu stricto, the *Babesia microti* clade and *Theileria* and *Cytauxzoon* species.

Cerebral babesiosis has frequently been reported in cattle (e.g., caused by *Babesia bovis*)[33] and dogs (e.g., caused by *Babesia rossi*).[34,35] To our knowledge, this serves as a first report of cerebral babesiosis in a domestic cat.

CONCLUSION

All clinical cases of feline babesiosis in South Africa are not necessarily caused by *B. felis*. Other *Babesia* species, e.g., *B. lengau*, may be incriminated in clinical cases, especially those occurring outside the known endemic area. As a routine, the identity of the *Babesia* species involved should ideally be confirmed by molecular techniques when specimens from suspected feline babesiosis cases are submitted to diagnostic laboratories. Examining brain smears or sections is also recommended. Since the vector of *B. felis* has not yet been confirmed, our findings emphasize the urgency of further investigations to enhance understanding the epidemiology of this enigmatic disease.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

AMB carried out the molecular genetic studies, participated in the sequence alignment and wrote the first draft of the manuscript; MCO supervised the laboratory work and participated in the sequence alignment and construction of the phylogenetic trees; EHV co-supervised the laboratory work; JCAS and TAG performed the necropsies and histological diagnostic investigation on Cases 1 and 2, respectively; BLP coordinated the investigation, conducted literature searches and wrote the final version of the report. All authors read and approved the final manuscript.

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Seroprevalence and risk factors for *Rickettsia felis* exposure in dogs from Southeast Queensland and the Northern Territory, Australia

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BACKGROUND

The recent detection of *Rickettsia felis* DNA in dogs in Australia suggests that dogs are potential mammalian reservoir hosts for this emerging rickettsia. To date, there is no published report addressing the seroprevalence of *R. felis* in dogs in Australia.

METHODS

Antigens for *R. felis* were produced by inoculating confluent XTC-2 monolayer cell cultures with three pools of cat flea (*Ctenocephalides felis*) homogenates. Infection was confirmed by real-time (qPCR), conventional, or nested PCRs targeting the ompB, gltA, 17kDa, and ompA genes. 292 dogs from Southeast Queensland and the Northern Territory were tested for the presence of *R. felis* antibodies using a microimmunofluorescence (IF) test and the seroprevalence and associated risk factors for exposure determined using both uni- and multivariate analyses.

RESULTS

R. felis was successfully isolated in cell culture from all three cat-flea pools. 148 dogs (50.7 %) showed seropositivity with titres ≥ 64 and 54 (18.5 %) with titres ≥ 128. At antibody titre ≥ 128, adult dogs were more likely to be seropositive to *R. felis*. At antibody titres ≥ 64, intact female dogs were significantly associated with seropositivity. Dogs with active ectoparasite control were less likely to be exposed to *R. felis*.

CONCLUSIONS

This first reported isolation of *R. felis* in cell culture in Australia allowed for the production of antigen for serological testing of dogs. Results of this serological testing reflect the ubiquitous exposure of dogs to *R. felis* and advocate for owner vigilance with regards to ectoparasite control on domestic pets.

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>>> Baneth *et al.*, *Parasites & Vectors* 2013, **6**:102 – http://www.parasitesandvectors.com/content/6/1/102

Redescription of *Hepatozoon felis* (Apicomplexa: Hepatozoidae) based on phylogenetic analysis, tissue and blood form morphology, and possible transplacental transmission

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ABSTRACT

BACKGROUND: A *Hepatozoon* parasite was initially reported from a cat in India in 1908 and named *Leucocytozoon felis domestici*. Although domestic feline hepatozoonosis has since been recorded from Europe, Africa, Asia, and America, its description, classification, and pathogenesis have remained vague and the distinction between different species of *Hepatozoon* infecting domestic and wild carnivores has been unclear. The aim of this study was to carry out a survey on domestic feline hepatozoonosis and characterize it morphologically and genetically.

METHODS: *Hepatozoon* sp. DNA was amplified by PCR from the blood of 55 of 152 (36%) surveyed cats in Israel and from all blood samples of an additional 19 cats detected as parasitemic by microscopy during routine hematologic examinations. *Hepatozoon* sp. forms were also characterized from tissues of naturally infected cats. Results: DNA sequencing determined that all cats were infected with *Hepatozoon felis* except for two infected by *Hepatozoon canis*. A significant association ($p = 0.00001$) was found between outdoor access and *H. felis* infection. *H. felis* meronts containing merozoites were characterized morphologically from skeletal muscles, myocardium and lungs of *H. felis* PCR-positive cat tissues and development from early to mature meront was described. Distinctly shaped gamonts were observed and measured from the blood of these *H. felis*-infected cats. Two fetuses from *H. felis* PCR-positive queens were positive by PCR from fetal tissue including the lung and amniotic fluid, suggesting possible transplacental transmission. Genetic analysis indicated that *H. felis* DNA sequences from Israeli cats clustered together with the *H. felis* Spain 1 and Spain 2 sequences. These cat *H. felis* sequences clustered separately from the feline *H. canis* sequences, which grouped with Israeli and foreign dog *H. canis* sequences. *H. felis* clustered distinctly from *Hepatozoon* spp. of other mammals. Feline hepatozoonosis caused by *H. felis* is mostly subclinical as a high proportion of the population is infected with no apparent overt clinical manifestations.

CONCLUSIONS: This study aimed to integrate new histopathologic, hematologic, clinical, epidemiological, and genetic findings on feline hepatozoonosis and promote the understanding of this infection. The results indicate that feline infection is primarily caused by a morphologically and genetically distinct species, *H. felis*, which has predilection to infecting muscular tissues, and is highly prevalent in the cat population studied. The lack of previous comprehensively integrated data merits the redescription of this parasite elucidating its parasitological characteristics.

KEYWORDS: *Hepatozoon felis*, *Hepatozoon canis*, Domestic cat, Transplacental transmission

BACKGROUND

Hepatozoon species are apicomplexan parasites with a hematophagous arthropod final host and a vertebrate intermediate host. They are transmitted by ingestion of the final host containing mature oocysts by the intermediate host.[1] The gamont stage of the parasite is found in leukocytes or erythrocytes of the intermediate host and infects the final host during the blood meal. Additional transmission pathways have been described in some *Hepatozoon* spp. including intrauterine transmission and carnivorism of the intermediate host by an intermediate host of a different species.[2–5] More than 340 species of *Hepatozoon* have been described to date in amphibians, reptiles, birds, marsupials, and mammals.[1,6] A *Hepatozoon* parasite was reported for the first time from the blood of a domestic cat in India by Patton in 1908 and named *Leucocytozoon felis domestici*.[7] The feline parasite was later transferred to the genus *Hepatozoon* and it was suggested that *Hepatozoon* parasites from the cat, jackal, and hyena are indistinguishable from *Hepatozoon canis*, which infects dogs, due to the similarity in morphology of the gamont stage seen in the blood of these animals.[8] The classification of the *Hepatozoon* parasites found in domestic cats has thereafter been uncertain and most studies have carefully referred to *Hepatozoon*-like parasites or *Hepatozoon* sp. without committing to a certain species.[9–15] With the advent of molecular techniques, PCR using genus-specific primers for *Hepatozoon* spp. was used to amplify 18S rRNA gene DNA from the blood of a collection of wild and domestic animals including 2 cats from Spain. Although no parasites were morphologically described in the cat's blood, the sequences from these cats were designated as *H. felis* and deposited in GenBank.[14,16]

Domestic cat hepatozoonosis has been reported from several countries worldwide including: India, South Africa, Nigeria, the USA, Brazil, Israel, Spain, and France.[7,9,11,12,17–20] Most studies have focused on reporting the detection of feline hepatozoonosis and almost no information has been published on its pathogenesis, transmission, life cycle, and epidemiology. In that context, the aims of this study were to carry out a survey on domestic feline hepatozoonosis, characterize its causative agents genetically and morphologically in blood and tissues, and evaluate its possible transplacental transmission.

METHODS

Collection of positive samples detected during routine laboratory evaluation

Anticoagulated blood in EDTA tubes from 19 domestic cats, in which *Hepatozoon* sp. gamonts were detected by May Grunwald Giemsa-stained blood smear microscopy at the Hebrew University Veterinary Teaching Hospital (HUVTH) and at the private Pathovet Veterinary Pathology Laboratory in Israel during routine blood tests, were collected from 2002 to 2011 and stored at – 80 °C.

Cat survey

Blood samples were collected in EDTA tubes during 2010 and 2011 from multiple locations and sources in Israel. These included convenience sampling of cats whose *Hepatozoon* infection status was unknown from animal shelters in 5 cities in Central Israel (Tel-Aviv, Jerusalem, Rehovot, Beit

Dagan, Rishon Le-Zion), cats brought for routine spay to HUVTH, cats admitted to private veterinary clinics in 5 cities and villages in Israel (Haifa, Nahariya, Carmiel, Kfar Vradim, Yodfat) and to the HUVTH in Central Israel whose samples were taken for routine diagnostic purposes. Serum samples were also collected from the same cats. Data collected on the cats included: sex, age, source of cats (e.g., shelter or private ownership), geographic location, indoors or outdoors access, and feline immunodeficiency (FIV) status as tested during this study.

Cats tissues for evaluation of *Hepatozoon* sp. infection

Formalin-fixed paraffin-embedded tissues of 3 cats in which structures of *Hepatozoon* sp. meronts were detected by histopathology were included in the study. One cat was a patient at the HUVTH and detected antemortally as being parasitemic with *Hepatozoon* sp. It died with hepatitis and pancreatitis and was necropsied at the Kimron Veterinary Institute (KVI) pathology department. Fresh tissues from multiple organs were also collected from this cat at necropsy and stored at −80 °C. A second cat was diagnosed with feline panleukopenia at the HUVTH and necropsied at the KVI following its death. Tissues including myocardial and skeletal muscle from a third cat diagnosed with *Hepatozoon* sp. infection in France[11] were submitted to the Hebrew University by its attending veterinarian.

Fetuses from shelter queens brought to the HUVTH for spaying and found to be pregnant during the neutering procedure were frozen at −80 °C and if the queen blood was found to be positive for *Hepatozoon* sp. by PCR, fetal tissues were dissected using separate sterile scalpels for each tissue and submitted for *Hepatozoon* PCR in order to detect possible intrauterine transmission.

DNA extraction

DNA from the blood of cats was extracted using the illustra blood genomicPrep Mini Spin KitW (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. Samples from paraffin-embedded tissues were deparaffinized and DNA was extracted using the QIAamp DNA FFPE tissue kitW (QIAgen, Valencia, CA, USA) according to the manufacturer's instructions. DNA from frozen tissues was extracted using the guanidine thiocyanate technique as previously described.[21]

PCR for the detection of *Hepatozoon* spp. and *Toxoplasma gondii*

A partial fragment of the 18S rRNA gene of *Hepatozoon* spp. was amplified by PCR using primers piroplasmid-F CCAGCAGCCGCGGTAATT and piroplasmid-R CTTT CGCAGTAGTTYGTCTTTAACAAATCT.[20] The following conditions were used: 94 °C 3 min, 35 cycles of [94 °C 30 s, 64 °C 45 s, 72 °C 30 s] 72 °C 7 min. PCR was performed using the Syntezza PCR-Ready High Specificity kit (Syntezza Bioscience, Israel). Positive *H. canis* control samples (5 µl DNA) from naturally infected dogs positive by blood smear and by PCR and sequencing of the PCR product, and negative controls were run with each PCR. This PCR protocol was used for all of the samples included in the study. Other sets of primers tried for amplification of a partial fragment of the 18S rRNA gene such as the HEP-F and HEP-R primers[22] were less successful in amplifying samples from domestic cats with hepatozoonosis.

A second PCR assay was performed in some of the samples positive by the piroplasmid PCR to amplify a larger segment (approximately 1,400 bp) of the 18S rRNA gene of *Hepatozoon* spp. for further phylogenetic analysis. This assay used primers HAM-1 F GCCAGTAGTCATATGCTTGTC and HPF-2R GACTTCTCCTTCGTCTAAG.[16] The amplification conditions for this reaction were: 95 °C, 5 min; (34x [95 °C 20 s, 56 °C, 30 s, 72 °, 90 s]; 72 °C, 5 min). Positive *H. canis* control samples (5 µl DNA) from naturally infected dogs positive by blood smear and by PCR and sequencing of the PCR product, and negative controls were run with each PCR reaction. In order to rule out possible misdiagnosis of *Hepatozoon* tissue forms with *Toxoplasma gondii* cysts, a PCR for *T. gondii* was carried

out on DNA extracted from tissues of the 3 cats in which *Hepatozoon* sp. meronts were detected by histopathology. PCR was performed to amplify a 529 bp fragment *T. gondii* repeat sequence using primers TOX4 CGCTGCAGGGAGGAAGACGAAAGTTG and TOX5 CGCTGCAGACACAGTGCATCTGGATT[23] as previously described.[24]

Sequencing and phylogenetic analysis

All PCR amplicons amplified from positive cats included in each part of the study were sequenced. The DNA products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Perkin-Elmer/Applied Biosystems) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequences were evaluated with the ChromasPro software version 1.33 and compared to sequence data available from GenBank using the BLAST 2.2.9 program (http://www.ncbi.nlm.nih.gov/BLAST/). The *Hepatozoon* species identity found was determined according to the closest BLAST match with an identity of 97–100 % to an existing GenBank accession. Only samples that produced amplicons with a sequence compatible with a *Hepatozoon* sp. were considered positive for *Hepatozoon* and included accordingly in the study analysis.

A phylogenetic analysis, which included DNA sequences from the blood of 18 cats from the study, was carried out to compare these sequences to *Hepatozoon* spp. described in other animal hosts and in domestic cats and had previously been deposited in GenBank. Sequences were analyzed using the MEGA version 3.0 (www.megasoftware.net), and a phylogenetic tree was constructed by the maximum-likelihood, minimum-evolution, and the neighbor-joining algorithms using the Kimura2-parameter model. Bootstrap replicates were performed to estimate the node reliability, and values were obtained from 1,000 randomly selected samples of the aligned sequence data.

Parasite morphology and sizes

The sizes of parasites found in blood smear and histologic specimens were measured by a manual micrometer and light microscope.

FIV serology

Detection of antibodies for FIV in cat sera was performed using the Feline Immunodeficiency Virus Immuno Run Antibody Detection assayW (Biogal Galed Labs, Israel).

Statistical analysis

Data were analyzed using the chi-square, Fisher's exact and the Mann-Whitney tests. A p value < 0.05 was considered statistically significant. The study was approved by the HUVTH research projects study evaluation committee.

RESULTS

Evaluation of positive blood smear *Hepatozoon* spp. sample collection

All 19 cat samples identified by blood smear microscopy during 2002–2011 and collected at the HUVTH were positive by PCR for *Hepatozoon* sp. using the piroplasmid primers followed by DNA sequencing.

Cat survey

Hepatozoon sp. DNA was amplified from the blood of 55 out of the 152 (36.2 %) surveyed cats whose *Hepatozoon* infection status was unknown using the Piroplsamid PCR protocol.

The sex of the cat was recorded for 146 of the 152 survey cats. 75 were males (51.4 %) of which 30 (40 %) were PCR+ for *Hepatozoon* and 71 were female (48.6 %) of which 24 (33.8 %) were PCR+.

Sex was not found to be significantly associated (p = 0.438) with positivity for *Hepatozoon* infection in the surveyed cats using the chi-square test. The age was known for 126 surveyed cats for which the mean age and standard deviation (SD) were 4.6 ± 4.2 years with a range of 4 months to 18 years. No significant association (p = 0.453) was found between age and *Hepatozoon* positivity using the Mann-Whitney test.

Information on access to the outdoors was available for 132 cats. 92 (68.7 %) of the cats were outdoors or spent a part of the day outdoors of which 45 (48.9 %) were PCR+ for *Hepatozoon*, whereas 42 (31.3 %) were strictly indoor cats of which only 4 (9.5 %) were PCR+. A significant association (p = 0.00001) was found between outdoor access and *Hepatozoon* positivity using the chi-square test.

Of the 101 cats belonging to private owners, 39 (38.6 %) were PCR+ for *Hepatozoon* and of the 51 non-privately owned cats, 16 (31.4 %) were PCR+. No significant association (p = 0.475) was found between cat ownership status and *Hepatozoon* positivity using the chi-square test. Likewise, when dividing the cats to those who originated from northern Israel (from Haifa northward) and to those who were from Central Israel, 29 of 67 (43.3 %) cats from northern Israel were PCR+ for *Hepatozoon* and 26 of 85 (30.6 %) cats from Central Israel were PCR+ with no significant association (p = 0.854) found between the cat's geographic origin and *Hepatozoon* positivity using the chi-square test.

Sera were only available from 64 cats for FIV testing. 54 of these cats (84.4 %) were negative for FIV of which 22 (40.7 %) were PCR+ for *Hepatozoon*, and 10 (15.6 %) cats were positive for FIV of which 6 (60 %) were *Hepatozoon* PCR+. No significant statistical association (p = 0.312) was found between FIV and *Hepatozoon* infections using the Fisher's exact test.

PCR on cat tissues

Hepatozoon sp. DNA was amplified from paraffin-embedded tissues of 3 cats using PCR with the piroplsamid primers. The first cat was detected antemortally at the HUVTH as being parasitemic with *Hepatozoon* sp. and at necropsy followed by tissue histopathology, it was described to be cachectic with chronic hepatitis and chronic pancreatitis. *Hepatozoon* sp. meronts were detected by histopathology in the skeletal muscles (semi-membranosus muscle of the hind limb) and in the myocardium of this cat. PCR carried out on DNA of paraffin-embedded tissues from both the semi-membranosus and myocardial muscles was positive and sequenced. In addition, *Hepatozoon* PCR of fresh tissues collected at necropsy was positive for the lingual muscle (tongue), diaphragm muscle, longissimus dorsi muscle, semi-membranosus muscle, myocardium, liver, pancreas, spleen, kidney, lung, mesenteric lymph node, and bone marrow. *Hepatozoon* PCR was also positive from blood taken antemortally.

The second cat diagnosed with feline panleukopenia had severe necrotizing enteritis and pneumonia by histopathology. *Hepatozoon* sp. meronts were detected in its myocardium and lungs by histopathology and these two tissues were also positive for *Hepatozoon* by PCR and sequencing.

Tissues from the third cat were sent from France, where it was reported to have feline leukemia virus (FeLV) infection, *Hepatozoon* sp. parasitemia, and forms compatible with *Hepatozoon* meronts in its muscular tissues by histopathology.[11] Both the myocardium and skeletal muscles were positive for *Hepatozoon* by PCR and sequencing in the current study.

Tissues from all the 3 cats positive for *Hepatozoon* by PCR were PCR-negative for *T. gondii*.

Detection of *Hepatozoon* DNA in fetal samples

Fetuses from 3 shelter queens brought to the HUVTH for spaying and found to be pregnant during neutering were included in the survey and tested by PCR for *Hepatozoon* DNA using the piroplasmid primers. Uteri from 2 cats contained 3 and 4 relatively developed fetuses, respectively, from which lungs, liver, spleen, skeletal muscle from a back limb, cardiac muscle, naval tissue, and amniotic fluid were collected from each embryo. The third uterus contained 4 small less developed fetuses from which abdominal cavity organ material and amniotic fluid were sampled. None of the fetuses from the uterus with 3 large embryos were positive for *Hepatozoon* by PCR. However, one developed fetus from the uterus with 4 fetuses was positive in the lung and amniotic fluid, and one of 4 less developed fetuses from the third uterus was positive in the amniotic fluid. In total, tissues from 2 of 11 fetuses were positive for *Hepatozoon* sp. by PCR.

Genetic identity and phylogenetic analysis GenBank accessions

A 358 bp fragment of the *Hepatozoon* 18S rRNA gene was amplified by PCR using the piroplasmid primers from the blood and tissues of all cats considered infected in this study. DNA sequencing revealed that all positive cats, except for two, had a sequence whose closest match by BLAST, with an identity of 97–100%, was a *H. felis* GenBank accession. Most sequences were 98–100% identical to *H. felis* [GenBank: AY628681] (*H. felis* isolate Spain 2[16]). The DNA sequences similar to *H. felis* included those obtained from the 55 positive survey cats, sequences from all cats from which paraffin-embedded or fresh frozen tissues were tested, and sequences from 17 of the 19 cats included in the collection of samples identified by blood smear microscopy during 2002–2011. The *Hepatozoon* sequence obtained from the blood of the cat in which infection was detected antemortally and also in necropsy tissue was 99% identical to *H. felis* [GenBank: AY628681]. The sequence amplified from the paraffin-embedded myocardial tissue of the French cat with hepatozoonosis was also 99% identical to *H. felis* [GenBank: AY628681] as was the sequence amplified from the paraffin-embedded myocardial tissue of the local cat with panleukopenia, and a sequence amplified from the amniotic fluid of a fetus from a blood-positive queen. Only 2 cats had DNA sequences that matched *H. canis* and not *H. felis*. Samples 9617 and 9618 from the collection of positive blood

Table 1. Description of new *Hepatozoon* spp. sequences from the study deposited in GenBank

Source and sample number	*Hepatozoon* species	Primers used for amplification of 18S rRNA gene fragment	GenBank accession number
Cat 9617	*H. canis*	HAM-1 F and HPF-2R*	KC138531
Cat 9618	*H. canis*	HAM-1 F and HPF-2R*	KC138532
Cat 8533	*H. felis*	HAM-1 F and HPF-2R*	KC138533
Cat 1	*H. felis*	HAM-1 F and HPF-2R*	KC138534
Dog 7243	*H. canis*	HAM-1 F and HPF-2R*	KC138535
Cat 9685	*H. felis*	piroplasmid-F and piroplasmid-R**	KC138536
Dog 6417	*H. canis*	piroplasmid-F and piroplasmid-R**	KC138537
Dog 8672	*H. canis*	piroplasmid-F and piroplasmid-R**	KC138538
Cat 9617	*H. canis*	piroplasmid-F and piroplasmid-R**	KC138539
Cat 9618	*H. canis*	piroplasmid-F and piroplasmid-R**	KC138540
Cat 1778	*H. felis*	piroplasmid-F and piroplasmid-R**	KC138541
Cat 8987	*H. felis*	piroplasmid-F and piroplasmid-R**	KC138542

* Ref. 16; ** Ref. 20

smear spp. were 99 % identical to *H. canis* [GenBank: EU289222] and other *H. canis* accessions. Although the piroplasmid primers can also amplify DNA from piroplasms such as *Babesia* spp., none of the amplified sequences were positive for *Babesia*, *Theileria*, or *Cytauxzoon* spp.

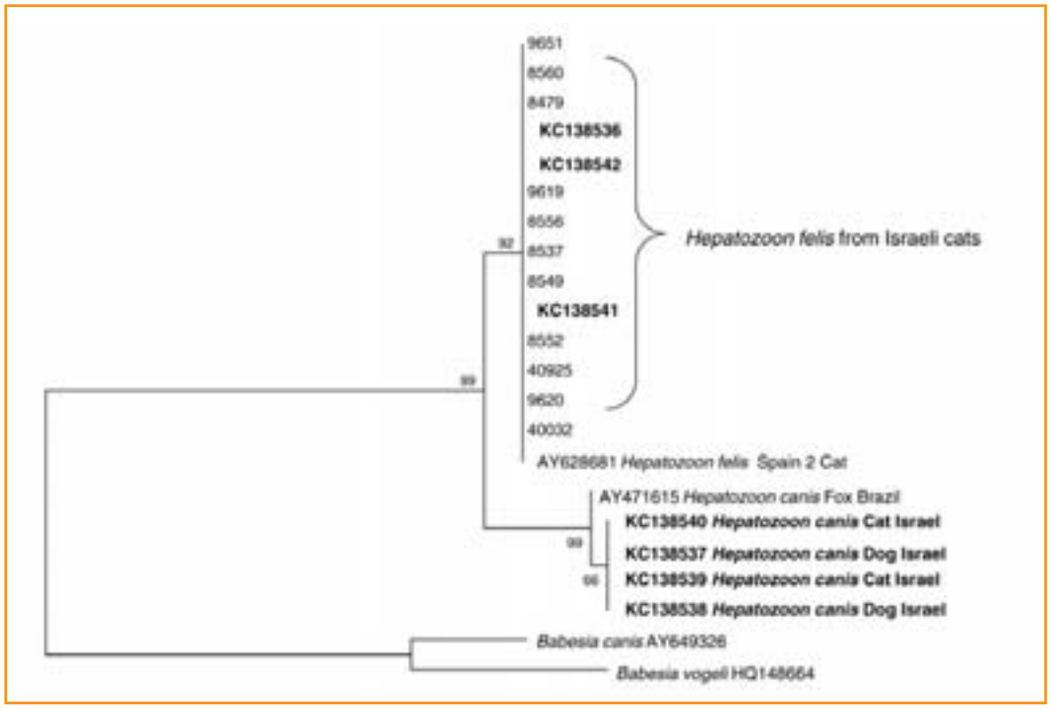

Figure 1. Neighbor-joining 18S rRNA tree. A neighbor-joining tree phylogram comparing 345 bp 18S rRNA DNA *Hepatozoon* sequences from Israeli cats to other *Hepatozoon* GenBank deposited sequences with *Babesia vogeli* and *Babesia canis* as outgroups. The GenBank accession numbers, species of infected animals, and country of origin from which the sequences were derived are included for each sequence. New GenBank accessions derived from the present study are indicated in bold letters.

Further investigation of the genetic identity of *Hepatozoon* parasites found in cats from this study was carried out using the HAM-1 F and HPF-2 R primers which amplified a 1,400 bp fragment of the *H. felis* 18S rRNA gene. Amplification of this large fragment of the 18S rRNA gene was carried out for selected samples included in the phylogenetic analyses. In addition to the DNA sequences from cats, fragments of *H. canis* from local Israeli dogs were also amplified using the two sets of primers and sequenced in order to compare *H. felis* and *H. canis* from cats with a local *H. canis* from dogs. Details on *Hepatozoon* sequences from this study submitted to GenBank are included in Table 1.

A neighbor-joining phylogenetic tree based on 345 bp from the shorter 18S rRNA fragment amplified by the piroplasmid primers (Figure 1) indicated that sequences from 14 Israeli cats detected as infected with *H. felis* clustered with *H. felis* [GenBank: AY628681] (*H. felis* isolate Spain 2), whereas the *H. canis* sequences found in 2 cats from the study clustered together with *H. canis* from dogs. Similar clustering was obtained also with the maximum-likelihood and the minimum-evolution algorithms. A Maximum-Likelihood tree based on 970 bp from the longer 18S rRNA fragment amplified by primers HAM-1 F and HPF-2 R (Figure 2) also indicated that *H. felis* sequences from Israe-

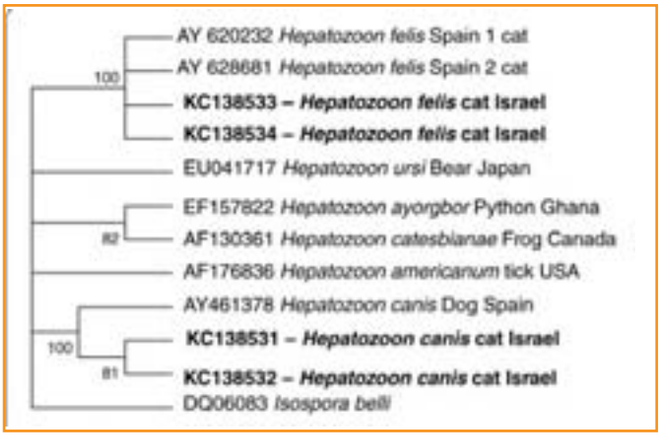

Figure 2. Long 18S rRNA segment minimum-evolution tree. A minimum-evolution tree phylogram comparing 970 bp 18S rRNA DNA *Hepatozoon* sequences from Israeli cats to other *Hepatozoon* GenBank deposited sequences with *Isospora belli* as outgroup. The GenBank accession numbers, species of infected animals and country of origin from which the sequences were derived are included for each sequence. New GenBank accessions derived from the present study are indicated in bold letters.

Table 2. Sizes of *Hepatozoon felis* life stages

Stage	Source of sample	Mean size (µm) with SD	Shape index (length/width ratio)	Number measured
Gamont	Cat blood	10.5 ± 0.6 x 4.7 ± 0.8	2.2	13
Gamont nucleus	Cat blood	4 ± 0.3 x 3.2 ± 0.5	1.2	12
Meront	Skeletal muscle; myocardium; lung	39 ± 5 x 34.5 ± 3.8	1.1	13
Meront capsule width	Skeletal muscle; myocardium; lung	1.4 ± 0.5	Not applicable	13
Merozoites	Skeletal muscle; myocardium; lung	7.5 ± 0.6 x 1.9 ± 0.3	3.9	14
Merozoite nucleus	Skeletal muscle; myocardium; lung	2.4 ± 0.5 x 1.6 ± 0.3	1.5	14

li cats clustered together with the *H. felis* Spain 1 and Spain 2 sequences. Likewise, *H. canis* from 2 Israeli cats clustered together with *H. canis* [GenBank: AY461378] from a dog in Spain. These were separated from *Hepatozoon americanum* and *Hepatozoon* spp. reported from other animal hosts.

Parasite morphology and sizes

The life stages visible in the blood and tissues of cats positive for *H. felis* by PCR were visualized by microscopy and measured (Table 2). Gamonts observed in stained blood smears were located in the cytoplasm of neutrophils and monocytes sometimes compressing the lobulated host cell nucleus. Gamonts were elongated, enveloped by a visible membrane and possessed a round acentric nucleus (Figure 3). Some gamonts contained basophilic staining granules. In comparison to *H. canis* gamonts from dog blood, gamonts of *H. felis* were different due to their generally round nucleus, which is dissimilar to the more elongated horse-shoe-shaped *H. canis* nucleus. They were also relatively shorter with a mean length of 10.5 µm compared with the *H. canis* gamont which is 11 µm long[25] and less conspicuous within the feline leukocyte than *H. canis* within its canine counterpart. The *H. felis* gamont was often hardly apparent within the host cell cytoplasm and almost concealed by its nucleus.

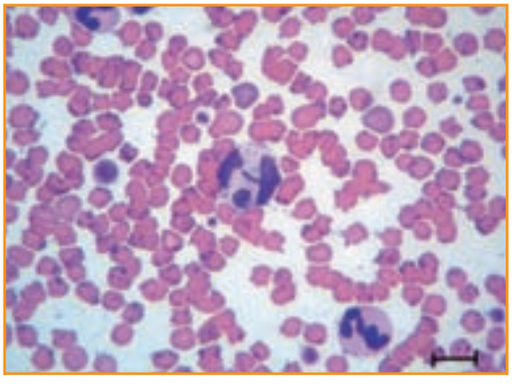

Figure 3. *Hepatozoon felis* gamont. A *H. felis* gamont in a monocyte from the blood smear of a cat co-infected with *Mycoplasma hemofelis*. May Grunwald-Giemsa stain, bar = 10 µm.

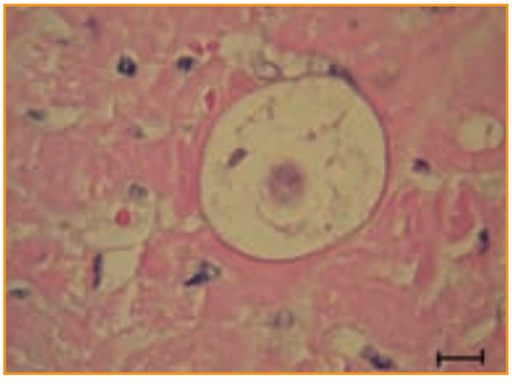

Figure 4. Early *Hepatozoon felis* meront. An early *H. felis* meront in the myocardial muscle of a domestic cat. Hematoxylin and Eosin stain, bar = 10 µm.

The *H. felis* meront is round to oval with a mean length of 39 µm by 34.5 µm and surrounded by a thick membrane separating it from the surrounding tissue (Table 2). The early *H. felis* meronts contain amorphous material without obvious zoites (Figure 4), and as they mature, they form nuclei

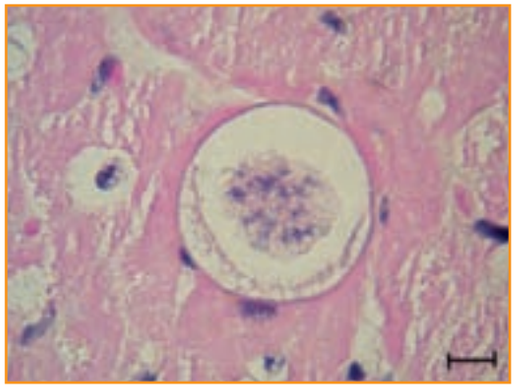

Figure 5. Maturing *Hepatozoon felis* meront. A maturing *H. felis* meront in the myocardial muscle of a domestic cat. Note that merozoite nuclei are visible but distinct merozoites are not formed yet at this stage. Hematoxylin and Eosin stain, bar = 10 µm.

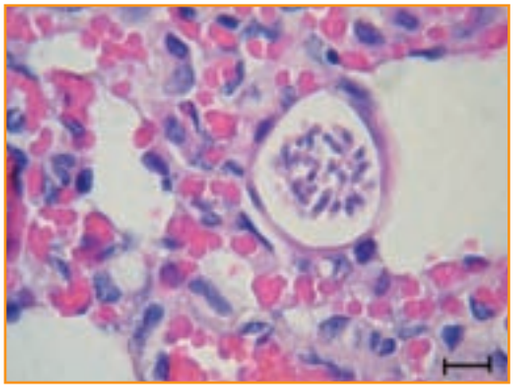

Figure 7. Mature *Hepatozoon felis* meront in lung. A mature *H. felis* meront in the lung of a cat with pneumonia and panleukopenia. Note the individual nucleated separated merzoites and thick external capsule. Hematoxylin and Eosin stain, bar = 10 µm.

(Figure 5), which develop further into distinct intact long merozoites with a rectangular nucleus that assumes the whole width of the merozoite (Figures 6 and 7). The *H. felis* merozoites are dispersed within the meront without an obvious pattern of arrangement. Early *H. felis* meronts appear to have thinner capsules that widen and become thicker as the meront matures. No apparent inflammatory response was found associated with the presence of intact meronts in muscular tissues (Figure 8).

The *H. felis* meront is larger in size than the *H. canis* meront which is 30.6 x 28.9 µm,[26] found in muscular tissues such as the myocardium and skeletal muscles, unlike *H. canis* which infects hemolymphoid and parenchymal tissues, but not muscles. Furthermore, the *H. felis* meront does not form the typical wheel spoke shape of the *H. canis* meront with merozoites arranged in a circle along the meront circumference around a central core.[26]

DISCUSSION

Although hepatozoonosis of domestic cats was initially reported in 1908, the same year when the type species *Hepatozoon muris* was described from a laboratory rat and its life cycle described,[27] it has almost been overlooked since and little has been published on its pathogenesis. This study aimed to integrate new histopathologic, hematologic, clinical, epidemiological, and genetic

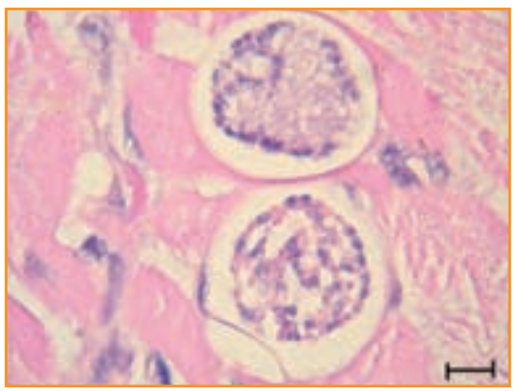

Figure 6. *Hepatozoon felis* meronts. Two maturing *H. felis* meronts in the myocardial muscle of a domestic cat. Individual merozoites with separate nuclei are visible in the lower meront. Hematoxylin and Eosin stain, bar = 10 µm.

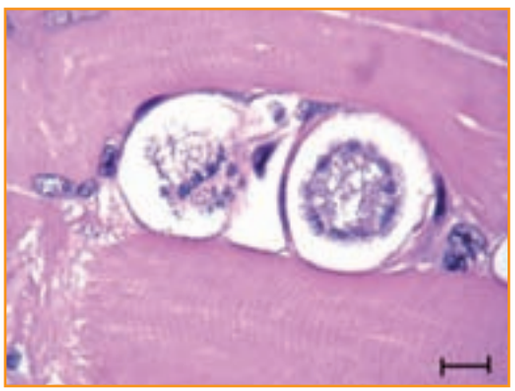

Figure 8. *Hepatozoon felis* meronts in striated muscle. Two maturing *H. felis* meronts in the semi-membranosus skeletal muscle. No host inflammatory cells are visible in the tissue adjacent to the parasites. Hematoxylin and Eosin stain, bar = 10 µm.

findings on feline hepatozoonosis and promote the understanding of this infection. The results clearly indicate that feline infection is primarily caused by a morphologically and genetically distinct species, *H. felis*, which has predilection to infecting muscular tissues, and is highly prevalent in the cat population studied. The lack of previous comprehensively integrated data merits the redescription of this parasite elucidating its parasitological characteristics.

Surveys of *Hepatozoon* sp. infection in domestic cats describe variable rates of infection in different areas. A study of myocardium specimens from 100 cats brought to necropsy in Israel found that 36 % of cats harbored cardiac *Hepatozoon*-like meronts.[9] Interestingly, this was the same rate of infection found in Israel almost 40 years later in the current study, indicating that feline hepatozoonosis is not a new emerging infection in this country. Studies using PCR detection from Spain have shown diverse prevalence rates with 0.6 % in one study,[16] 16 % in a cat colony from Barcelona,[14] and 4 % in cats from the Barcelona area.[20] A comparative study carried out in several districts of Bangkok, Thailand, where both canine and feline hepatozoonosis are prevalent, has reported a high infection rate of 32 % in 300 cats by PCR. A positive association was found between the rates of infected dogs and cats in the same districts and 18S rDNA sequences from cats and dogs were closest to *H. canis*.[28] These findings encouraged the authors to hypothesize that *H. canis* was the cause of both canine and feline infections. However, the genetic analyses in this study were probably made before the sequences designated as *H. felis* were deposited in GenBank.[16] Therefore, the identity of some feline sequences may have been misinterpreted. A study from Brazil evaluated 200 blood samples from Sao Luis in Brazil and found only one cat infected with a *Hepatozoon* sp. which clustered with *H. felis* on a phylogenetic analysis.[15]

The lack of association between age and infection found in the study supports the possibility of transplacental transmission and kittens being born already infected. It may also suggest intensive exposure at a young age. The highly significant association found between infection and access to outdoors suggests the possibility of transmission by arthropod vectors, such as fleas, ticks, or mites, which are common ectoparasites of cats globally, or by carnivorism as described for a number of *Hepatozoon* spp.[2–5] So far, no arthropod vector has been described for *H. felis*. Other *Hepatozoon* spp. have been demonstrated to be transmitted by fleas, ticks, mites, lice, mosquitoes, and sandflies[1] and it is therefore expected that *H. felis* would also be transmitted by an hematophagous arthropod. The existence of more than one route of transmission for *H. felis* is also optional as other *Hepatozoon* spp. have been shown to be transmitted both by arthropod vectors and by additional routes, for instance *H. canis* is transmitted by the tick *Rhipicephalus sanguineus* as well as transplacentally,[3,29] and *H. americanum* is transmitted by the tick *Amblyomma maculatum* and by carnivorism.[5,30]

The level of parasitaemia is usually low in feline hepatozoonosis with less than 1 % of the neutrophils and monocytes containing gamonts.[10] A survey from Thailand found that parasitemia was detected by light microscopy of blood smears in only 0.7 % of 300 cats, while 32 % were positive by sensitive PCR.[28] Furthermore, none of the blood smears from 100 Israeli cats of which 36 % were positive for cardiac *Hepatozoon* sp. meronts had evident gamonts.[9] This study also found a higher infection rate in the myocardium of apparently healthy cats compared to sick cats.[9] Feline hepatozoonosis seems to be mostly subclinical as a high proportion of the population is infected with no apparent overt clinical manifestations. The seven cats described in a case series of feline hepatozoonosis suffered from various other infections including FIV, FeLV, and hemotropic mycoplasmosis.[10] Although no significant association was found in the present study between FIV infection and *Hepatozoon* infection, this could be due to the small number of samples included, as proportionally, more FIV positive cats were infected with *Hepatozoon* than FIV negative

cats. FIV or FeLV infections have also been described in conjunction with feline hepatozoonosis in other studies.[11,14] It is therefore probable that *Hepatozoon* infection may escape control by the immune system in immune-suppressed cats, allowing the intensification of parasitemia and increasing the likelihood of detection by blood smear microscopy.[10,11] The reason for not testing the cats in the study for FeLV infection is the relatively low prevalence of this infection in local Israeli cats.[31]

Feline hepatozoonosis is associated mostly with infection of muscle tissues. *Hepatozoon* sp. meronts have been reported in the muscles of domestic cats with hepatozoonosis,[9,11] and elevated activities of the muscle enzyme creatine kinase were found in the majority of cats with hepatozoonosis in a retrospective study of this infection.[10] The genetic and morphologic findings of this study clearly showed that it is *H. felis* which infects myocardial and skeletal cat muscles, and not another *Hepatozoon* sp. However, other tissues such as the lungs were also infected with meronts, and PCR detected the presence of the parasite's DNA in hemolymphoid organs such as the spleen, bone marrow and lymph nodes, and in the liver, pancreas, and kidney, as well as in fetal lungs and amniotic fluid. No substantial inflammatory response surrounding meronts was seen in the muscles examined in this study, as well in the other reports[9,11] This is in agreement with the generally subclinical nature of this infection. Infection of muscle tissues by *Hepatozoon* sp. has also been reported in wildlife felids and carnivores where *H. felis* or closely related species are responsible for myositis and myocarditis.[32–35] *H. americanum* infection of dogs and wildlife in the USA also has a predilection to muscle tissue, like *H. felis*, differing substantially from *H. canis* in dogs, which is found mostly in the hemolymphoid tissues and does not directly infect muscle. However, *H. americanum* induces the formation of muscle cysts which are much larger than *H. felis* meronts, are composed of concentric layers of muco-polysaccharide material surrounding a core zoite in a formation described as "onion skin" cysts, and elicit severe and painful pyogranulomatous myositis following merogony.[24,36,37]

The phylogenetic placement of 18S rRNA *Hepatozoon* sequences amplified from domestic cats in this study revealed that both *H. felis* and *H. canis* infect cats in Israel, although *H. felis* is by far more common. Israeli *H. felis* sequences are indistinguishable from those reported from Spain and Brazil, and closely related to those reported in wildlife felids and carnivores from India, Korea, Japan, Tanzania, Brazil, and Argentina.[15,16,33–35,38–40] It is obvious that *H. felis* clusters away from *H. canis* and other *Hepatozoon* species, and is responsible for infection of domestic cats and likely also of other carnivores.

It is most likely that *H. felis* is the predominant species of *Hepatozoon* that infects domestic cats and wild felids globally. Its wide geographic distribution could be due to transmission by some ubiquitous vector such as a common flea, mite, or tick species, or to highly successful alternative routes of transmission such as transplacental transmission or carnivorism of a yet unknown wildlife intermediate host. *H. canis* has been shown to spread rapidly in a young dog shelter population, and it is possible that *H. felis* may also spread rapidly under similar conditions.[41]

CONCLUSIONS

Merging of morphologic and genetic findings on *H. felis* from multiple tissues and blood of the same cats, in conjunction with a broad-based epidemiological study, facilitated detailed characterization and redescription of this species in its intermediate host, the domestic cat. Further studies are needed to elucidate its definitive host, likely an arthropod vector, and other transmission pathways with transplacental transmission as a probable option.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

GB designed the study, analyzed data, wrote the manuscript; AS collected samples, performed PCR, and analyzed data; SH performed and analyzed the histopathology; JPB contributed samples and assisted in writing the manuscript; YA analyzed collected samples and blood smears; DTE performed PCR on histopathological specimens, performed genetic analyzes, and assisted in writing the manuscript. All authors read and approved the final version of the manuscript.

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3 NEW INSIGHTS INTO THE ZOONOTIC IMPACT OF BARTONELLOSIS

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Bartonella henselae bacteremia in a mother and son potentially associated with tick exposure

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ABSTRACT

BACKGROUND: *Bartonella henselae* is a zoonotic alpha-Proteobacterium, historically associated with cat scratch disease (CSD), but more recently associated with persistent bacteremia, fever of unknown origin, arthritic and neurological disorders, and bacillary angiomatosis, and peliosis hepatis in immunocompromised patients. A family from The Netherlands contacted our laboratory requesting to be included in a research study (NCSU-IRB#1960), designed to characterize *Bartonella* spp. bacteremia in people with extensive arthropod or animal exposure. All four family members had been exposed to tick bites in Zeeland, southwestern Netherlands. The mother and son were exhibiting symptoms including fatigue, headaches, memory loss, disorientation, peripheral neuropathic pain, striae (son only), and loss of coordination, whereas the father and daughter were healthy.

METHODS: Each family member was tested for serological evidence of *Bartonella* exposure using *B. vinsonii* subsp. *berkhoffii* genotypes I-III, *B. henselae* and *B. koehlerae* indirect fluorescent antibody assays and for bacteremia using the BAPGM enrichment blood culture platform.

RESULTS: The mother was seroreactive to multiple *Bartonella* spp. antigens, and bacteremia was confirmed by PCR amplification of *B. henselae* DNA from blood, and from a BAPGM blood agar plate subculture isolate. The son was not seroreactive to any *Bartonella* sp. antigen, but *B. henselae* DNA was amplified from several blood and serum samples, from BAPGM enrichment blood culture, and from a cutaneous striae biopsy. The father and daughter were seronegative to all *Bartonella* spp. antigens, and negative for *Bartonella* DNA amplification.

CONCLUSIONS: Historically, persistent *B. henselae* bacteremia was not thought to occur in immunocompetent humans. To our knowledge, this study provides preliminary evidence supporting the possibility of persistent *B. henselae* bacteremia in immunocompetent persons from Europe. Cat or flea contact was considered an unlikely source of transmission and the mother, a physician, reported that clinical symptoms developed following tick exposure. To our knowledge, this is the first time that a *B. henselae* organism has been visualized in and amplified from a striae lesion. As the tick bites occurred three years prior to documentation of *B. henselae* bacteremia, the mode of transmission could not be determined.

KEYWORDS: *Bartonella*, BAPGM, Bacteremia, Striae, Neuropathy, Neurological disorder

BACKGROUND

Due to complex nutritional requirements and slow dividing times, which necessitate a prolonged incubation period for successful isolation, members of the genus *Bartonella* are highly fastidious microorganisms that are difficult to document microbiologically in patient blood or tissue specimens.[1] Over the past decade, evolving evidence supports the fact that these bacteria can induce long-lasting intravascular infections in animals that serve as reservoir-adapted hosts, whereas, more recently a number of *Bartonella* sp. have been associated with persistent bacteremia in immunocompetent human patients experiencing a spectrum of symptoms and somewhat diverse disease pathologies.[2,3] On a comparative medical basis, *Bartonella* infection induces similar pathology in dogs, people, and other animals.[3,4] Infection with one or more *Bartonella* sp. has also been associated with fever of unknown origin,[5–9] and arthritic and neurological disorders.[10–12]

In an effort to overcome limitations associated with the molecular detection and isolation of *Bartonella* spp. from animals and immunocompetent human patients, our laboratory developed a novel diagnostic platform based on biochemical optimization of a modified insect-cell culture liquid medium (*Bartonella* alpha-Proteobacteria Growth Medium or BAPGM).[13] The BAPGM platform consists of PCR amplification of *Bartonella* DNA from the patient's extracted blood and serum samples before and after BAPGM enrichment culture and from isolates, if obtained, following subculture of pre-enriched samples onto blood agar plates. Research utilization of this testing platform has facilitated the documentation of *B. henselae* bacteremia in patients from Australia,[14] England,[14] and the United States.[10–12,15] In addition, utilization of the BAPGM enrichment blood culture platform has facilitated the isolation or molecular detection of other *Bartonella* spp. including *Bartonella vinsonii* subsp. *berkhoffii* genotypes I and II,[10–12,15,16] *Bartonella koehlerae*,[10,12,14,17] '*Candidatus* Bartonella melophagi',[18] and a novel *Bartonella* sp. related to *Bartonella volans*[19] from the blood of immunocompetent humans.[10,11,13] Standardized precautions are routinely used in our laboratory to avoid DNA contamination and negative controls are used in each step of the testing platform, including culture, DNA extraction, and PCR-negative controls.

All conventional PCR amplicons are sequenced to confirm the *Bartonella* species and 16S-23S ITS strain type. In this study, four members of a family from The Netherlands were tested for serological, microbiological (isolation) or molecular (PCR amplification and DNA sequencing) evidence of *Bartonella* exposure, or bacteremia, using five indirect fluorescent antibody assays and the *Bartonella* alpha-Proteobacteria growth medium (BAPGM) enrichment culture platform, respectively. In addition, skin biopsies were obtained surgically from the son for BAPGM enrichment culture, histopathology, and laser confocal immunohistochemistry.

METHODS

PATIENTS AND SAMPLES

While reviewing recently published literature relative to potential transmission of *Bartonella* species by ticks,[20–24] the mother, a 58-year-old anesthesiologist from The Netherlands contacted one of the investigators in our laboratory (EBB) by email and requested BAPGM enrichment blood culture testing to determine if she and her son could have been infected with a *Bartonella* sp. following tick bites. It was ultimately decided that the family would be tested in conjunction with an ongoing research study designed to determine the prevalence of bacteremia in people with animal and arthropod exposure. This research study was reviewed and approved by the North Carolina State University Institutional Review Board (NCSU IRB#1960) to assure conformity with all confidentiality and patient assurance laws in the United States. In July 2008, prior to the onset of illness in the mother and son, all four family members had experienced tick bites at the family vacation home in Zeeland, located in southwestern Netherlands, where they reported a large deer population.

Tick attachments had also occurred during subsequent summer vacations. The family did not own a cat or dog, and all family members denied exposure to cats and cat fleas. During the three years prior to testing in the Intracellular Pathogens Research Laboratory (IPRL), the mother reported fatigue, headaches, memory loss, irritability, disorientation, chest pain, syncope (two episodes), fine tremors, shoulder pain, joint pain, loss of coordination, and peripheral neuropathic pain involving the arms. The son (an 18-year-old student) reported an illness of three-year duration, with symptoms including: fatigue, headaches, fine tremors, red conjunctivae, cervical lymphadenopathy, and striae involving both legs and the buttock. In the context of this manuscript, striae are irregular bands, stripes, or lines in the skin. Between July 2009 and July 2011, both the mother and son underwent multiple diagnostic evaluations for infectious and non-infectious diseases. Both had been treated for a potential *Borrelia* sp. infection with multiple antibiotic combinations (azithromycin, cefuroxime, metronidazole, rifampin, tetracycline) for at least 6 weeks duration, on several occasions. The historical response to antibiotics was inconsistent and difficult to assess, but in general, symptoms would diminish while receiving antibiotics and reoccur weeks to months after antibiotic administration ended. The last date in which the mother and son took antibiotics was May 24, 2011 and July 7, 2011, respectively.

The father (64-year-old investment banker) and daughter (20-year-old medical student) were not symptomatic prior to or at the time of sample collection. As a physician, the mother decided that shipping samples from Europe to the United States might compromise sample integrity, thereby contributing to false negative test results. Therefore, after flying to North Carolina, the mother's and son's blood samples were aseptically collected during the last week of July 2011, whereas the father's and daughter's samples were collected the first week of August 2011. After the two week sample collection period, the family flew back to The Netherlands to await research testing results, which required months to complete. Three aseptically collected blood and serum samples were obtained during a one-week period from the mother, father, son, and daughter.

Three skin biopsy samples, which included a blue nevus located over the right deltoid, and a striae lesion (0.4 x 0.3 cm, excised to a depth of 0.5 cm) located on the left thigh, were surgically obtained from the son at a hospital in Raleigh, North Carolina, during the same time frame as blood samples were being collected. Blood, serum, and skin biopsy samples were processed and tested in the IPRL, as described below. Histopathological examination of hematoxylin- and eosin-stained tissue sections was performed by the consulting human pathologist for the local hospital. Frozen tissues were sent to the Cutaneous Imaging Center, Department of Dermatology and Center for Drug Design, University of Minnesota, to determine if *Bartonella* organisms could be visualized.

Bartonella spp. IFA testing

Antibody seroreactivity to *B. vinsonii* subsp. *berkhoffii* genotypes I, II, and III, *B. henselae* (strain Houston I), and *B. koehlerae* were determined using previously described indirect fluorescent antibody tests.[10,11,25]

BAPGM enrichment culture

The BAPGM enrichment culture platform, which has been employed by several research laboratories,[26–28] was used to assess for *Bartonella* sp. bacteremia. Briefly, the procedure included inoculation of 2 ml of blood and up to 2 ml of serum collected the same day into a culture flask containing 10 ml of BAPGM. Cultures were incubated for up to 14 days at 36 °C with 5 % CO_2 and 100 % humidity with constant agitation. A milliliter of each enrichment culture sample was sub-inoculated onto blood agar plates (10 % rabbit blood, TSA II) at 7 and 14 days after incubation of the BAPGM

flask for potential colony formation. For each patient sample tested using the BAPGM platform, an un-inoculated BAPGM culture was processed in an identical and simultaneous manner to monitor for potential laboratory contamination (quality assurance).

DNA extraction, PCR amplification and DNA sequencing

Using established assays, PCR targeting the *Bartonella* 16S-23S intergenic spacer (ITS) region was used to amplify *Bartonella* spp. DNA from extracted blood, serum, skin biopsies, BAPGM enrichment cultures and isolates.[10] All PCR products obtained after amplification of extracted DNA from blood, serum, and tissue samples, after BAPGM enrichment, and from agar plate colonies were sequenced directly or after cloning. Bacterial species and strain were defined by comparing DNA sequence similarities with other sequenced bacteria deposited in the GenBank database using the Basic Local Alignment Search Tool (Blast version 2.0).

***Bartonella* confocal microscopy**

An excised skin biopsy sample from the son (striae lesion) was drop-fixed in formalin and stored at room temperature. As a negative control, a scalp skin 4-mm punch biopsy was drop-fixed in Zamboni's fixative (0.03 % (w/v) picric acid and 2 % (w/v) paraformaldehyde) for 48 h at 4 °C and then transferred to a 20 % sucrose solution with 0.05 % sodium azide in PBS for storage. Processing and multi-staining of tissue specimens were performed according to a previously published procedure.[29,30] Vertical sections, 60 microns thick, were mounted in and cut on a cryostat. Floating sections were then incubated with primary antibodies to Collagen Type IV at a 1:200 dilution (Southern Biotech, 1340-01), donkey anti-goat Cy3 at 1:500 dilution (Jackson Immunoresearch, West Grove, PA), and a mouse antibody to *B. henselae* at a 1:100 dilution (Abcam, ab704-250) plus donkey anti-mouse Cy5 at a 1:500 dilution (Jackson Immunoresearch). Washed samples were subsequently fixed to cover slips in agar, dehydrated in ethanol, cleared with methyl salicylate, and mounted in DEPEX (Electron Microscopy Sciences, Hatfield, PA).

RESULTS

The son was not seroreactive to *B. henselae*, *B. koehlerae*, or *B. vinsonii* subsp. *berkhoffii* genotype I, II, or III antigens by IFA testing (Table 1). However, *B. henselae* (16S-23S SA2 strain type) DNA was amplified and successfully sequenced from his blood, serum, a BAPGM enrichment blood culture,

Table 1. Indirect fluorescent serum antibody results for the four family members

Patient	Collection date	*Bvb* TI	*Bvb* TII	*Bvb* TIII	*Bh*	*Bk*
Son	7/25/2011	< 16	< 16	< 16	< 16	< 16
	7/27/2011	< 16	< 16	< 16	< 16	< 16
	7/29/2011	< 16	< 16	< 16	< 16	< 16
Mother	7/25/2011	< 16	64	< 16	64	64
	7/27/2011	< 16	64	64	64	64
	7/29/2011	< 16	64	< 16	64	64
Daughter	8/1/2011	< 16	< 16	< 16	32	< 16
	8/2/2011	< 16	< 16	< 16	32	< 16
	8/5/2011	< 16	< 16	< 16	32	< 16
Father	8/1/2011	< 16	< 16	< 16	32	< 16
	8/2/2011	< 16	< 16	< 16	32	< 16
	8/5/2011	< 16	< 16	< 16	32	< 16

Bh: *B. henselae*; *Bk*: *B. koehlerae*; *Bvb*: *B. vinsonii* subsp. *berkhoffii*
TI,TII, TIII denote *B. vinsonii* subsp. *berkhoffii* genotypes I, II, and III, respectively

Table 2. ***Bartonella*** **spp. blood, serum, BAPGM enrichment blood culture, subculture, and tissue PCR testing results for the four family members**

Patient	Sample type	Collection date	PCR sample	PCR culture	PCR isolate
Son	Serum	7/29/2011	Neg	Neg	Neg
	Blood	7/29/2011	Neg		
	Blue nevus	7/25/2011	Neg	Neg	Neg
	Normal skin	7/25/2011	Neg	Neg	Neg
	Striae	7/25/2011	*Bh*SA2	Neg	Neg
	Serum	7/25/2011	*Bh*SA2	Neg	Neg
	Blood	7/25/2011	*Bh*SA2		
	Serum	7/27/2011	*Bh*SA2	*Bh*SA2	Neg
	Blood	7/27/2011	*Bh*SA2		
Mother	Serum	7/25/2011	Neg	Neg	Neg
	Blood	7/25/2011	Neg		
	Serum	7/27/2011	Neg	Neg	*Bh*SA2
	Blood	7/27/2011	*Bh*SA2		
	Serum	7/29/2011	Neg	Neg	Neg
	Blood	7/29/2011	Neg		
Daughter	Serum	8/2/2011	Neg	Neg	Neg
	Blood	8/2/2011	Neg		
	Serum	8/2/2011	Neg	Neg	Neg
	Blood	8/2/2011	Neg		
	Serum	8/5/2011	Neg	Neg	Neg
	Blood	8/5/2011	Neg		
Father	Serum	8/2/2011	Neg	Neg	Neg
	Blood	8/2/2011	Neg		
	Serum	8/2/2011	Neg	Neg	Neg
	Blood	8/2/2011	Neg		
	Serum	8/5/2011	Neg	Neg	Neg
	Blood	8/5/2011	Neg		

and from the striae biopsy sample (Table 2). *Bartonella* spp. DNA was not amplified from the biopsy of the son's blue nevus. In contrast to her son, the mother was seroreactive to *B. henselae* (at an endpoint titer of 1:64), *B. koehlerae* (1:64), and *B. vinsonii* subsp. *berkhoffii* genotypes II and III antigens (both 1:64), but was not seroreactive to *B. vinsonii* subsp. *berkhoffii* genotype I. *B. henselae* SA2 bacteremia was confirmed in the mother by PCR amplification and DNA sequencing (460/460 bp homology with GenBank AF369529) of 16S-23S ITS PCR amplicons from an extracted blood sample and from colonies obtained on blood agar plates following BAPGM enrichment[10,11,18] from the same collection date. Isolation of *B. henselae* from the mother's blood and amplification of *B. henselae* DNA after enrichment blood culture from the son supported active infection with viable intravascular bacteria. The father and daughter were not seroreactive to the *Bartonella* spp. test antigens used in this study and *B. henselae* DNA was not amplified from any of their blood, serum, or BAPGM enrichment blood cultures. As detailed in previous studies,[10,11] *Bartonella* DNA was not amplified from any of the simultaneously processed BAPGM-negative culture controls (results not shown).

The gross appearance of the son's striae are depicted in Figures 1A and 1B. Histopathology of the right deltoid skin lesion contained features of a blue nevus. The striae lesional biopsy from the

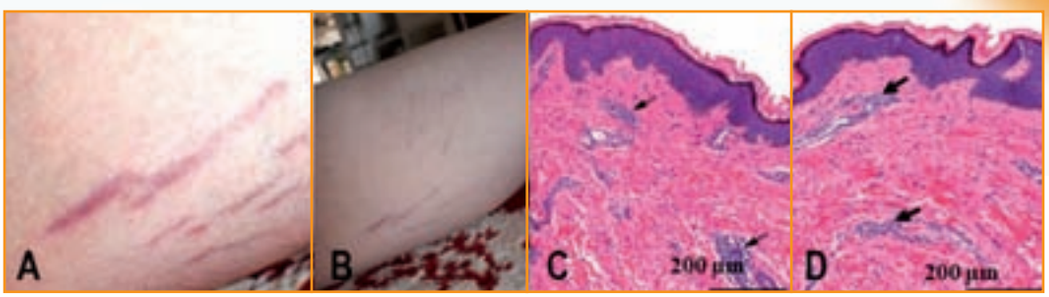

Figure 1. Gross appearance of striae located on the thigh of the son (A & B, photographs provided by the family) and the hematoxylin/eosin-stained striae tissue biopsy (C & D) illustrating (arrows) minimal non-specific superficial perivascular chronic inflammation.

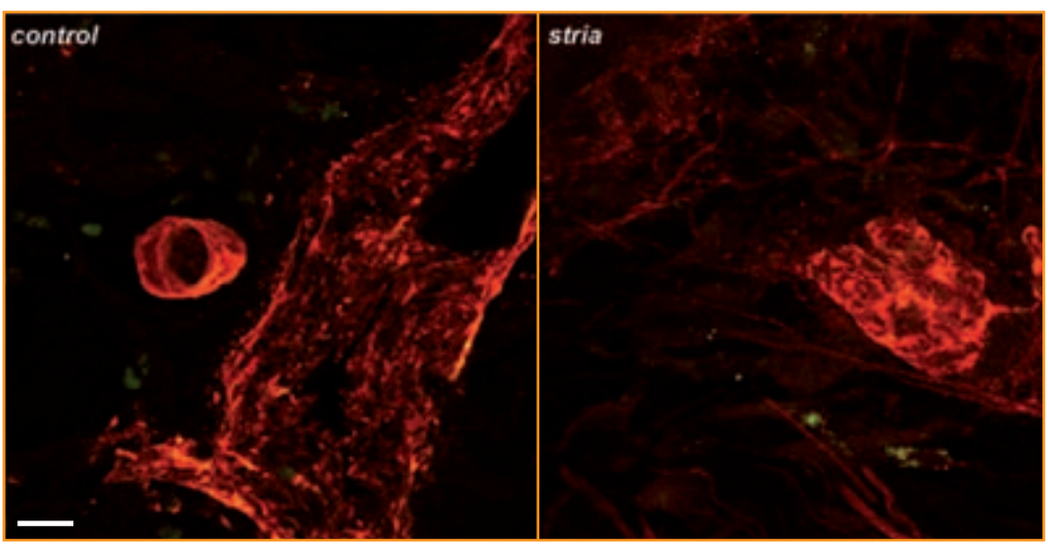

Figure 2. Photomicrographs captured using laser scanning confocal microscopy demonstrating immunoreactive *B. henselae* organisms in the striae skin biopsy obtained from the son's thigh (right panel, small green particles). Left panel is scalp skin from a non-infected subject also immunostained with *B. henselae* antibody. No bacteria were visualized in control sections, although relatively large auto-fluorescent red blood cells are partially visible (pseudo-colored green). Both samples also stained with collagen IV to highlight immunoreactive vascular tissue (pseudo-colored red). Note bacteria within skin appear external to vascular tissues. Images are projections of 31, 0.48 micron-thick optical sections, total thickness 15 microns. Scale bar = 10 microns.

left thigh contained minimal non-specific superficial perivascular chronic inflammation (Figures 1C and 1D). Immunostaining of the striae tissue from the son's skin biopsy revealed *B. henselae* immuno-positive staining within the dermis and by confocal microscopy imaging, the bacteria were external to vascular tissue (Figure 2).

After these results were obtained, the son elected to not be treated with additional antibiotics. During 2012 he remained mostly healthy, and passed Propedeuse exam Cum Laude. The striae regressed spontaneously leaving slight, colorless, and painless marks. Conjunctivitis and the fine motor tremor have persisted. Psoriasis was diagnosed in 2012 for which he is being treated with vitamin supplementation and immune stimulants. The mother also did not seek treatment for *B. henselae* bacteremia until several months after test results became available, when she developed severe myalgia, tendinitis involving several joints and paresthesias. She was treated with intraveneous ceftriaxone 4 gram twice a week for six months. After three months of antibiotic therapy, *Bartonella* serology was reported as positive (IFA titer 1:32 – normal < 32). During the final two months of antibiotic therapy, rifampin, hyperthermic therapy every day (sauna), and hyperbaric oxygen therapy by mask in a high-pressure cabin 2.4 bar for 75 minutes each day were administered for 45 days. None of these therapies decreased the woman's symptoms. As of March 2013, the father and daughter have remained healthy.

DISCUSSION

In this study, *B. henselae* bacteremia was confirmed in the two symptomatic family members, but not in the two historically healthy family members. Although the symptoms that developed in 2008 as reported by the physician and her son were similar to questionnaire respondent symptoms reported by other patients with *B. henselae* bacteremia,[10,11] it is not possible to determine whether the symptoms in these two family members were due in part or total to infection with this organism. Unless re-infection was occurring, prior efforts to eliminate *B. henselae* with antibiotics were not successful, further supporting the possibility that antibiotic treatment failure can occur in a subset of *B. henselae*-bacteremic patients.[14,31] As our research group was not involved in patient management decisions prior to or after *B. henselae* bacteremia was confirmed, the purpose of this report was to provide evidence supporting *B. henselae* bacteremia in two immmunocompetent individuals from Europe, who believed that tick transmission was the most likely source of their infections. During the previous three years, all four family members reported tick bites while vacationing in southwestern Netherlands and all denied exposure to cats. As *B. henselae* DNA was successfully amplified and sequenced from one of the son's BAPGM enrichment blood cultures and from a subculture isolate obtained from the mother's blood, bacteremia (viable bacteria) was confirmed in both individuals despite prior administration of antibiotics. Only one of three blood culture sample sets from the mother and the son resulted in enrichment culture or isolation evidence to support the presence of bacteremia, which is consistent with prior experience with the BAPGM platform when testing sequentially obtained blood samples from sick human patients.[10,11,16,17,19,32] Presumably, failure to document infection in two of the three sample sets is potentially due to a relapsing pattern of bacteremia in humans,[33–36] as has been reported in experimentally infected cats[37,38] and rodents.[33,39,40] Due to diagnostic limitations associated with serology, direct blood plating methods, and PCR following DNA extraction directly from patient samples, our research group developed and have used a combined method that incorporates PCR from extracted blood, serum, and BAPGM enrichment blood culture.[10,11,17] This study further supports the enhanced diagnostic sensitivity of the BAPGM enrichment blood culture platform for documentation of *Bartonella* spp. bacteremia in immunocompetent patients, and provides additional support for the need to test three sample sets obtained during a one-week collection period.[32]

In addition to documentation of bacteremia, immunohistochemical confocal microscopy was used to visualize *B. henselae* organisms in the son's striae tissue biopsy. This observation provides preliminary evidence to support a potential association between *B. henselae* infection and striae. Although lay publications have frequently reported[41,42] that striae are caused by *Bartonella* sp. infections, to our knowledge there are no scientific publications that have investigated, reported, or confirmed this possibility. Clearly, additional immunohistochemical studies of striae would be of interest to determine if persistent *Bartonella* sp. bacteremia could contribute to the development of these skin lesions in human patients.

As is true throughout much of the world, *B. henselae* infection in immunocompetent people has been reported previously in The Netherlands in association with the diagnosis of typical or atypical cases of cat scratch disease (CSD).[43,44] Cats, infected with *B. henselae* by cat fleas (*Ctenocephalides felis*), develop a bacteremia that can persist for years.[37,45–49] The term cat scratch disease is clearly of historical medical importance, but continued use of the term as a sole reference to *B. henselae* or other *Bartonella* sp. infections is potentially detrimental for patient diagnosis and patient management decisions. Briefly, *B. henselae*, the acknowledged cause of CSD, has also been documented (by PCR or isolation) in dogs,[13,25,50–54] dolphins,[55,56] feral swine,[57] horses,[58–60] and Beluga whales.[61] In North America, *B. henselae* is the most frequent *Bartonella* species isolated from bacteremic sick dogs[54] and people.[10,11,62] In a recent report of a patient with neuroretinitis in Australia, a well-

documented ocular pathology induced by *B. henselae*, bartonellosis was diagnosed following the bite (sting) of a bull ant (genus *Myrmecia*).[21] Those authors also advocated for medical use of the more inclusive term bartonellosis. Thus, because of alternative vectors, numerous accidental or reservoir hosts, and the seemingly broad spectrum of disease manifestations, referring to all *B. henselae* infections as CSD is a contradiction in fact, and the use of the more globally applicable term such as bartonellosis is suggested. In addition, CSD is considered a self-limiting infection for which antibiotic therapy is not recommended, whereas more recent evidence indicates that antibiotic elimination of *B. henselae* bacteremia can be extremely challenging, and potentially difficult to achieve.[19,31] Interestingly, molecular differences among *B. henselae* isolates based on 16S rDNA sequences and multiple-locus variable number tandem repeat analysis (MLVA) have documented the presence of two different *B. henselae* genotypes, one more frequently observed in association with human infections (genotype I), and the second (genotype II) most often isolated from bacteremic cats, suggesting that genotype II isolates may be minimally or non-pathogenic for humans (CSD) as compared with a more pathogenic and zoonotic genotype I.[63,64]

In recent years, a substantial number of European studies have reported the presence of *B. henselae* DNA in ticks, including *I. ricinus*,[24,65–76] suggesting that ticks may act as an important ecological reservoir for this *Bartonella* species. In addition, several recent publications have provided indirect[22,23] or experimental vector competence evidence[24] to support transmission of *Bartonella* spp. (including *B. henselae*, *B. birtlesii*, and *B. vinsonii* subsp. *berkhoffii*) by ticks (including *I. ricinus*).[22–24] Two previous studies have not documented *Bartonella* spp. DNA in ticks from The Netherlands,[46,77] where this family experienced tick exposures. Although not reported specifically in the results, *B. henselae* SA2 strain DNA was amplified and sequenced from two *I. ricinus* ticks from Klasdorf, Brandenburg, Germany, by targeting the 16S-23S rRNA intergenic spacer region[73] (Kempf VA, Maggi R, unpublished data). The DNA sequences derived from these two ticks were identical to the *B. henselae* DNA sequences obtained from the two bacteremic people in this report. Interestingly, the remaining eight ticks that were sequenced as part of that study to confirm the *Bartonella* species and strain type contained a *B. henselae* 16S-23S Houston 1 DNA and were collected in southern Germany, France, and Portugal, suggesting the possibility of *B. henselae* strain variation among ticks. It is also important to consider the tick life stage, as in that study the odds of detecting *B. henselae* DNA was 14-fold higher in nymphal ticks as compared to adult ticks.[73] In addition, differences in PCR sensitivity among studies and differences in *Bartonella* sp. gene targets used to test tick DNA extractions can contribute to divergent findings among laboratories, when testing ticks from the same location. Most previous studies designed to detect and identify *Bartonella* species in *I. ricinus* ticks from Europe have targeted the citrate synthase (gltA) and 16S rRNA genes. Unfortunately, these genes have very limited genetic variability and therefore are not optimal to differentiate among *B. henselae* strains. Clearly the role of ticks as potential vectors for transmission of *Bartonella* sp. to animals and humans deserves additional research consideration.

CONCLUSION

Although *B. henselae* infection was documented by PCR amplification and DNA sequencing in both of the sick members of this family, there may or may not be a causal relationship between the bacteria and the reported symptoms. *Bartonella* serology was supportive of *B. henselae* infection in the mother, but not in the son, further supporting reports of seronegative *Bartonella* sp. bacteremia in human patients.[10,11,78,79] Also, despite improvements in PCR sensitivity, immunohistochemical methods are useful to facilitate the visualization of *Bartonella* species in patient tissue specimens. Finally, as ticks removed from the patients were not saved for PCR testing, it is not clear whether infection with *B. henselae* was acquired by tick bites or by another mode of transmission.

CONSENT

Written informed consent was obtained from the patients for publication of this report and any accompanying images.

ABBREVIATIONS

ITS: Intergenic Spacer; BAPGM: *Bartonella* Alpha-Proteobacteria Growth Medium; IPRL: Intracellular Pathogens Research Laboratory.

COMPETING INTERESTS

In conjunction with Dr. Sushama Sontakke and North Carolina State University, Dr. Breitschwerdt holds U.S. Patent No. 7,115,385; Media and Methods for cultivation of microorganisms, which was issued October 3, 2006. He is the chief scientific officer for Galaxy Diagnostics, a company that provides diagnostic testing for the detection of *Bartonella* species infection in animals and human patients. Dr. Ricardo Maggi has led research efforts to optimize the BAPGM platform and is the Scientific Technical Advisor for Galaxy Diagnostics. All other authors have no potential conflicts.

AUTHORS' CONTRIBUTIONS

RM and PM performed the BAPGM enrichment blood culture and PCR testing of the patient samples, performed DNA sequencing and alignments, and generated the first draft of the manuscript. ME performed the immunohistochemistry and confocal microscopy. JB assisted in sample acquisition and serological testing. EB coordinated various aspects of the investigation and helped to draft the final manuscript. All authors read and approved the manuscript.

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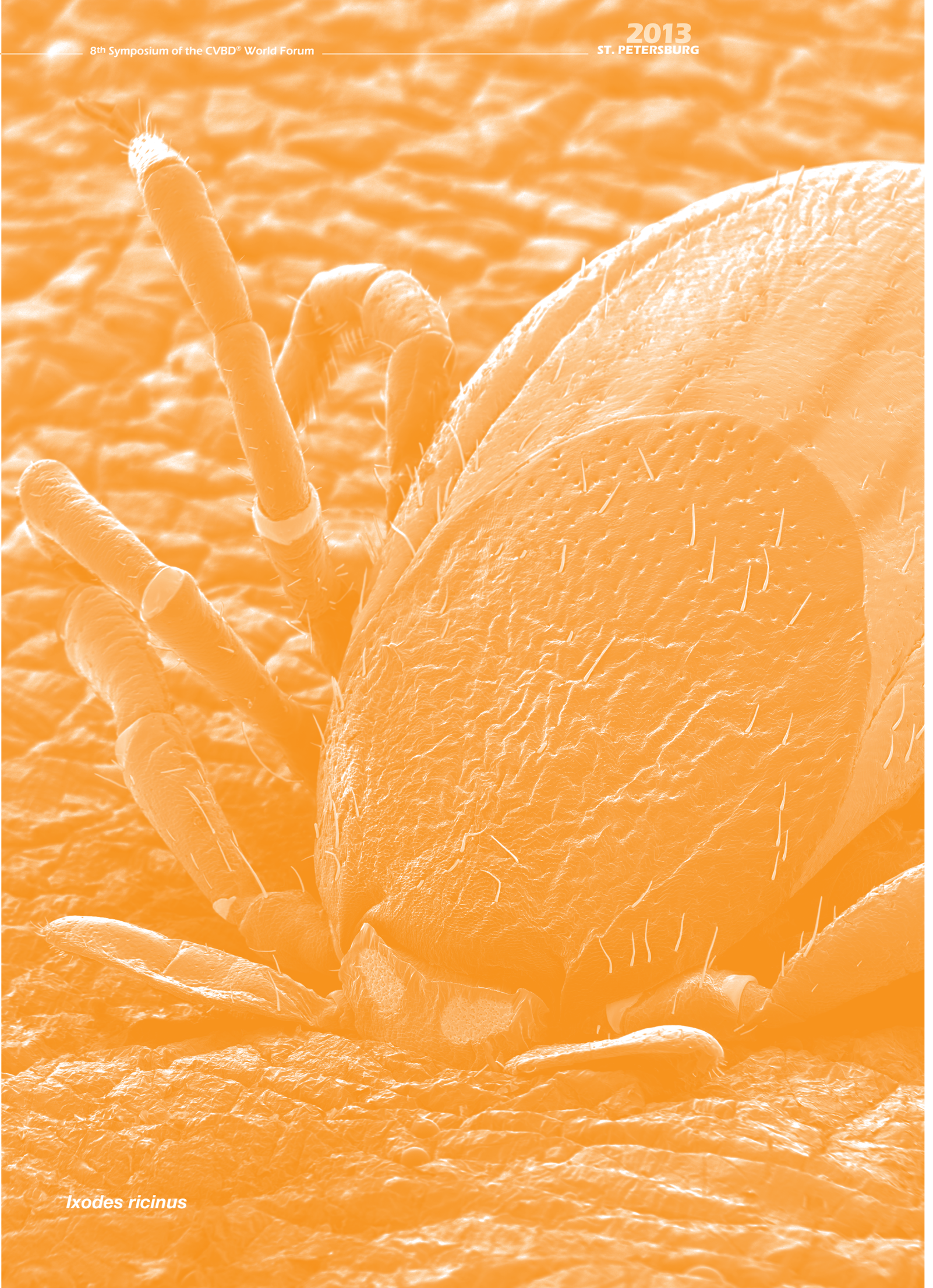

Ixodes ricinus

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Bartonella henselae infection in a family experiencing neurological and neurocognitive abnormalities after woodlouse hunter spider bites

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ABSTRACT

BACKGROUND: *Bartonella* species comprise a group of zoonotic pathogens that are usually acquired by vector transmission or by animal bites or scratches.

METHODS: PCR targeting the *Bartonella* 16S-23S intergenic spacer (ITS) region was used in conjunction with BAPGM (*Bartonella* alpha-Proteobacteria growth medium) enrichment blood culture to determine the infection status of the family members and to amplify DNA from spiders and woodlice. Antibody titers to *B. vinsonii* subsp. *berkhoffii* (*Bvb*) genotypes I–III, *B. henselae* (*Bh*), and *B. koehlerae* (*Bk*) were determined using an IFA test. Management of the medical problems reported by these patients was provided by their respective physicians.

RESULTS: In this investigation, immediately prior to the onset of symptoms, two children in a family experienced puncture-like skin lesions after exposure to and presumptive bites from woodlouse hunter spiders. Shortly thereafter, the mother and both children developed hive-like lesions. Over the ensuing months, the youngest son was diagnosed with Guillain-Barre (GBS) syndrome followed by Chronic Inflammatory Demyelinating Polyradiculoneuropathy (CIDP). The older son developed intermittent disorientation and irritability, and the mother experienced fatigue, headaches, joint pain, and memory loss. When tested approximately three years after the woodlouse hunter spider infestation, all three family members were *Bartonella henselae*-seroreactive and *B. henselae* DNA was amplified and sequenced from blood, serum, or *Bartonella* alpha-Proteobacteria (BAPGM) enrichment blood cultures from the mother and oldest son. Also, *B. henselae* DNA was PCR amplified and sequenced from a woodlouse and from woodlouse hunter spiders collected adjacent to the family's home.

CONCLUSIONS: Although it was not possible to determine whether the family's *B. henselae* infections were acquired by spider bites or whether the spiders and woodlice were merely accidental hosts, physicians should consider the possibility that *B. henselae* represents an antecedent infection for GBS, CIDP, and non-specific neurocognitive abnormalities.

KEYWORDS: *Bartonella*, Spiders, Neurological disease, Guillain-Barré syndrome, Serology, PCR

BACKGROUND

The genus *Bartonella* is composed of fastidious, Gram-negative, and aerobic bacilli belonging to the alpha-Proteobacteria group. *Bartonella* species (spp.) are hemotropic, arthropod-borne bacteria that cause long-term bacteremia in mammalian reservoir hosts.[1,2] During the past decade, there has been a dramatic increase in the number of new *Bartonella* species that have been discovered among diverse animal reservoir hosts in geographical regions throughout the world. Since 1990, over thirty *Bartonella* species and subspecies have been characterized and named, with many other putative species yet to be described. Globally, these bacteria reside in diverse ecological niches; many cause persistent intravascular infection in reservoir hosts and 17 *Bartonella* spp. have been associated with an expanding spectrum of human and animal diseases, ranging from acute febrile illnesses to more severe disease manifestations, including encephalopathy, endocarditis, myocarditis, sensory and motor neuropathies, pleural and pericardial effusion, pneumonia, granulomatous hepatitis, and hemolytic anemia.[3–7]

The natural history for seemingly all *Bartonella* spp. consists of one or more reservoir hosts and one or more transmission-competent arthropod vectors. A vertebrate, generally a mammal, sustains a chronic intravascular infection, which in some instances is associated with a relapsing pattern of bacteremia. The persistently infected host serves as the blood reservoir for perpetuation of the transmission cycle, with an arthropod vector transferring the bacteria from the reservoir host to a susceptible uninfected host.[8] Most vectors for *Bartonella* spp. are arthropods. The vector for *B. quintana* is the body louse (*Pediculus humanus* and potentially *Pediculus capitis*) and for *B. bacilliformis* is the sandfly (*Lutzomyia verrucarum*). Fleas (for example *Ctenocephalides felis* on cats and dogs) play a major role in the natural transmission cycle for many bartonellae, especially *B. henselae* among pets and wildlife.[9,10] There is also a growing spectrum of arthropods that have been implicated as potential vectors for *Bartonella* species. Genetic diversity and bacterial strain variability appear to enhance the ability of *Bartonella* spp. to infect not only specific reservoir hosts, but also accidental hosts, as has been shown for *B. henselae*.[11]

Because neurological abnormalities developed in both children after woodlouse hunter spider bites were suspected by their parents, a family from Kentucky was directed to our laboratory for inclusion in a *Bartonella* research study. Three family members were *B. henselae*-seroreactive and *B. henselae* DNA was amplified and sequenced from the mother's and older son's blood, and from a woodlouse and woodlouse hunter spiders.

Family historical summary

Prior to moving to a new apartment housing location in suburban Louisville, Kentucky, on May 1, 2008, all four family members were healthy and had normal sleep patterns. Two months earlier, while in a previous apartment, a bat was removed by an exterminator. Although the bat was flying free within the apartment when the family awakened, there was no indication of bite wounds. The family's dog had also experienced a flea infestation prior to moving to the new apartment. The family dog was the only pet, there was no history of family members experiencing bites or scratches and no flea infestations were reported after moving into the new apartment or subsequently to a new house in the same neighborhood.

In July 2008, their new apartment flooded, after which there was a large influx of woodlice (order Isopoda). Subsequently, the mother reported seeing occasional woodlouse hunter spiders (*Dysdera crocata*) in the apartment, including in the children's beds and on the children. During August 2008, the parents suspected that both sons (5 months old and 5 years old, respectively)

were bitten by woodlouse hunter spiders. The mother (41 years old) did not knowingly experience any spider bites. After the apartment was treated by an exterminator, no woodlice or woodlice hunter spiders were observed in or around the apartment. Subsequently, the mother and both sons developed recurrent rash-like skin lesions, disruptive sleep patterns, and both boys developed anxiety accompanied by episodes of inconsolable crying, irritability, and panic attacks. In July 2009, the oldest son was examined by a surgeon because of enlarged lymph nodes in the neck. Over the ensuing months, the mother developed symptoms, including fatigue, headaches, joint pain, eye pain, insomnia, memory loss, disorientation, irritability, weakness in the upper extremities, and loss of sensation to both legs. In May 2010, as detailed in the case report below, the youngest son was diagnosed with Guillain-Barre syndrome (GBS), and subsequently with Chronic Inflammatory Demyelinating Polyneuropathy (CIDP).

After contacting the corresponding author and describing the family's medical history, the mother elected to enter her sons and herself into an ongoing study regarding *Bartonella* spp. infection in patients with arthropod and animal exposures. (North Carolina State University Institutional Review Board approval IRB 1960-11). Beginning in August 2011, blood and serum samples from the mother, both sons, and the dog were submitted for *Bartonella* testing. As the parent's initial concerns related to the youngest son's CIDP diagnosis, this child was tested in August 2011, followed by the mother and dog in November, and the oldest son in April 2012. The father did not recall being bitten by a spider himself, remained healthy during the course of this investigation and was never tested for evidence of *Bartonella* sp. infection. Over a one-year period (2011–2012), spiders, identified as woodlouse hunter spiders (*Dysdera crocata*), and several woodlice (Isopoda order) collected from around the family's new house (located three miles from the spider-infested apartment) were submitted by express mail for manual DNA extraction and *Bartonella* PCR.

METHODS

Samples

Aseptically obtained EDTA (ethylene diaminetetraacetic acid)-anti-coagulated blood and serum samples from the family, their dog, whole spiders, and woodlice collected from around the apartment in Kentucky were submitted to the North Carolina State University College of Veterinary Medicine Intracellular Pathogens Research Laboratory (NCSU-CVM-IPRL) for *Bartonella* testing. Collection and analyses of these data were conducted in conjunction with North Carolina State University Institutional Review Board approval (IRB no. 1960-11).

Serological analyses

Bartonella vinsonii subsp. *berkhoffii* genotypes I, II, III, *B. henselae* (Houston 1 strain), *B. henselae* (San Antonio 2 strain), and *Bartonella koehlerae* antibodies were determined in the Intracellular Pathogens Research Laboratory (IPRL) following traditional immunofluorescence antibody assay (IFA) practices with fluorescein conjugated goat anti-human IgG (Pierce Biotechnology Rockford IL), as described in previous studies from our laboratory.[12–14] *Bartonella* organisms of feline isolates of *B. koehlerae* (NCSU 09FO-01) and *B. henselae* H1 (NCSU 93FO-23), *B. henselae* SA2 (NCSU 95FO-099), and canine isolates of *B. vinsonii berkhoffii* genotype I (NCSU 93CO-01), II (NCSU 95CO-08), and III (NCSU 06CO-01) were passed from agar grown cultures into cell cultures to obtain antigens for IFA testing. Heavily infected cell cultures were spotted onto 30-well Teflon-coated slides (Cel-Line/Thermo Scientific), air-dried, acetone-fixed, and stored frozen. Serum samples were diluted in phosphate-buffered saline (PBS) solution containing normal goat serum, Tween-20, and powdered nonfat dry milk to block non-specific antigen binding sites. Sera were screened at dilutions of 1:16 to 1:8,192. To avoid confusion with possible non-specific binding found at low dilutions and

to standardize with other laboratories such as the CDC, a cut-off titer of 1:64 was used to define a seroreactive titer.

DNA extraction, PCR assay, and DNA sequencing

A previously described approach that combines PCR amplification of *Bartonella* spp. DNA from blood, serum, and enrichment BAPGM (*Bartonella* alpha-Proteobacteria growth medium) enrichment blood culture was used to test EDTA-anti-coagulated whole blood and centrifuged serum samples.[4,13,15–17] DNA was automatically extracted from 200 µl of EDTA-anticoagulated blood, from serum, and from 200 µl of BAPGM enrichment blood culture, using a BioRobot Symphony Workstation and MagAttract DNA blood kit (Qiagen, Valencia, CA). Prior to extraction of DNA from the woodlice and woodlouse hunter spiders, each individual specimen was washed twice using 2 ml of dH_2O followed by a single wash with 95 % ethanol. For DNA extraction, the entire body of each arthropod was pulverized to a fine powder by bead-beating using stainless steel beads. DNA from spiders and woodlice were manually extracted using DNeasy blood and tissue mini kit following manufacturer's instructions (Qiagen, Valencia, CA). *Bartonella* DNA was amplified using conventional *Bartonella* genus PCR primers targeting the 16S-23S intergenic spacer region (ITS) as previously described.[18,19] *Bartonella* genus PCR was performed using oligonucleotides 425 s (50CCGGGGAAGGTTTTCCGGTTTATCC 30), 325 s (50 CCTCAGATGATGATCCCAAGCCTTTTGGCG 30) and 1,000 as (50 CTGAGCTACGGCCCCTAAATCAGG 30) as forward and reverse primers, respectively. Amplification was performed in a 25-µl final volume reaction containing 12.5 µl of MyTaq Premix (Bioline), 0.2 µl of 100 µmol/l of each forward and reverse primer (IDTWDNA Technology, Coralville, IA, USA), 7.3 µl of molecular grade water, and 5 µl of DNA from each sample tested. Conventional PCR was performed in an Eppendorf Mastercycler EPgradientW (Eppendorf, Hauppauge, NY, USA) under the following conditions: a single cycle at 95 °C for 2 s, followed by 55 cycles with DNA denaturing at 94 °C for 15 s, annealing at 66 °C for 15 s, and extension at 72 °C for 18 s. The PCR reaction was completed by a final cycle at 72 °C for 30 s. All PCR reactions were analyzed by 2 % agarose gel electrophoresis. Amplicons obtained from arthropod and human samples were sequenced to identify the *Bartonella* sp. and ITS strain type. Bacterial species and strains were defined by comparing similarities with other sequences deposited in the GenBank database using the Basic Local Alignment Search Tool (Blast version 2.0).

RESULTS

Individual medical histories

Youngest son

In August 2008, the youngest son, a developmentally normal child with precocious motor skills, sustained puncture-like bite lesions in the skin overlying the mid humerus and proximal femur (Figure 1). Based upon the exposure history and appearance of the lesions, spider bites were diagnosed by the child's pediatrician. Subsequently, the boy developed intermittent rashes that were initially diagnosed as food allergy and was concurrently diagnosed with chronic sinusitis. At ap-

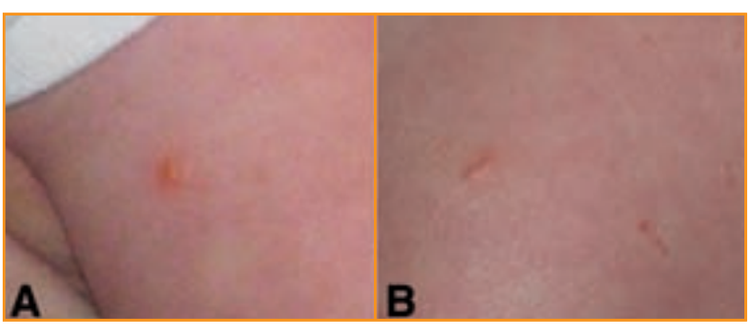

Figure 1. **Photographs taken by the parents during the woodlouse and woodlouse hunter spider infestations.** Puncture-like bite lesions were observed in the skin overlying the mid humerus (A) and proximal femur (B).

proximately 24 months of age (February 2010), his parents first noted that he would stumble. He also awakened at night crying and complaining of pain in his legs. Other concerns included early morning awakenings, constipation, intermittent complaints of dizziness, and seeing "spots."

In May 2010, about a month after an upper respiratory infection, the boy was unable to climb stairs. His parents brought him to an emergency room, where it was noted that he was unable to stand from a seated position on the floor. A lumbar puncture revealed an elevated CSF protein of 110 (normal 15–45) with 4 white blood cells. An MRI of the spine demonstrated enhancement of the ventral nerve roots and pial enhancement from the 11th thoracic vertebrae through the remainder of the spinal cord. Neurological examination was significant for areflexia and weakness in the lower extremities. Guillain-Barré syndrome was diagnosed and he was treated with 2 grams per kilogram of intravenous gammaglobulin (IVIG) over 4 days. He improved rapidly and was discharged following the infusions.

When re-examined one month later, his leg strength was judged to be improved to 85 % of normal and no further treatment was given. By July 2010, he was less able to walk without stumbling and was unable to stand from a seated position. He also complained of tingling and discomfort around his mouth and pain in the legs. IVIG was administered at a dose of 1 gram/kilogram on each of 2 days. Electromyelogram (EMG) findings were consistent with chronic sensory motor demyelinating polyneuropathy with secondary axonal features and conduction block. CIDP was diagnosed and IVIG treatments were re-instituted every 4 weeks, along with a 4-week course of prednisone and gabapentin for pain. By May 2011, following internet searches, his mother became concerned that the boy's symptoms might be related to *Bartonella* infection. Due to this concern, treatment with azithromycin was initiated for 10 days, and it was felt that this was associated with some improvement. *Bartonella henselae* IgM and IgG antibodies were not detectable at a 1:16 dilution (ARUP Laboratories, Salt Lake City, Utah). By July 2011, despite medical therapy, pain and the right foot drop had worsened.

When he had another relapse of muscle weakness, IVIG was administered on August 19, 2011, ten days prior to obtaining blood for additional *Bartonella* testing at the NCSU-CVM-IPRL. While awaiting test results, treatment with azithromycin for 30 days was reinitiated. The family felt that they noted almost immediate improvement of the patient's symptoms and requested that the next IVIG treatment be delayed. The boy was seroreactive to multiple *Bartonella* spp. antigens (see **Table 1**). A decision was made to decrease the dose of the IVIG to 1 g/kg and stretch the infusions to every 6 weeks with the first infusion at this dose being administered in October 2011. It was rapidly clear that this would not be successful as weakness and symptoms of burning pain in his legs returned. Infusions were then reinitiated at a dose of 2 g/kg every 4 weeks and then stretched to every 5 weeks in July 2012. The addition of prednisone was avoided due to concern that immune suppression might interfere with therapeutic elimination of the suspected *Bartonella* infection. After IVIG infusion was reinitiated at the full dose in late 2011, the patient's muscle strength continued to improve. In May 2012, treatment with clarithromycin 125 mg twice daily and rifampin 150 mg twice daily was instituted. By July 2012, his deep tendon reflexes had returned and his strength was normal. As his pain was diminished, gabapentin was discontinued. Clarithromycin and rifampin have been well tolerated and both drugs were continued through November 2012. As of this writing, the child is ambulating normally, but still occasionally complains of stiffness and joint pain in his legs, particularly when he wakes up in the morning. As of January 31, 2013, polyneuropathy remains in remission and IVIG has not been administered since September 14, 2012. The parents report that the child is actively socializing with other children and now runs and plays like he had never done before.

Mother

The mother, who takes care of her children at home full-time, was healthy prior to August 2008. She reported limited exposure to cats, wildlife or production animals, but did allow the family dog to sleep in her bed. Prior vector exposure was infrequent, but included fleas, ticks, and mosquitoes. She did not recall being bitten by a spider. Subsequent to the spider infestation of the apartment, she developed fatigue, memory difficulties, headaches, irritability, eye pain, insomnia, chest pain, blurred vision, shortness of breath, rash and skin lesions, and anxiety attacks. She had also experienced a loss of sensation in her legs, joint pain involving the shoulders and ankles, ear pain, and she frequently had a sore throat. In July 2009, she was examined due to an abdominal rash and shingles was tentatively diagnosed. The mother reported that her symptoms persisted between 2008–2011, without notable improvement or deterioration, during which time she sought care from her family physician, an otolaryngologist and a neurologist. Using blood and serum samples

Table 1. Serological, PCR and culture results from the three patients and their family dog

Family members	Date	Sample	PCR/BAPGM culture results[a]		*Bartonella* IFA reciprocal titers					
			Direct extraction	Enrichment culture	*Bvb* I	*Bvb* II	*Bvb* III	*Bh* H1	*Bh* SA2	*Bk*
Youngest Son	8/29/2011	Serum	Neg	N/D	< 16	< 16	< 16	256	256	128
		Blood	Neg	Neg						
	8/31/2011	Serum	Neg	N/D	64	1,024	128	512	512	2,048
		Blood	Neg	Neg						
	9/2/2011	Serum	Neg	N/D	32	512	128	256	256	512
		Blood	Neg	Neg						
	11/21/2012	Serum	Neg	Neg	< 16	< 16	< 16	< 16	64	< 16
		Blood	Neg	Neg						
Mother	11/14/2011	Serum	Neg	N/D	< 16	32	< 16	< 16	64	< 16
		Blood	Neg	Neg						
	11/16/2011	Serum	*Bh*	N/D	32	128	64	64	64	32
		Blood	Neg	Neg						
	11/18/2011	Serum	*Bh*SA2	N/D	32	256	32	32	64	64
		Blood	Neg	Neg						
Oldest Son	4/16/2012	Serum	Neg	N/D	< 16	256	< 16	< 16	128	< 16
		Blood	Neg	Neg						
	4/18/2012	Serum	Neg	N/D	< 16	256	< 16	< 16	128	< 16
		Blood	Neg	*Bh*SA2						
	4/20/2012	Serum	Neg	N/A	< 16	256	< 16	< 16	128	< 16
		Blood	*Bh*SA2	Neg						
Dog	11/23/2011	Serum	N/D	N/D	< 16	< 16	< 16	< 16	< 16	< 16
		Blood	Neg	Neg						

a: Denotes 16S-23S ITS DNA sequence results;
Bh = *Bartonella henselae*; *Bh* SA2 = *Bh* strain San Antonio; *Bh* H1 = *Bh* strain Houston 1;
Bvb I, II, and III = *Bartonella vinsonii* subsp. *berkhoffii* genotype I, II, and III; *Bk* = *Bartonella koehlerae*;
N/D = Not done

submitted in November 2011, infection with *B. henselae* (SA2 strain) was confirmed serologically and by PCR amplification and DNA sequencing. Between February and July 2012, she was treated with doxycycline 200 mg once daily and rifampin 300 mg twice a day. Following this antibiotic course, the mother reported substantial overall improvement and was almost symptom-free. However, she continues to experience occasional irritability, confusion, dizziness, nausea, and pain involving the shoulder, hip, and the bottoms of her feet.

Oldest son

In August 2008, the older son, was examined by his pediatrician due to suspected spider bites and a rash. This child also had occasional exposure to cats and dogs and had exposure to fleas and mosquitoes. Subsequently, the boy complained of a sore throat, occasional ear pain, and pain in the thigh region. In July 2009, the boy's parents sought medical consultation with a dentist and surgeon for a swollen lymph node in the neck that had persisted for approximately 3 months. The lymph node regressed in size without therapy. During 2009–2012, the parents indicated that the boy experienced episodes of unexplained depression, irritability, and anxiety, but was otherwise healthy. Infection with *B. henselae* SA2 strain was confirmed by serology and BAPGM enrichment blood culture PCR in April 2012. Treatment with clarithromycin 250 mg twice daily was instituted in May 2012. On August 29, 2012, rifampin 300 mg twice daily was added to the treatment regimen, which has continued through November, without known adverse side effects. The sore throat, ear and eye pain resolved by October; however, during the fall 2012 school year, his parents reported increased irritability and rage episodes. In addition, the boy's teacher indicated a lack of attention during class, and suggested that the child might have an Attention Deficit Hyperactivity Disorder (ADHD). After consultation with the attending physician and a psychiatrist, the parents declined therapy for ADHD.

Bartonella spp. serology and BAPGM enrichment PCR results

Bartonella serology and PCR results for the three family members and the dog are summarized in Table 1. The youngest son was seroreactive to *B. henselae* SA2, *B. henselae* H1, and between August 29th and August 31st, there was a fourfold or greater increase in antibody titers to *B. koehlerae* and to *B. vinsonii* subsp. *berkhoffii* genotypes I, II, and III. *Bartonella* sp. DNA was not amplified from his blood, serum, or BAPGM enrichment blood cultures. The mother was seroreactive to *B. henselae* H1, *B. henselae* SA2, *B. vinsonii* subsp. *berkhoffii* genotypes II and III, and *B. koehlerae*. *B. henselae* bacteremia was confirmed in the mother by PCR amplification from two BAPGM enrichment blood cultures. Based upon the amplified DNA sequences, the *B. henselae* strain in the mother's samples were 99.8 % and 100 % similar, respectively, to *B. henselae* SA2 (GenBank accession AF369529). The oldest son was seroreactive to *B. henselae* SA2 and *B. vinsonii* subsp. *berkhoffii* genotype II. Based upon the DNA sequences amplified from a blood sample and BAPGM enrichment blood culture sample, the oldest son was bacteremic with a *B. henselae* SA2 strain (99.2 % and 99.6 % homology with GenBank accession AF369529). The dog was seronegative to all *Bartonella* spp. antigens and no *Bartonella* DNA was amplified from blood or the BAPGM enrichment blood culture. Following BAPGM enrichment culture, no subculture isolates were obtained from any family member. When retested in November 2012, the youngest son was only seroreactive to the *B. henselae* SA2 strain and *Bartonella* sp. DNA was not amplified from blood, serum, or the BAPGM enrichment blood culture.

PCR testing of woodlice and woodlouse hunter spiders

Bartonella henselae SA2 DNA (97.0 % homology with GenBank accession AF369529) was amplified and sequenced from pooled woodlice (Table 2). Of the thirteen woodlouse spiders tested, *B. henselae* SA2 DNA (100 % and 99.3 % homology, GenBank accession AF369529) was amplified

and sequenced from two spiders and *B. vinsonii* subsp. *berkhoffii* genotype III DNA (98.6 % homology, GenBank accession DQ059765) from one spider. As the family had moved from the apartment in which the bites occurred into a house, all of the spiders and woodlice were collected approximately 3 miles from the original suspected spider bite location.

DISCUSSION

PCR amplification and sequencing of *B. henselae* SA2 DNA from two family members, woodlouse hunter spiders, and a woodlouse collected at least three years after family members were exposed to and the children were presumably bitten by similar spiders was unexpected. To the best of our knowledge, this is the first report of the presence of *Bartonella* spp. DNA in spiders or in woodlice. Although *B. henselae* DNA was amplified from two spiders collected 13 months apart, a woodlouse, and from serum, blood, and BAPGM enrichment culture samples from two family members, these results should be interpreted with caution, as it is not clear whether *Bartonella* was acquired at the time of the infestation and spider bites or whether the spiders and woodlice are accidental hosts for *Bartonella* spp. Since the woodlouse hunter spider is thought to feed exclusively on woodlice (a land-dwelling crustacean), the amplification of *Bartonella* DNA from spiders and woodlice suggests that the *B. henselae*-infected spiders fed on infected woodlice. Preliminary results (unpublished data) obtained in our laboratory indicates that washed woodlice can become PCR-positive for *B. henselae* after feeding on food contaminated with the bacteria. Although the length of time that *B. henselae* can remain viable within the environment has not been investigated to any degree, the bacteria remains viable in flea feces for several days. Whether bacteria ingested by woodlice remain viable, whether replication can occur, how long a *Bartonella* sp. can be retained within the isopod and whether a spider feeding on this crustacean can acquire or transmit *Bartonella* are subjects for future studies. Although the family experienced a flea infestation prior to moving into the new apartment, the family dog was not seroreactive to *Bartonella* sp. antigens and was PCR-negative in blood and BAPGM enrichment blood culture, making the dog and potentially fleas a less likely source of *B. henselae* transmission to family members. To date, *B. henselae* has not been reported in bats to the author's knowledge, no family member experienced a bat bite, and the bat exposure occurred several months before the onset of illness in the children and mother.

Table 2. *Bartonella* PCR from spiders and pooled woodlice

Spider #	Collection Date	PCR results[a]	Woodlouse #	Collection Date	PCR results[a]
1	9/28/2011	*Bh* SA2	1	9/8/2011	Neg
2		Neg	2		Neg
3		*Bvb* III	3		Neg
4		Neg	4		*Bh* SA2
5		Neg			
6	6/1/2012	Neg			
7		Neg			
8		Neg			
9	9/12/2012	Neg			
10		Neg			
11	10/8/2012	*Bh* SA2			
12		Neg			
13		Neg			

a: Denotes 16S-23S ITS DNA sequencing results;
Bh SA2 = *Bh* strain San Antonio; *Bvb* III = *Bartonella vinsonii* subsp. *berkhoffii* genotype III

Bartonella DNA has also been amplified from non-hematophagous arthropods, such as honey bees.[20] Those authors hypothesized that honey bees ingested or acquired *Bartonella* organisms

through environmental contact. In a recent report, a patient with neuroretinitis, a well-documented ocular pathology induced by *B. henselae*, was diagnosed with bartonellosis following the bite (sting) of a bull ant (genus *Myrmecia*) in Australia.[21] These authors suggested that *B. henselae* was probably transmitted to the patient via the stinger or mandibles, which provided a portal for bacterial entry into the skin. These recent publications indicate that physicians should routinely review a patient's medical history for arthropod exposure. Based upon recent clinical and research observations, there appears to be a growing spectrum of arthropods that might serve as vectors for *Bartonella* species, thereby emphasizing the critical importance of and the need for additional experimentally controlled vector competence studies. In addition, localizaton of *Bartonella* sp. replication within arthropods, further documentation of other potential animal reservoirs, and the determination of trans-ovarian transmission in various arthropod species represent other important issues that require scientific attention.

From a clinical perspective, the non-specific symptoms reported in the mother are consistent with previous reports of *Bartonella* sp. bacteremia in immunocompetent patients.[4,22] Although less well characterized, the behavioral and neurocognitive abnormalities that predominated in the older son have also been reported in *Bartonella*-bacteremic children.[14,23,24] Interestingly, and as reported in a small subset of patients in two case series, *B. henselae* DNA was only amplified from the mother's extracted serum samples, whereas *B. henselae* DNA was amplified from both blood and a BAPGM enrichment blood culture from the oldest son.[4,22] The reason(s) for these observations remain unclear, but one study has reported progressive increases in serum DNA concentration in association with prolonged sample storage times in certain pathologic conditions.[25] *Bartonella* DNA was never amplified from a negative control, and DNA from a *B. henselae* H1 strain (not *B. henselae* SA2 as found in this study) was used as a positive control for all PCR testing, therefore, laboratory contamination is an unlikely explanation for the PCR and DNA sequencing results reported in this study. Due to the fact that *B. henselae* induces a relapsing bacteremia in cats[26] and *B. birtlesii* induces a relapsing bacteremia in experimentally infected rodents[27], three blood samples obtained at approximately 2-day intervals were tested for each patient. For the mother and oldest son, only two dates yielded positive PCR results, potentially supporting the possibility of a relapsing pattern of *B. henselae* bacteremia in human patients. Also, as reported previously from our laboratory,[28] there was considerable variability in the mother's and youngest son's antibody titers when serum samples obtained within a one-week time frame were tested using an IFA technique. The mother had low antibody titers with up to fourfold variations in four of the six *Bartonella* spp. antigens over a one-week period. The youngest son had identical antibody titers to *B. henselae* strains H1 and SA2, but seemingly seroconverted to *B. vinsonii* subsp. *berkhoffii* genotypes I, II, III, and *B. koehlerae*. Administration of IVIG ten days prior to collection of the initial blood sample may well have influenced the youngest son's serological results, particularly if IVIG has antibacterial properties.[29] In contrast, the oldest son's antibody titers were identical for all six antigens at all three time points. In the context of antigenic specificity, he had antibodies to *B. henselae* SA2 strain, but not to a *B. henselae* H1 strain. All serum sample sets from each patient were tested at the same time, by the same experienced technician, using the same conjugate and IFA antigen slides. Whether these serological discrepancies are related to sample collection and storage issues, a prozone effect associated with excess antigen, IVIG, or other unknown factors requires additional investigation.

Similar to the initial diagnosis in the youngest son, GBS due to neurobartonellosis was diagnosed in a 10-year-old girl, who was hospitalized due to progressive leg weakness.[30] Seven days earlier, the girl had a self-limiting episode of fever and vomiting of one-day duration. Four days later, she had difficulty walking, became irritable, and complained of severe myalgia in the lower limbs.

Laboratory findings were not remarkable. Nerve conduction studies identified decreases in motor conduction velocity and amplitude, consistent with axonal damage. An exhaustive search for known causes of GBS was negative. The girl was treated with IVIG for 5 days, and within two weeks her neurological status had normalized. There was no history of cat scratches, no palpable lymphadenopathy, and no hepatic or splenic lesions on an abdominal ultrasound; however, because she lived in a rural area and played with kittens, *B. henselae* serology was requested. Her *B. henselae* IgG titer was 1:1,024 and a specific IgM titer was "positive", although a value was not reported. Her convalescent IgM titer was negative and the IgG antibody titer had decreased. To date, CIDP has not been associated with *Bartonella* infection. Although serology supported *Bartonella* exposure in the younger son, prior administration of IVIG complicates interpretation of his antibody titers and potentially his BAPGM enrichment culture PCR test results. It is possible that the source of *Bartonella* antibodies was the IVIG and that repeated immunoglobulin administration suppressed the level of bacteraemia below the level of successful PCR amplification. CIPD, also referred to as relapsing polyneuropathy, is a neurological disorder characterized by progressive weakness and impaired sensory function in the legs and arms. As was true in the boy in this report, CIPD is often diagnosed as the chronic counterpart of GBS. Prior infection or vaccination can precipitate GBS, and *Campylobacter jejuni* has become the most well-recognized antecedent infection.[31] Consideration should be given to *B. henselae* as an antecedent infection for GBS and CIPD. Physicians should pursue the medical history in these patients to determine if they have experienced animal bites or scratches or arthropod bites or stings.

As scientists, physicians, and veterinarians learn more about the medical importance of the genus *Bartonella*, there has been enhanced focus on known and suspected arthropod vectors. Because of their ability to reside within erythrocytes of a diverse number of mammalian hosts in conjunction with their diverse ecological niches, there is the potential opportunity for various *Bartonella* spp. to be transmitted by a variety of arthropod vectors. Several blood-feeding arthropods, *Lutzomyia verrucarum*, *Pediculus humanus humanus*, *Ctenocephalides felis,* and some rodent fleas (*Ctenophthalmus nobilis*) have been confirmed to be competent vectors for transmission of *Bartonella* species.[32] Tick transmission of *Bartonella* spp. has been a controversial subject in recent years;[33,34] however, vector competence for tick (*Ixodes ricinus*) transmission of a *Bartonella* sp. was recently demonstrated experimentally, thus supporting the possibility that *Ixodes* sp. ticks are transmitting *Bartonella* spp. throughout the Northern Hemisphere.[35]

Previous studies from Europe and North America have documented the presence of *B. henselae* DNA in *Ixodes ricinus,*[36] *Ixodes scapularis,*[37] and *Ixodes pacificus.*[38] In conclusion, it must be stressed that there is an important difference between the vector competence and vector potential of arthropods from which *Bartonella* spp. DNA is amplified. The amplification of *Bartonella* spp. DNA in the woodlouse hunter spiders in this study does not provide definitive proof of vector competence and may merely represent an accidental infection associated with ingestion of *Bartonella*-infected blood from an infected host (isopod). Although *B. henselae* was amplified and sequenced from woodlouse hunter spiders and from their associated prey, the woodlouse, definitively establishing the source of bacterial transmission to this family was not possible.

CONCLUSIONS

There appears to be a growing spectrum of arthropods that might serve as vectors for various *Bartonella* species. The location of *Bartonella* replication within arthropods, the documentation of other potential reservoirs, and the determination of trans-ovarian transmission in various arthropod species represent important public health issues that need to be resolved. As *B. henselae* SA2 DNA was amplified from woodlouse spiders and from a woodlouse collected nearly three years

after the reported bites, it is not clear if the *B. henselae* infections in this family were acquired by spider bites or whether spiders and woodlice were accidental hosts. Also, additional studies are needed to determine whether *B. henselae* bactermia can predispose patients to GBS, CIDP, and neurocognitive abnormalities.

CONSENT

Written informed consent was obtained from the patient for publication of this report and any accompanying images. The parents contacted the investigators to be entered into an ongoing IRB-approved research study and were full supportive of investigations described in this manuscript.

ABBREVIATIONS

GBS: Guillain-Barré syndrome; CIDP: Chronic Inflammatory Demyelinating Polyneuropathy; BAPGM: *Bartonella* Alpha-Proteobacteria Growth Medium; EDTA: Ethylene Diaminetetraacetic Acid; IPRL: Intracellular Pathogens Research Laboratory; EMG: Electromyelogram; IVIG: Intravenous Gammaglobulin; ADHD: Attention Deficit Hyperactivity Disorder; IFA: Immunofluorescence Antibody Assay.

COMPETING INTERESTS

In conjunction with Dr. Sushama Sontakke and North Carolina State University, Dr. Breitschwerdt holds U.S. Patent No. 7,115,385; Media and Methods for cultivation of microorganisms, which was issued October 3, 2006. He is the chief scientific officer for Galaxy Diagnostics, a company that provides diagnostic testing for the detection of *Bartonella* species infection in animals and human patients. Dr. Ricardo Maggi has lead research efforts to optimize the BAPGM platform and is the Scientific Technical Advisor for Galaxy Diagnostics. Dr. Robert Mozayeni was one of the attending physicians for the patients described in this study and has recently joined Galaxy Diagnostics as the chief medical officer. All other authors have no potential conflicts.

AUTHORS' CONTRIBUTIONS

PM and RM performed the BAPGM enrichment blood culture and PCR testing of the patient samples, woodlice, and spiders, performed DNA sequencing and alignments, and generated the first draft of the manuscript. SH is a pediatric neurologist who cared for the youngest son and drafted his case report. BRM is a rheumatologist who cared for the mother and oldest son and drafted their case reports. CT performed PCR testing of the woodlice and spiders. JB and BH assisted in sample acquisition and serological testing. EB coordinated various aspects of the investigation and helped to draft the final manuscript. All authors read and approved the manuscript.

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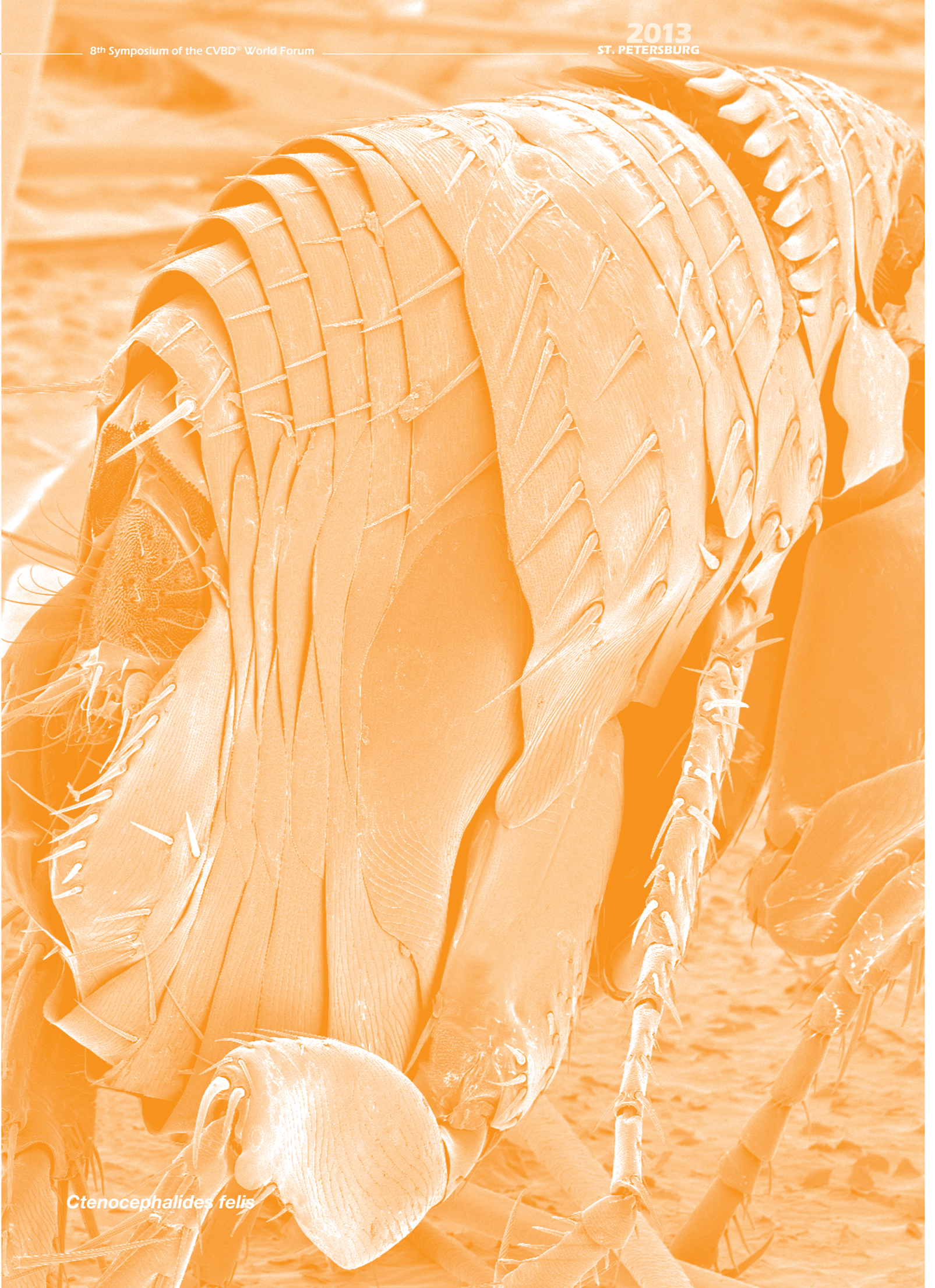

Ctenocephalides felis

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Co-infection with *Anaplasma platys*, *Bartonella henselae*, and '*Candidatus* Mycoplasma haematoparvum' in a veterinarian

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ABSTRACT

BACKGROUND: During a two-year period, a 27-year-old female veterinarian experienced migraine headaches, seizures, including status epilepticus, and other neurological and neurocognitive abnormalities. Prior to and during her illness, she had been actively involved in hospital-based work treating domestic animals, primarily cats and dogs, in Grenada and Ireland and anatomical research requiring the dissection of wild animals (including lions, giraffe, rabbits, mongoose, and other animals), mostly in South Africa. The woman reported contact with fleas, ticks, lice, biting flies, mosquitoes, spiders, and mites and had also been scratched or bitten by dogs, cats, birds, horses, reptiles, rabbits, and rodents. Prior diagnostic testing resulted in findings that were inconclusive or within normal reference ranges and no etiological diagnosis had been obtained to explain the patient's symptoms.

METHODS: PCR assays targeting *Anaplasma* spp. *Bartonella* spp. and hemotopic *Mycoplasma* spp. were used to test patient blood samples. PCR positive amplicons were sequenced directly and compared to GenBank sequences. In addition, *Bartonella* alpha Proteobacteria growth medium (BAPGM) enrichment blood culture was used to facilitate bacterial growth and *Bartonella* spp. serology was performed by indirect fluorescent antibody testing.

RESULTS: *Anaplasma platys*, *Bartonella henselae,* and '*Candidatus* Mycoplasma haematoparvum' DNA was amplified and sequenced from the woman's blood, serum or blood culture samples. Her serum was variably seroreactive to several *Bartonella* sp. antigens. Despite symptomatic improvement, six months of doxycycline most likely failed to eliminate the *B. henselae* infection, whereas *A. platys* and '*Candidatus* M. haematoparvum' DNA was no longer amplified from post-treatment samples.

CONCLUSIONS: As is typical of many veterinary professionals, this individual had frequent exposure to arthropod vectors and near daily contact with persistently bacteremic reservoir hosts, including cats, the primary reservoir host for *B. henselae,* and dogs, the presumed primary reservoir host for *A. platys* and '*Candidatus* M. haematoparvum'. Physicians caring for veterinarians should be aware of the occupational zoonotic risks associated with the daily activities of these animal health professionals.

KEYWORDS: *Bartonella*, *Mycoplasma*, *Anaplasma*, Headache, Migraines, Seizures, Serology, PCR

BACKGROUND

Many veterinary professionals (veterinarians, veterinary technicians, and veterinary support personnel) are occupationally exposed to a spectrum of domestic, production, and wild animals, a subset of which can be persistently infected with bacteria, protozoans, and viruses. In addition to extensive contact with infected animals and their biological fluids and tissues while performing clinical and necropsy examinations, veterinarians are frequently exposed to arthropod vectors, such as fleas, lice, and ticks that infest healthy, sick, and dead animals. Also, veterinarians involved in the care of production animals, wildlife or zoological collections have additional environmental exposure to biting flies, chiggers, mosquitoes, spiders, and other arthropods while working in terrestrial and marine coastal environments throughout the world. Although it is well recognized that approximately 60 % of all human pathogens and 75 % of emerging pathogens are zoonotic in nature,[1–3] little attention has been focused on the occupational risks associated with the daily professional activities of veterinarians.

Recently, infections with various *Bartonella* species, including *Bartonella henselae*,[4–6] *Bartonella koehlerae*,[6,7] *Bartonella vinsonii* subsp. *berkhoffii* genotypes I and II,[4,6,8,9] and '*Candidatus* Bartonella melophagi'[10] have been reported among veterinary professionals. Two studies have also supported potential needle stick transmission of *B. vinsonii* subsp. *berkhoffii* and *B. henselae* to veterinarians, respectively.[11,12] In a study in which many of the participants were veterinary professionals,[6] *Bartonella* spp. seroreactivity or bacteremia was documented in 49.5 % (n = 95) and 23.9 % (n = 46) of 192 patients, respectively; however, IFA antibodies were not detected in 30.4 % (n = 14) of bacteremic patients. Thus, seronegative *Bartonella* bacteremia is not an uncommon phenomenon. Co-infection with *B. henselae* and *Mycoplasma ovis* was also recently described in a veterinarian, who reported frequent bites or scratches from cats, dogs, rodent pocket pets, and an assortment of wild and zoo animals.[13] On numerous occasions, that veterinarian had traveled for professional activities to Central America and Colombia. Also, while working in Texas, he was exposed to sheep, goats, llamas, camels, and had frequent deer contact throughout his career. The exposure history and travel experiences reported by the Texas veterinarian and the veterinarian described in this report are typical of the experiences of many veterinary professionals working around the world throughout their careers.

Anaplasma platys, transmitted by the Brown Dog Tick (*Rhipicephalus sanguineus*), is an obligate intracellular rickettsial organism that infects platelets.[14,15] In 1978, this bacterium was first described in the southeastern United States as the cause of canine infectious cyclic thrombocytopenia. Historically, *A. platys* was thought to only infect dogs, however, a recent report from Brazil implicated *A. platys* infection in a cat.[16]

In 2004, Sykes and colleagues described a novel hemotropic *mycoplasma* in the blood of a splenectomized immunocompromised dog with haemic lymphoid neoplasia and proposed the name '*Candidatus* Mycoplasma haematoparvum'.[17] We now report the medical history for a sick veterinarian from Grenada, who was infected with *A. platys*, *B. henselae*, and '*Candidatus* Mycoplasma haematoparvum'.

CASE REPORT

Prior to the onset of her symptoms, a 27-year-old female veterinarian had been actively involved in hospital-based work treating domestic animals, primarily cats and dogs, and anatomical research dissecting wild animals (including lions, giraffe, rabbits, mongoose, and other animals). These activities had occurred in Grenada, Ireland, and South Africa. The woman reported contact with fleas, ticks, lice, biting flies, mosquitoes, spiders, and mites. She also reported scratches or

bites by dogs, cats, birds, horses, reptiles, rabbits, and rodents. Beginning in September 2010, headaches of approximately two weeks duration preceded a fainting episode, photophobia, generalized muscle fasciculations and the onset of tonic-clonic seizures. When hospitalized in South Africa, a Complete Blood Count (CBC) and serum chemistry profile were unremarkable and a contrast brain CT revealed no structural abnormalities or evidence of vascular damage. A few days later, encephalitis was diagnosed based on MRI lesions that were considered characteristic for Herpes simplex encephalitis. The patient was admitted to the Intensive Care Unit (ICU), and over the next 10 days was maintained on a sedative, lorazepam (Ativan), anticonvulsants including sodium valproate (Epilim) and phenytoin sodium (Dilantin) which was stopped after an adverse reaction, and pain medications including tramadol hydrochloride (Ultram ER) and paracetamol (Perfalgan), as required. Amoxicillin, cephazolin and doxycycline were also administered for 2 weeks. A 24-hour electroencephalogram (EEG) revealed no electrical abnormalities and CSF analysis was not indicative of inflammation or infection. Electron microscopy of CSF revealed protein fibrils suspected to be associated with "tick-bite fever". Due to the potential of a transmissible infectious agent, the National Institute of Communicable Diseases (NICD) in South Africa tested for Ebola hemorrhagic fever (Ebolavirus), Lyme disease (*Borrelia burgdorferi*), spotted fever group rickettsioses (*Rickettsia africae* and *Rickettsia conorii*), Rift Valley Fever (Phlebovirus), Equine Viral Encephalitis (Arbovirus), African horse sickness (Orbivirus), and Rabies (Lyssavirus), all of which were negative. The patient was also treated presumptively because of potential *Streptococcus suis* exposure, as she had case contact a few days prior to hospitalization with a piglet that had cerebral signs and a positive brain culture. When discharged from ICU to the ward, she was treated with sodium valproate and carbamazepine (Tegretol). One month post discharge, the patient continued to experience tremors, mild seizures, ataxia and memory loss and had left-sided weakness. An analgesic containing paracetamol, codeine phosphate, caffeine, and meprobmate (STILPNEW Capsules) was added to the treatment regimen. During the next two weeks, seizure frequency, severity, and duration increased, with seizures becoming more violent and lasting at least 10 minutes. When referred to a neurologist, the patient was monitored on the neurology ward using a three-day camera linked to an EEG, which documented seizures occurring four to seven times a day. As seizures accompanied ringing noises (drip alarms, jiggling bottles on trolleys, etc.), anticonvulsants were discontinued on the premise that seizures were induced by sound hypersensitivity and were potentially being accentuated by the medications. Sertraline hydrochloride, a selective serotonin reuptake inhibitor (SSRI) was administered for noise hypersensitivity, post-encephalitic depression, disorientation, and irritability. Results of repeat CBC, serum chemistry panel, EEG and MRI were negative or within reference ranges. The neurologist concluded that the lesion(s) inducing the seizure focus was/were microscopic and deeper than detectable by EEG. While hospitalized, a psychologist recommended further personal counseling, as he felt that the patient needed to come to terms with post-encephalitic seizures and inability to be active and to live the life she experienced before becoming ill. Before the onset of symptoms, the woman was actively involved in windsurfing, diving, surfing, running, and sailing and had completed the Dublin marathon less than a year before the onset of her illness.

One month later, headaches continued, migraines were diagnosed, and vertigo had developed especially when in a moving vehicle, however, the left-sided weakness had improved and repeat memory testing documented substantial improvement in short-term memory with residual deficits in math skills. Amitriptyline (Elavil) and clonazepam (Rivotril) were administered for treatment of the migraines and anxiety, respectively. By four months after the onset of illness, the patient associated her seizures with loud noises, bright light, or long car rides, all of which she attempted to avoid. One month later, she was again hospitalized due to severe migraines, inability to enunciate words, and left-sided weakness. A CBC, serum chemistry panel, and a CT scan were negative or within reference ranges. Sodium valproate was restarted after consultation with a second neurologist. A

combination drug containing tramadol hydrochloride and acetaminophen (Tramacet) were added to the treatment regimen for the migraines. When discharged five days later, amitriptyline, sodium valproate, sertraline hydrochloride, and Tramacet were continued until August 2011, at which time the patient elected to discontinue medications against medical advice. One week later, she was hospitalized for debilitating migraines, which persisted for seven days despite administration of multiple medications. Again, there were no MRI abnormalities. She was discharged by her neurologist with instructions to take clobazam (Urbanol), agomelatine (Valdoxin), and amitriptyline for depression and management of the noise hypersensitivity, Tramadol for headaches, and topiramate 100 mg bid (Topamax) as an anticonvulsant, and lorazepam (Ativan SL) for emergency seizure control. Between September 2011 and January 2012, these medications were continued and the patient experienced remarkable improvement, with decreased sensitivity to flashing lights, increased tolerance to loud noises, increased energy and improved mental capacities. However, in November 2011, she was admitted to the emergency room following the development of joint pain, particularly severe in the knees, left wrist, right elbow, and lower back. Standard view radiographs of her left wrist, which was the most severely affected source of pain, revealed no structural abnormalities, CBC values were within reference ranges and an ANA panel, uric acid level and Rheumatoid Factor assay were negative. Initial treatment included intravenously administered dexamethasone, followed by oral steroid maintenance therapy for five days, and Etoricoxib (Arcoxia) 90 mg bid for two weeks. Joint and back pain abated after two weeks.

While in transit from South Africa to Grenada in December 2011, the patient had a seizure in JFK airport in New York, after which she continued to experience severe tonic-clonic seizures while in Grenada. When examined by a neurologist on January 6, 2012, there was a mildly abnormal EEG pattern characterized by bilateral sharp activity, and interpreted as consistent with cerebral irritability in a patient receiving anticonvulsant therapy. Medications now included topiramate (200 mg bid), lamotragine (Lamictal), clonazepam, amitriptyline, agomelatine (Valdoxane), and Tramadol as needed. Over the next two months, the seizures decreased in frequency and severity. Concurrently, the woman's migraines increased in frequency and duration, often lasting 5 days. On March 1, the patient was airlifted from Grenada to Trinidad in status epilepticus. She remained hospitalized until March 8, during which time therapy targeted seizures and migraines, but would not have addressed an underlying infection. After discharge, the migraines continued, requiring periodic administration of buprenorphine (Temgesic), which rapidly stopped the migraine within minutes of administration.

Twenty months after the onset of the patient's illness, one of the authors visited St George's University to participate in a research collaboration involving canine ehrlichiosis, which is a highly endemic canine tick-borne disease on the island of Grenada. After reciting her medical history during a casual conversation, the woman elected to enter an ongoing study regarding *Bartonella* spp. infection in high-risk patients, i.e., veterinary professionals (North Carolina State University Institutional Review Board approval IRB 1960-11). Written permission was given to also test for other vector-borne organisms.

METHODS

Serology

For this study, all serum samples were tested by IFA assays using a panel of *Bartonella* antigens. Briefly, antibody responses to *Bartonella henselae* strain Houston 1, *B. henselae* strain San Antonio 2, *B. vinsonii* subspecies *berkhoffii* genotype I, *B. vinsonii* subspecies *berkhoffii* genotype II , *B. vinsonii* subspecies *berkhoffii* genotype III, and *B. koehlerae* were tested by IFA as previously described.[4–6] Seropositive samples were defined as having endpoint titers ≥ 1:64 using a twofold scale of 1:16 – 1:8192.

Molecular testing

Bartonella testing was performed using the BAPGM platform, as previously described.[4–8] The BAPGM platform incorporates 4 separate PCR testing time points, each representing a different component of the testing process for each patient sample: 1) and 2) PCR amplification of *Bartonella* spp. following DNA extraction from whole blood and from serum; 3) PCR following BAPGM enrichment of whole blood culture incubated for 7 and 14 days; and 4) PCR from subculture isolates if obtained after subinoculation from the BAPGM flask onto plates containing trypticase soy agar with 10 % sheep whole blood that are incubated for 4 weeks. PCR specimen preparation, DNA extraction, and PCR amplification and analysis were performed in three separate rooms with unidirectional work flow to avoid DNA contamination. In addition, BAPGM cultures were processed in a biosafety cabinet with Hepa filtration in a limited access Biosafety Level II laboratory. PCR-negative controls were prepared using 5 µL of DNA from the blood of a healthy dog, and *B. henselae* (Houston 1 strain) at a concentration of 1 genome copy/µL was used as a PCR-positive control during the entire course of this study. To assess for potential contamination during blood sample processing into BAPGM, an un-inoculated BAPGM culture flask was processed simultaneously and in an identical manner with each batch of patient blood and serum samples tested. In addition, molecular testing aiming at amplify *Anaplasma* (16SrRNA and GroEl genes), *Babesia* (18SrRNA), *Ehrlichia* (16SrRNA and GroEl genes), and hemotropic *Mycoplasma* (16SrRNA and RNaseP genes) was performed on DNA extracted from blood and serums samples using primers as previously described.[18–21]

RESULTS AND DISCUSSION

In April 2012, whole blood and serum sample sets were obtained on Monday, Wednesday, Friday, and the following Monday and shipped overnight express to the North Carolina State University, College of Veterinary Medicine, Intracellular Pathogens Research Laboratory (NCSU-CVM-IPRL), for *Bartonella* spp. serology and inoculation into *Bartonella* alpha-Proteobacteria growth medium (BAPGM).

The patient was seroreactive to *B. vinsonii* subsp. *berkhoffii* genotype II (titer 1:256) and *B. henselae* antigens (1:64), but was not seroreactive to *B. vinsonii* subsp. *berkhoffii* genotypes I and III or to *B. koehlerae* antigens at the lowest testing dilution of 1:16. *Bartonella* spp. DNA was not amplified from four blood, four serum, or six enrichment blood culture samples obtained at 7 and 14 days post-incubation and no subculture isolates on blood agar were obtained. For all components of the BAPGM platform (PCR from blood, serum, enrichment cultures at 7 and 14 days, and subcultures), PCR-negative controls remained negative throughout the course of the study. In addition, subcultures of un-inoculated BAPGM medium (culture control) at 7 and 14 days did not yield bacterial growth.

In contrast, by targeting a conserved region of the 16S rRNA gene, *A. platys* DNA was PCR-amplified and sequenced from all four serum and 2 of 4 blood-extracted DNA samples (**Table 1**). The six amplified sequences were identical to each other and had 99.7 % (350/351 bp) homology with *A. platys* (M82801) deposited in GenBank. Similarly, amplification of the GroEL gene generated a 450 bp product that was 98.3 % similar to *A. platys* GenBank AY008300, and 98.9 % (444/446) similar to *A. platys* GenBank accessions AF478129, and AF399916. In addition, '*Candidatus* M. haematoparvum' DNA was amplified and sequenced from two of the patient's serum samples using primers targeting a conserved region of the 16S rRNA and the RNaseP genes.[21] The 16S rRNA gene sequences were 99.8 % (400/401 bp) similar to GenBank accession GQ129113 and the RNAseP sequences were 100 % (128/128 bp) similar to GenBank accession AY380803 of '*Candidatus* M. haematoparvum', respectively. As the authors were unable to identify a research or commercial laboratory that could provide *A. platys* IFA antigen slides, *A. platys* serology was not possible. Also,

Table 1. Chronological PCR and *Bartonella* spp. serology results for a veterinarian infected with *Anaplasma platys*, '*Candidatus* Mycoplasma haematoparvum', and *Bartonella henselae*

Sample date	Sample	PCR						Serology					
		Bartonella			*Anaplasma*		Hemot. *Mycoplasma*	*Bvb* I	*Bvb* II	*Bvb* III	*Bh*	*Bh* SA2	*Bk*
		ITS			GEP	GroEl	16S rDNA and RNaseP	IFA reciprocal titers					
		Original	BAPGM										
			7 d	14 d									
4/16/2012	Serum	Neg			***A. platys***	***A. platys***	Neg	< 16	256	< 16	64	< 16	< 16
	Blood	Neg	Neg	Neg	Neg	Neg	**M. haematoparvum**						
4/18/2012	Serum	Neg			***A. platys***	Neg	Neg						
	Blood	Neg	Neg	Neg	***A. platys***	Neg	Neg						
4/20/2012	Serum	Neg			***A. platys***	***A. platys***	**M. haematoparvum**						
	Blood	Neg	Neg	Neg	Neg	Neg	Neg						
4/23/2012	Serum	Neg			***A. platys***	Neg	Neg						
	Blood	Neg	Neg	Neg	***A. platys***	Neg	Neg						
5/7/2012	Serum	Neg			***A. platys***	***A. platys***	***A. platys***	< 16	64	32	< 16	128	64
	Blood	Neg	Neg	Neg	Neg	***A. platys***	***A. platys***						
5/9/2012	Serum	Neg			Neg	Neg	Neg						
	Blood	Neg	Neg	Neg	Neg	Neg	Neg						
5/11/2012	Serum	Neg			Neg	Neg	Neg						
	Blood	Neg	Neg	Neg	Neg	Neg	Neg						
5/14/202	Serum	Neg			***A. platys***	Neg	Neg						
	Blood	Neg	Neg	***Bh*SA2**	Neg	Neg	Neg						
12/10/2012*	Serum	Neg			Neg	Neg	Neg	< 16	256	128	128	128	< 16
	Blood	Neg	Neg	Neg	Neg	Neg	Neg						
12/12/2012*	Serum	Neg			Neg	Neg	Neg						
	Blood	***Bh*SA2**	Neg	Neg	Neg	Neg	Neg						
12/14/2012*	Serum	Neg			Neg	Neg	Neg						
	Blood	Neg	Neg	Neg	Neg	Neg	Neg						

Bh SA2 = *Bartonella henselae* San Antonio 2 strain; *Bvb* = *Bartonella vinsonii* subsp. *berkhoffii*; *Bk* = *Bartonella koehlerae*; Hemot. = hemotropic; N/D = not determined; * = post treatment; Neg = negative

as cell-wall-deficient hemotropic *Mycoplasma* species have not been isolated to date, hemoplasma serology was not performed. *Babesia* and *Ehrlichia* genus PCR assays did not result in DNA amplification.

When the *A. platys* and '*Candidatus* M. haematoparvum' PCR results became available, an additional set of aseptically obtained blood and serum samples were submitted for repeat *Anaplasma*, *Bartonella*, and hemotropic *Mycoplasma* spp. testing. Four sample sets collected between May 5 and May 14, 2012 were shipped to the NCSU-CVM-IPRL by overnight express carrier. The patient was again seroreactive to *Bartonella* spp. antigens by IFA testing (Table 1). *Bartonella henselae* (SA2 strain type) DNA was amplified and sequenced from a 14-day BAPGM enrichment blood culture. *Bartonella* ITS PCR was negative for 4 blood, 4 serum, 4 seven-day enrichment blood cultures and 3 of 4 14-day enrichment blood cultures. No subculture agar plate isolates were obtained at any testing time points (April and May 2012). *Anaplasma platys* DNA was again successfully amplified

and sequenced from two of the patient's four serum samples. The *A. platys* DNA sequences were identical to the sequences derived from the April blood and serum samples. '*Candidatus* M. haematoparvum' DNA was not amplified from the May blood or serum samples.

Following NCSU-CVM-IPRL confirmation of infection with *A. platys*, '*Candidatus* M. haematoparvum', and *B. henselae,* the patient returned to South Africa before initiating antimicrobial treatment on July 18, 2012. Based on the longevity of her illness, the attending physician requested a standard echocardiogram, CBC, C-reactive protein and Lyme serology (negative) be repeated. The only hematological abnormality was a mild increase in C-reactive protein. When the echocardiogram revealed slight thickening of the mitral valve, a trans-esophageal echocardiogram was obtained under deep sedation. Thickening of the mitral valve was attributed to age-related myxoedamatous degeneration. Treatment was initiated with doxycycline (100 mg bid) for 6 months. Concurrent administration of azithromycin or rifampin was not attempted due to concerns that these antibiotics could interfere with the anticonvulsant medications, resulting in patient destabilization. During the first week of doxycycline administration, the patient experienced several days in which severe tonic-clonic seizures of a few minutes duration occurred up to three times per day. Seizures were followed by disorientation and severe migraines, with the latter only responsive to buprenorfine (Temgesic SL). Following a week of doxycycline treatment, the patient reported less frequent seizures, more clarity in her thoughts. Also, the historical lethargy, which had been a constant symptom since contracting encephalitis in September 2010, had substantially resolved.

Approximately one month before commencing antibiotic treatment, the patient injured her right wrist during a seizure. After a series of radiographs and an MRI scan, a scapholunate ligament tear was confirmed. The MRI also identified minute osteolytic lesions involving the joint surfaces of numerous bones within the wrist along with generalized osteopenia. The scapholunate ligament tear required surgical correction. Within two weeks of surgery, the patient developed complex regional pain syndrome (CRPS), requiring a follow-up bone scintigraphic scan in August 2012 that identified increased uptake in the scaphoid, lunate and pisiform bones of the right wrist and the periarticular joints distal to the right wrist. There was also moderate to intense linear uptake in the right distal ulna. A consulting orthopedic surgeon suggested that the patient's osteolytic lesions might be similar to lesions reported in immune-compromised patients with *Bartonella* infections.

In December 2012, after 6 months of doxycycline therapy, three aseptically obtained whole blood and serum sample sets were collected in Grenada and shipped overnight express to the NCSU-CVM-IPRL, for *A. platys* PCR, *Bartonella* sp. serology, BAPGM enrichment blood culture/PCR, and '*Candidatus* M. haematoparvum' PCR, as described above. The patient was seroreactive to *B. vinsonii* subsp. *berkhoffii* genotypes II and III (titers 1:256 and 1:128, respectively) and *B. henselae* antigens (1: 128), but was not seroreactive to *B. vinsonii* subsp. *berkhoffii* genotype I or to *B. koehlerae* antigens at the lowest testing dilution of 1:16. *A. platys* and '*Candidatus* M. haematoparvum' DNA was not amplified by the respective PCR assays. *B. henselae* SA2 DNA was amplified and sequenced from one of three blood sample sets, suggesting that the *B. henselae* infection may not have been eliminated by the doxycycline therapy. As *Bartonella* spp. DNA was not amplified from BAPGM enrichment blood cultures, the presence of viable bacteria was not documented.

Clinically, following the six months of doxycycline therapy, the patient was more alert, enjoyed a more active life-style and cognition had greatly improved. However, following periods of overexertion, she continues to develop lethargy, followed by severe migraines, which require treatment with analgesics or bed rest. The patient is being transitioned off of the anti-epileptic medications.

Beginning in November 2011, because the patient was experiencing severe insomnia, lamotrogine was tapered and completely withdrawn without an increase in seizure frequency. Topiramate is being tapered gradually until a lowest effective dose is found, or the drug can be withdrawn completely. The patient was advised that the amitriptyline can be stopped once the frequency and intensity of the migraines decrease, whereas valdoxane will be continued until the patient is less sensitive to noise. If topiramate can be tapered, the treatment plan is to add rifampin and continue doxycycyline.

Documentation of co-infection with three vector-borne organisms in the same patient, two of which (*A. platys* and '*Candidatus* M. haematoparvum') have not been described in association with human blood-borne infections, represents a medically important observation derived from sequential testing of blood samples provided by this veterinarian. As is typical of many veterinary professionals, this woman had frequent exposure to arthropod vectors and near daily contact with persistently bacteremic reservoir hosts, including cats, the primary reservoir host for *B. henselae*, and dogs, the primary reservoir host for *A. platys* [22,23] and '*Candidatus* M. haematoparvum'.[21] Based upon serological evidence, this veterinarian may have been exposed to both *B. henselae* and *B. vinsonii* subsp. *berkhoffii*. A previous study from Grenada documented a 19.2 % *A. platys* PCR prevalence and an 8.2 % *B. vinsonii* subsp. *berkhoffii* seroprevalence among dogs.[24] Those dogs were not tested serologically for *B. henselae* exposure, nor was BAPGM enrichment blood culture/PCR, which increases molecular diagnostic sensitivity,[25,26] performed in that study, as was done with blood and serum specimens from this patient. It is obvious from the results summarized in **Table 1** that consistent PCR amplification of each of these three organisms from blood, serum, or enrichment blood culture samples represents an ongoing challenge for molecular diagnostic laboratories. Presumably, the patient's *B. henselae* infection was missed when she was first tested in April 2012, and only one of three BAPGM enrichment cultures documented viable *B. henselae* infection when retested one month later and only after a 14-day BAPGM incubation period. Recently, we reported a statistical increase in the molecular detection or isolation of *Bartonella* spp. when three blood sample sets were tested from a one-week collection period, as compared to testing a single blood sample.[27] *Bartonella* detection in blood by PCR and/or following enrichment blood culture remains difficult to achieve, due to the potential for very low numbers of bacteria in the patient's blood at the time of sample collection and because of the suspected relapsing nature of the bacteremia in immunocompetent individuals.[27] As *B. henselae* SA2 strain DNA was again sequenced from a single blood sample obtained in December 2012, after six months of doxycycline therapy, it seems likely that the woman remained infected, although it is possible that the amplified DNA in September was from dead or nonviable bacteria, whereas growth in enrichment culture in May would reflect the presence of viable blood-borne bacteria. Treatment failure seems more likely in this patient, as doxycycline alone is not a consistently effective antibiotic for the elimination of *B. henselae* bacteremia and bacterial DNA does not persist for months in the blood, after the infection has been eliminated.[13] An ongoing limitation of the BAPGM enrichment blood culture platform is the failure to obtain isolates from most patients following subculture at 7 and 14 days of incubation.[6]

In addition to treating companion animals and wildlife on three different continents, this veterinarian had the added risk of performing frequent wildlife necropsies in Grenada and South Africa, including lions and mongoose (NCSU-CVM-IPRL, unpublished data) that could be a source of *B. henselae* exposure. Although NCSU-CVM-IPRL research testing efforts focused on vector-borne organisms of veterinary medical importance, it is possible that this woman was exposed to or infected with other pathogens that contributed to or influenced her clinical course of illness prior to or during the course of this investigation. Therefore, correlation of patient symptoms and di-

sease findings with the microbiological findings reported as a component of this study should be interpreted with caution. However, in association with improved diagnostic testing modalities, co-infections with more than one vector-borne pathogen are being reported frequently in dogs and occasionally in human patients. As cats and dogs are more often exposed to fleas, ticks, and other vectors as compared to their human counterparts, co-infections are commonly reported to occur in pet and working dogs with frequent flea and tick infestations. [28–31] These clinical observations have prompted veterinary researchers to study *A. platys* and *E. canis* co-infections in dogs experimentally.[32] Among other examples in the human medical literature, co-infection with *Ehrlichia chaffeensis* and a spotted fever group *Rickettsia* was reported in a 44-year-old man.[33] Of medical importance to physicians and veterinarians, co-infection with organisms that can potentially persist for months to years complicates a patient's clinical presentation, can substantially influence the historical progression of illness, and can make laboratory diagnosis much more challenging than an acute infection or an infection by a single pathogen. Also, in selected patients, co-infections can influence the choice of therapeutic agents, for example when a patient is infected with a bacterium and a protozoa.[34] Whenever possible, PCR amplification with DNA sequence confirmation, as was used in this study, should be the basis for diagnosis of a co-infection. Because microbial specific genes are targeted in well-designed PCR assays, this increasing useful diagnostic approach is applicable to both human and veterinary patient populations. In this study, all PCR amplicons were sequenced to assure specificity. Bacteremia with the two novel organisms for human infection (*A. platys* and '*Candidatus* M. haematoparvum') was confirmed by targeting two different genes, whereas DNA sequencing of the highly variable *Bartonella* 16S-23S ITS region was used to confirm infection with a SA2 strain of *B. henselae*.

During the two-year period prior to documentation of *A. platys*, *B. henselae,* and '*Candidatus* M. haematoparvum' infection in this patient, extensive diagnostic testing was pursued in conjunction with efforts to define the cause of the headaches, seizures, and other neurological and neurocognitive abnormalities. Unfortunately, those tests proved to be normal, negative, or inconclusive in the context of identifying an etiological diagnosis. The lack of fever, in conjunction with normal hematological, serum biochemical and cerebrospinal fluid findings, and several normal MRI examinations after the initial post-encephalitic MRI diagnosis argued against an ongoing infectious cause of the neurological symptoms in this patient. During the patient's initial illness, the first consulted neurologist was convinced that the MRI lesions were residual from an unusually virulent case of African tick-bite fever. He also believed that the patient would respond to the standard two weeks of doxycycline used to treat *Rickettsia conorii*, *Rickettsia africae,* and *Coxiella burnetii* infections in South Africa. Unfortunately, this treatment did not prove to be sufficient.

In veterinary medicine, *A. platys* and hemotropic *Mycoplasma* sp. are considered pathogens of low virulence, often documented in association with other infections or other diseases. Most dogs infected with *A. platys* are healthy, but experience a cyclic thrombocytopenia; however, strain variation in pathogenicity has been proposed due to more severe disease attributed to *A. platys* infections in dogs in Europe.[14,15] Despite development of thrombocytopenia, dogs experimentally infected with *A. platys* remained healthy throughout a study.[32] Currently, there is also minimal evidence to support an important pathogenic role for hemotropic *Mycoplasma* species infecting dogs[17,21] or people.[13] Hemotropic *Mycoplamsa* infections that are accompanied by disease manifestations occur most often in nutritionally deprived or immunologically compromised animals, such as retroviral infected cats. Infection with *Mycoplasma haemofelis* was reported in an HIV-positive human from Brazil.[35]

Recently, veterinary professionals have been identified as a high-risk group for *Bartonella* spp. bacteremia.[6] Based upon repeat testing, there was serological and BAPGM enrichment blood culture PCR evidence to support *B. henselae* infection in this veterinarian. Although the pathophysiological mechanisms remain essentially unstudied, headaches, seizures, and other neurological signs have been reported in patients with *Bartonella* spp. bacteremia.[4–8] Similar to the patient in this report, there is often no history of fever or hematological, biochemical, or cerebrospinal fluid indicators of infection in patients with neurobartonellosis.[5,8,36] In addition, co-infection with *B. henselae* and *Mycoplasma ovis*, a hemotropic *Mycoplasma* sp. that infects sheep, has been reported in a veterinarian with long-standing neurological disease.[13] Of the three organisms infecting this patient, *B. henselae* alone, or in combination with the two other intravascular bacteria may have contributed to the headaches, neurocognitive abnormalities, and seizures reported in this patient. The osteolytic bone lesions documented in this patient just prior to initiation of antibiotics are also consistent with lesions that have been increasingly described in immunocompromised HIV-infected patients and in children with cat scratch disease.[37,38] Despite the use of different combinations of anticonvulsant and antidepressant medications, effective control of the headaches and seizures was never achieved until treatment with doxycycline was instituted. Unfortunately, serology and PCR results following 6 months of antibiotic therapy supported the possibility of ongoing *B. henselae* infection.

Nearly two decades ago, investigators in Venezuela described inclusions in human platelets ultrastructurally consistent with *A. platys*.[39,40] As those observations predated the use of PCR amplification and DNA sequencing, confirmation that the platelet inclusions were in fact *A. platys* was not possible and no subsequent report of human *A. platys* infection has been published in the English literature. Although vector competence has not been proven, there is substantial epidemiological support for *R. sanguineus* as the vector and the dog as the primary reservoir host for *A. platys*[29] and potentially *M. haematoparvum*[21] and *B. vinsonii* subsp. *berkhoffii*.[41] Dogs throughout tropical and subtropical regions of the world are frequently infested with *R. sanguineus*, commonly referred to as the "Brown Dog Tick" or "kennel tick" because all three life stages (larvae, nymph, and adult) prefer to feed on dogs, and these ticks are frequently found in kennel environments, veterinary hospitals, and homes in tropical and subtropical regions of the world. Grenada, located approximately 100 miles from Venezuela, shares very similar rural and urban ecosystems, each of which supports frequent and severe *R. sanguineus* infestations in dogs. In fact, *R. sanguineus* is the only tick known to infest dogs on the island of Grenada and is a commonly encountered tick on dogs in South Africa. Although an important vector for transmission of *Babesia canis* and *Ehrlichia canis* throughout the world, historically, the human medical importance of this tick has been underappreciated. Recently, *R. sanguineus* has been implicated in the transmission of *Rickettsia rickettsii* on Indian reservations in Arizona, resulting in fatal cases of Rocky Mountain spotted fever.[42] *R. sanguineus* is also the vector for *Rickettsia conorii*, the cause of Mediterranean spotted fever in dogs and people in southern Europe and northern Africa. Recently, persistent *R. conorii* bacteremia has been demonstrated experimentally in dogs infected by tick (*R. sanguineus*) attachment.[43] Although the timing and mode(s) of infection for this patient will remain unknown, the importance of *R. sanguineus* as a source of *A. platys* and '*Candidatus* M. haematoparvum' for dogs and people deserves additional research consideration. Similarly, fleas, the primary vector for transmission of *B. henselae* and likely other *Bartonella* spp. among cats and dogs, are currently underappreciated as a source of zoonotic bartonellosis among animals and human patients.[44]

CONCLUSION

As is typical of many veterinary professionals, this individual had frequent exposure to arthropod vectors and near daily contact with persistently bacteremic reservoir hosts, including cats, the primary reservoir host for *B. henselae*, and dogs, the presumed primary reservoir host for *A. platys* and '*Candidatus* Mycoplasma haematoparvum'. Due to frequent contact with ticks and fleas, and the animals that harbor intravascular vector-borne pathogens for months to years, veterinary professionals should use personal protective measures, such as gloves, hand washing, and optimal restraint to avoid bites and scratches. Rapid kill spray products should be used routinely to eliminate fleas and ticks from animals that are being examined at necropsy or cared for by veterinary professionals.

More importantly, physicians caring for veterinary professionals should be aware of the occupational zoonotic risks associated with the daily activities of animal health professionals.

CONSENT

Written informed consent was obtained from the patient for publication of this report and any accompanying images.

ABBREVIATIONS

NICD: National Institute of Communicable Diseases; CRPS: Complex Regional Pain Syndrome; BAPGM: *Bartonella* Alpha-Proteobacteria Growth Medium; NCSU-CVM-IPRL: North Carolina State University College of Veterinary Medicine, IPRL: Intracellular Pathogens Research Laboratory.

COMPETING INTERESTS

In conjunction with Dr. Sushama Sontakke and North Carolina State University, Dr. Breitschwerdt holds U.S. Patent No. 7,115,385; Media and Methods for Cultivation of Microorganisms, which was issued October 3, 2006. He is the chief scientific officer for Galaxy Diagnostics, a company that provides diagnostic testing for the detection of *Bartonella* species infection in animals and human patients. Dr. Ricardo Maggi has lead research efforts to optimize the BAPGM platform and is the Scientific Technical Advisor for Galaxy Diagnostics. All other authors have no potential competing interest.

AUTHORS' CONTRIBUTIONS

RM and PM performed the BAPGM enrichment blood culture, PCR testing of the patient's samples, DNA sequencing and alignments, and helped generate the first draft of the manuscript. LH and VN assisted in sample acquisition, provided medical information and helped to write the case history. EB coordinated various aspects of the investigation and helped to draft the final manuscript. All authors read and approved the manuscript.

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4 HIGHLIGHTS ON FILARIOIDS

8th CVBD WORLD FORUM Symposium

Heartworm resistance to macrocyclic lactones in the United States – a brief look

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ABSTRACT

At the beginning of 2011, a paper appeared in the Journal of Veterinary Internal Medicine reporting that one dog in each of two groups of animals treated one month after challenge with an isolate of heartworms designated as MP3 were found infected when examined after the worms had had time to develop to adulthood. A second paper that year revealed that using this same isolate, a product combining milbemycin oxime and spinosad required three monthly treatments after infection to produce groups of dogs that were all protected against heartworm infection by MP3.

During product development with a stand-alone ivermectin product, Bayer had also done research where they could not protect all dogs from infection with this isolate using a single treatment at the standard 6–12 microgram per kilogram dose nor at a dose one-and-one-half times higher. This led to a trial where this same MP3 isolate was used to infect dogs that were then treated with selamectin, ivermectin plus pyrantel pamoate, milbemycin oxime, or topical moxidectin plus imidacloprid. In this trial, only the moxidectin-treated dogs were protected, while 7 out of 8 dogs in each of the other three groups developed infections with adult worms (maximum numbers in each group were 9, 7, and 6, respectively).

Other work reported on a dog imported into Canada from Louisiana, USA, that remained microfilaremic for two years after two treatments with melarsomine dihydrochloride, multiple bi-weekly treatments with preventive, treatment with ivermectin at 200 micrograms per kilogram, and two weeks where it received milbemycin oxime at 4 times the recommended minimum preventive monthly dose daily for each of two weeks. There are now additional isolates that have been collected with microfilariae that are resistant to macrocyclic lactones in various *in-vitro* assays, and worms are now being routinely monitored for resistance markers by groups in Canada and the United States. There are also rumors that there is another MP3-like isolate that has now been involved with testing that was acquired as was MP3 from a random-source dog in the southern United States that had no apparent history of drug pressure prior to microfilarial collection for growth in mosquitoes for propagation. It is expected that there will be a lot more information on this topic being brought forward in the near future.

On some neglected filarioids of dogs

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ABSTRACT

Among the most studied parasites of dogs, *Dirofilaria immitis* and *Dirofilaria repens* (Spirurida, Onchocercidae), causing cardiopulmonary and subcutaneous filarioses, respectively, are characterized by blood-circulating microfilariae and are regarded as causes of zoonoses in humans.

Aside of these two filarioids, dogs may be parasitized by other less known species (e.g., *Onchocerca lupi* and *Cercopithifilaria* spp.), whose larvae reside in subcutaneous tissues. In particular, recent studies on nematodes parasitizing dogs in Europe revealed that they are infested by at least three species belonging to the genus *Cercopithifilaria*. *Cercopithifilaria grassii* was described from a dog in Rome (Italy) and was characterized by long microfilariae (i.e., 650 µm; defined as "gigantesche"; from Italian, giant). This nematode was transferred to the genus *Cercopithifilaria* in 1982 but remained ignored until two occasional reports in ticks from dogs in Switzerland and in northern Italy.

At the meantime, in a very different locality of southeastern Brazil, *C. grassii* was reported in a dog. Two years later, the same authors, following a careful examination of the same material, described a new species, *Cercopithifilaria bainae*, which was characterized by short microfilariae (about 180 µm). Near 30 years later, microfilariae of a *Cercopithifilaria* sp. (referred to as *Cercopithifilaria* sp. I) were collected by chance from a dog in Sicily (Italy), being morphologically described and molecularly characterized. The microfilariae of *Cercopithifilaria* sp. I correspond well by their body length (185.18 µm) to those of *C. bainae*. Subsequently infestations by *Cercopithifilaria* sp. I were reported within dog populations in Spain, Greece, and southern Italy with prevalence rate up to 21.6% in some areas. Furthermore, it was demonstrated that the microfilariae of *Cercopithifilaria* sp. I complete their development to infective stage in the Brown Dog Tick *Rhipicephalus sanguineus* in about two months.

In addition to *Cercopithifilaria* sp. I, *C. grassii* and another undescribed species (microfilaria 264.4 ± 20.2 µm long) of *Cercopithifilaria* (i.e., *Cercopithifilaria* sp. II) were also morphologically and molecularlych aracterized.

Epidemiological data gathered so far suggest that microfilariae of *Cercopithifilaria* sp. I are the most prevalent in Europe, followed by *Cercopithifilaria* sp. II and *C. grassii*. Following the retrieval of adults of *Cercopithifilaria* sp., during the necropsy of a *Cercopithifilaria* sp. I-positive dog, the main morphological characters of males and females were compared with those of all other *Cercopithifilaria* spp. infesting dogs. Based on morphological comparisons between the type specimens of *C. bainae* and the adult nematodes collected, existence of *C. bainae* was ascertained.

5 CLINICAL IMPACT OF CO-INFECTIONS

8th CVBD WORLD FORUM Symposium

Parasites & Vectors

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Are vector-borne pathogen co-infections complicating the clinical presentation in dogs?

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ABSTRACT

BACKGROUND: InfInfection by two or more canine vector-borne disease (CVBD)-causing pathogens is common in subtropical and tropical regions where vectors are plentiful. Co-infections may potentiate disease pathogenesis, thereby altering clinical manifestations typically associated with singular infections. These factors complicate diagnosis, treatment and can adversely influence prognosis if the practitioner fails to suspect, document, and treat each concurrent infection. The spectrum of pathogens co-infecting dogs may change over time in a given practice location due to the rapid expansion of arthropods and their associated vectored agents, and international transit among pets and wild animals. This applies, for example, to *Dirofilaria immitis* and *Leishmania infantum*, the distributions of which have expanded from northern to southern Italy, and vice versa, respectively. Indeed, mixed infections by *D. immitis* and *L. infantum* have only been reported once in Italy, probably due to the fact that competent vectors for these infections do not usually occur in the same geographical areas. Thus, information that would help practitioners to identify clinical presentations in dogs co-infected by *D. immitis* and *L. infantum* and other CVBD-causing pathogens is scant.

FINDINGS: This manuscript describes the clinical history and physical examination of findings for 7 CVBD co-infected dogs that were examined because of a spectrum of clinical signs. Five dogs were co-infected with *L. infantum* and *Ehrlichia canis*, one dog with *L. infantum*, *E. canis* and *D. immitis* and the remaining dog with *L. infantum* and *D. immitis*.

CONCLUSIONS: The clinical signs and haematological abnormalities associated with the diagnostic evaluation and treatment of these dogs is discussed. Also, the usefulness of bone marrow specimens for the molecular diagnosis of CVBDs and for the enhanced monitoring of treatment response is emphasized.

KEYWORDS: Co-infection, *Dirofilaria immitis*, *Ehrlichia canis*, *Leishmania infantum*, Bone marrow

FINDINGS

Canine vector-borne diseases (CVBDs) are caused by a spectrum of pathogens that are transmitted by arthropods, including ticks, fleas, mosquitoes, and phlebotomine sandflies.[1] Dogs are reservoir hosts for several arthropod-borne pathogens, some of which are of major zoonotic concern.[2] In Italy, dogs may be infected by several CVBD-causing pathogens, such as *Leishmania infantum*, *Ehrlichia canis*, *Babesia* spp., *Bartonella* spp., *Cercopithifilaria* spp., *Hepatozoon canis*, *Anaplasma platys*, *Dirofilaria immitis,* and *Dirofilaria repens*.[3] Canine leishmaniosis (CanL) and canine monocytic ehrlichiosis (CME) are both severe systemic diseases and each can be characterized by a wide range of overlapping clinical signs (e.g., lymph node enlargement, weight loss, and splenomegaly). Dogs with CanL may also present skin alterations (alopecia, furfuraceous dermatitis, ulcers, and nodular lesions),[4] whereas dogs infected with *E. canis* may display haemorrhagic disorders.[5] Heartworm disease, caused by *D. immitis*, may be an asymptomatic infection or may be associated with pulmonary and cardiovascular illnesses (e.g., cough, dyspnoea, exercise intolerance, ascites, renal failure, and lethargy), with severity of disease mostly related to the nematode burden.[6] Molecular assays, serological testing, and cytology performed on different biological tissues (bone marrow and lymph node for CanL and blood for CME, blood for dirofilariosis) are the most frequently used tests for diagnosing the respective infections.[7,8] Recommended treatment for CanL is a combination of meglumine antimoniate or miltefosine with allopurinol,[9] doxycycline for CME,[10] and melarsomine dihydrochloride as adulticide treatment for dirofilariosis, followed by macrocyclic lactones as larvicide.[11]

In subtropical and tropical regions where vectors are plentiful, co-infections may potentiate disease pathogenesis, thereby altering clinical disease manifestations typically associated with singular infections. These factors complicate diagnosis, treatment and can adversely influence prognosis if the practitioner fails to suspect, document, and treat each concurrent infection.[12–16] To the authors' knowledge, co-infection with *D. immitis* and *L. infantum* has only been reported in a single dog from Italy,[17] probably due to the fact that the geographical distribution of the vectors for these infections does not usually overlap. Under the above circumstances, information regarding the clinical presentation of dogs co-infected by *D. immitis* and *L. infantum* is scant, thus representing a challenge for practitioners in their attempt to diagnose such conditions.

This manuscript describes the clinical history of seven dogs that were examined due to highly variable disease presentations. Co-infection with two or more CVBDs was diagnosed in all seven dogs.

From January to December 2011, 7 dogs were referred to the teaching hospital of The Department of Veterinary Medicine (University of Bari, Italy) for diagnostic evaluation (**Table 1**). Dogs were of different breeds, both sexes, and various ages and came from an area of southern Italy that is endemic for *L. infantum* and *E. canis*.[3] At admission, one dog (no. 5) had a severe tick infestation, whereas the other dogs were not infested at the time of examination, but had no history of acaricide treatment. When referred, clinical signs included depression (2/7), weight loss (2/7), hyperthermia (1/7), anorexia (3/7), exercise intolerance (1/7), pale mucous membranes (2/7), lymph node enlargement (5/7), tongue ulcers (1/7), furfuraceous dermatitis (1/7), ulcerative granulomatous dermatitis of prepuce (1/7), cough (1/7), lameness (1/7), epistaxis (1/7), and multiple mammary nodules (1/7). Complete blood counts (CBC) were obtained using an automated cell counter (Abbott Cell-Dyn 3700). Serum proteins were determined by agarose gel electrophoresis. The following haematological and serum biochemistry parameters were recorded: haemoglobin concentration (Hb), haematocrit (Hct), nucleated red blood cells (nRBC), white blood cells (WBC), platelet count (PLT), total serum protein (TP), albumin, and γ-globulin. Haematological reference ranges were previously determined.[18] Blood, buffy coat, lymph node, and bone marrow smears were prepared and

Table 1. Breed, sex, age (in years), description of clinical signs, laboratory abnormalities, and diagnostic test results for 7 dogs co-infected by two or more canine vector-borne diseases causing pathogens

Dog no./breed	Sex	Age	Clinical signs	Laboratory abnormalities	Cytology				PCR	
					Blood	Buffy coat	Lymph node	Bone marrow	Blood	Bone marrow
1/ Fila Brasileiro	F	5.5	Weight loss, anorexia, exercise intolerance, pale mucous membranes, lymph node enlargement, furfuraceous dermatitis	Anaemia, polyclonal gammopathy, low PLT	*Diro*	–	*Leish*	*Leish, Diro*	*Ehrl, Diro*	*Ehrl, Leish*
2/ Fila Brasileiro	M	6	Asymptomatic	Polyclonal gammopathy	*Diro*	–	–	*Diro*	*Ehrl, Diro*	*Ehrl*
3/ Mongrel	F	5	Tongue ulcers, lymph node enlargement	Polyclonal gammopathy	–	–	*Leish*	*Leish*	*Ehrl*	*Ehrl, Leish*
4/ English setter	M	9	Ulcerative granulomatous dermatitis of prepuce	Anaemia, polyclonal gammopathy, low PLT	nd	nd	*Leish*	*Leish*	*Ehrl*	*Ehrl, Leish*
5/ Siberian Husky	M	8	Coughing, anorexia, depression, hyperthermia, pale mucous membranes, lymph node enlargement	Anaemia, polyclonal gammopathy, low PLT	–	–	*Leish*	*Leish*	*Ehrl*	*Ehrl, Leish*
6/ Doberman	F	4	Depression, weight loss, lymph node enlargement, multiple mammary nodules	Anaemia, hyperproteinemia, polyclonal gammopathy, low PLT	–	–	*Leish*	*Leish*	*Ehrl*	*Ehrl, Leish*
7/ Schnauzer	F	5.5	Lameness, anorexia, lymph node enlargement, epistaxis	Anaemia, polyclonal gammopathy	–	–	*Leish*	*Leish*	*Ehrl*	*Leish*

Abbreviations: F: female; M: male; PLT: platelets; nd: not done; *Diro: Dirofilaria immitis; Leish: Leishmania infantum; Ehrl: Ehrlichia canis*

stained using the MGG Quick Stain (Bio Optica Spa, Italy) and stained-smears were examined by light microscopy for the presence of intracellular inclusions (or free forms) of common tick-borne pathogens and *L. infantum*.[19] All dogs had a polyclonal gammopathy. Anaemia and thrombocytopenia were found in five and four dogs, respectively. Cytological examination of lymph node and bone marrow smears resulted in the identification of *L. infantum* amastigotes in 6/7 dogs (nos. 1, 3, 4, 5, 6, and 7). In dogs 1 and 2, nematode microfilariae, identified as *D. immitis*, were initially confirmed by direct examination of blood smears and modified Knott's test.[20] *D. immitis* infection in these two dogs was further confirmed by the detection of circulating antigens using a commercial assay (SNAP heartworm antigen test; IDEXX Laboratories, Milano, Italy) and by PCR-specific amplification of *Dirofilaria* spp. DNA, that was performed as described elsewhere.[21] PCR on blood and bone marrow samples was also performed for detection of *L. infantum*, *Ehrlichia*, *Anaplasma* and *Babesia* species, as previously described.[19] Five dogs were co-infected with *L. infantum* and *E. canis*, one dog with *L. infantum*, *E. canis,* and *D. immitis* and the remaining dog with *E. canis* and *D. immitis*. The finding of microfilariae of *D. immitis* in the bone marrow cytology was most likely due to the presence of blood in the bone marrow sample, even if microfilariae have rarely been found in the bone marrow of dogs.[22]

Dogs co-infected by *L. infantum* and *E. canis* were treated with a combination of meglumine antimoniate (50 mg/kg/bid/SC) and allopurinol (10 mg/kg/bid/OS)[23] and with doxycycline (10 mg/kg/die/OS). Dogs 1 and 2 were also treated with melarsomine 2.5 mg/kg/IM for two days for heartworm infections.[6]

After therapy with doxycycline, whole blood PCR was negative for *E. canis*, whereas *E. canis* DNA was amplified from the bone marrow in five dogs (nos. 1, 2, 3, 4, and 6), which had normal platelet counts but failed to reduce gammopathy. For these dogs, an additional four weeks of treatment with doxycycline (10 mg/kg/die OS) was given. After two weeks, PCR assays were negative on both blood and bone marrow samples from all five dogs and remained negative when retested at 4 weeks post treatment.

DISCUSSION

Clinical evaluation of the seven co-infected dogs described in this case series illustrates the diagnostic, therapeutic, and management challenges imposed by the spectrum of CVBDs prevalent in southern Italy. These concurrent infections can induce clinical illness, as illustrated by dog 1, that was infected by *E. canis*, *L. infantum,* and *D. immitis*, as compared to asymptomatic infection in dog 2, that was infected with *E. canis* and *D. immitis*. Co-infections with *E. canis* and *L. infantum* are frequently detected in the Mediterranean region,[24] whereas to date, co-infection with *D. immitis* and *L. infantum* has only been reported in a dog from Italy.[17] The region from which both dogs originated is endemic for *L. infantum* and *E. canis*.[3] Documentation of two additional cases of heartworm disease indicates that *D. immitis* occurs in autochthonous foci in southern Italy (i.e., both dogs had lived in this area since they were puppies, and had no travel history), thus confirming the spread of this helminthic infestation in this region.[25,26] Undoubtedly, infection with two or more pathogens complicates the clinical presentation, which provides the basis for the veterinarian's diagnostic and therapeutic recommendations. In addition, co-infection with multiple CVBDs impacts the severity of haematological abnormalities in dogs, and poses challenges in terms of therapeutic strategies to be applied to each individual patient.[18] For example, *L. infantum* may impair the cellular and humoral immune response of the host, which may favour the establishment or the reactivation of a pre-existing *E. canis* infection.[27] Moreover, *E. canis* causes a reduction in major histocompatibility complex of class II receptors, which could ultimately enhance the clinical progression of CanL.[28]

Out of five dogs infected with *L. infantum*, only dog 1 had clinical signs suggestive of CanL (e.g., lymphadenopathy, dermatitis, and onychogryphosis), whereas the laboratory abnormalities (normocytic, normochromic non-regenerative anaemia, thrombocytopenia, and gammopathy) in the remaining *L. infantum*-infected dogs overlapped with laboratory abnormalities associated with CME.[19] This suggests that when infection with multiple pathogens is involved, the clinical presentation might be unpredictable and specific clinical or haematological abnormalities cannot be definitively ascribed to a single pathogen. For example, the polyclonal gammopathy detected in all seven dogs in this case series might be the consequence of a chronic antigenic stimulation[29] caused either by *L. infantum*, *E. canis* or *D. immitis,* or any combination of these three pathogens.

Diagnosis of single or multiple CVBDs should rely on epidemiological information, including travel history, on the clinical status of the dog and on a panel of laboratory diagnostic tests.[19] In the cases reported herein, a definitive diagnosis of co-infection required a combination of clinical suspicion in conjunction with documentation of abnormal laboratory findings, and diagnostic confirmation by microscopy, serology, and PCR, preferably using both blood and bone marrow specimens when attempting to confirm the diagnosis of *E. canis* and *L. infantum* infections. Accordingly, the tissues most frequently used for the diagnosis of *E. canis* infections are blood and bone marrow.[7,30] Monitoring the response following doxycycline administration in dogs suffering from ehrlichiosis is pivotal in distinguishing persistently infected sick dogs unsuccessfully treated, from dogs that achieve clinical and haematological recovery, but remain infected as compared to those dogs that recover and clear the pathogen.[10] Although preliminary and limited in quantity, data herein reported indicate that under natural exposure conditions, PCR on bone marrow aspirates may be a reliable method for the evaluation of the treatment response in dogs with CME. In our experience, when infections with *E. canis* and *L. infantum* occur in dogs with severe clinical or haematological abnormalities, these infections should be treated simultaneously to improve the dogs clinical status, before treating for *D. immitis*.

CONCLUSIONS

The atypical clinical signs and mild to severe haematological abnormalities described in these seven co-infected dogs highlight the importance of a holistic diagnostic approach that includes microscopy, serology, and PCR testing, when dealing with CVBDs in endemic areas. This report also supports the potential utility of using bone marrow specimens as a biological tissue for the molecular diagnosis of CVBDs and for monitoring of the treatment response in dogs with CME. Our results further stress the need for continued development of multi-pathogen detection methods for CVDBs in endemic and non-endemic regions.

COMPETING INTEREST

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

ASDT and DdC conceived the study and documented clinical cases. ASDT, DdC, DO, FDT, EBB, and GC drafted and revised of manuscript. All authors read and approved the final version of manuscript.

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6 SHARING ON PREVENTION, DIAGNOSIS, AND TREATMENT OF CVBDs

8th CVBD WORLD FORUM Symposium

SHARING ON PREVENTION, DIAGNOSIS, AND TREATMENT OF CVEDs

Current status of vaccination against canine leishmaniosis in Europe

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ABSTRACT

Canine leishmaniosis (CanL) is endemic in different European Countries, particularly in those belonging to Mediterranean Basin area. Current climatic and ecological changes have also permitted to different species of sandflies to colonize some continental European areas that till few years ago were considered *Leishmania*-free. Spreading of CanL is also possible because of the absence of severe limitation for the importation of infected dogs from endemic to non-endemic countries. For these reasons, CanL constitutes a veterinary and public health problem in most part of Europe. Main strategies for control of *Leishmania* infection are the large use of repellent against sandflies and the very recent introduction of a specific CanL vaccine, licensed with name of Canileish®. This product consists of *Leishmania infantum* cultured excreted/secreted proteins (ESP), the dominant antigen of which is the promastigote surface antigen (PSA). Canileish® is adjuvant with *Quillaja saponaria* purified extract (QA-21) that is demonstrated to induce a good cell-mediated response. The vaccine is indicated for the active immunization of *Leishmania*-negative dogs from 6 months of age and induces a 1-year duration of immunity. To date, there are few published studies about Canileish® efficacy. However, it seems clear that this vaccine produces a strong T helper 1 (Th1)-profile cell-mediated response within three weeks of completing the primary course, and that this response reduces the parasite load in pre-infected macrophages *in vitro*. Moreover, a two-year field trial, performed on 90 beagle dogs in Italy and Spain, showed that the vaccine, although did not protect dogs from initial infections, decreased the risk of developing progressive infections by about fourfold in natural conditions of high intensity of transmission. More than 1 million of vaccine doses have been sold in Europe, the most in Portugal, Spain, Italy, France, and Greece, that corresponds to about 1.4% of their canine population. To be applied on large scale, the most relevant points that remain to be clarified are the capacity of vaccine to protect dogs for long periods independently from the endemicity of the area, the difficulty to distinguish vaccinated from infected dogs by immunofluorescence method, and the infectivity to phlebotomine vectors of vaccinated dogs. Again, vaccination should be regarded as one of multiple strategies (including control of sandfly exposure and control of stray dogs) in the management of this disease within the population.

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CVBD Clinical Center: a possibility of interaction among veterinarians

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ABSTRACT

Canine Vector-Borne Diseases (CVBD) constitute a large group of infectious diseases transmitted by numerous blood-feeding ectoparasites, such as ticks, fleas, sandflies, and mosquitoes. CVBD have an increasing worldwide distribution because of various factors, the most important of which is the global climatic change that is conditioning the presence of CVBD transmitting insects in different areas of the world. Due to the complexity of their clinical pictures, CVBD diagnosis and management may constitute a difficult field for many veterinarians. CVBD Clinical Center (CVBD CC) has to be intended as an opportunity for all veterinarians to interact with a couple of CVBD "expert clinicians" that may help them to manage CVBD clinical cases or to solve possible doubts about prevention, diagnosis, therapy, and follow-up. CVBD CC is available through a linked banner on the web page www.advantix.it, directing in a web site that is exclusively available for veterinarians after the prerequisite registration. On this page, all registered veterinarians may ask questions about CVBD or charge data regarding their cases on an available clinical form. CVBD CC web page also allows to charge all types of pictures and files useful for description of imaging diagnosis. Further, all veterinarians may share among them all information that are on the mentioned web page and may contact again the experts after the first request without limitation of the number of accesses. CVBD CC started at beginning of September 2012 and to date has received more than 40 contacts from different areas of Italy. Particularly, the most frequent request of consultation has regarded Canine Leishmaniosis (CanL), because of its recent spread in non-endemic area of North Italy. Authors hope that in the near future CVBD CC will register a constant growing of requests and contacts to better improve the quality of this particular web veterinary medicine approach.

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8th CVBD WORLD FORUM Symposium

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