Broad-Range Survey of Tick-Borne Pathogens in Southern Germany Reveals a High Prevalence of *Babesia microti* and a Diversity of Other Tick-Borne Pathogens

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Abstract

Ticks harbor numerous pathogens of significance to human and animal health. A better understanding of the pathogens carried by ticks in a given geographic area can alert health care providers of specific health risks leading to better diagnosis and treatments. In this study, we tested 226 *Ixodes ricinus* ticks from Southern Germany using a broad-range PCR and electrospray ionization mass spectrometry assay (PCR/ESI-MS) designed to identify tick-borne bacterial and protozoan pathogens in a single test. We found 21.2% of the ticks tested carried *Borrelia burgdorferi* sensu lato consisting of diverse genospecies; a surprisingly high percentage of ticks were infected with *Babesia microti* (3.5%). Other organisms found included *Borrelia miyamotoi*, *Rickettsia helvetica*, *Rickettsia monacensis*, and *Anaplasma phagocytophilum*. Of further significance was our finding that more than 7% of ticks were infected with more than one pathogen or putative pathogen.

Key Words: Lyme borreliosis—*Borrelia*—*Babesia microti*—*Anaplasma phagocytophilum*—*Rickettsia helvetica*.

Introduction

TICKS CAN TRANSMIT A VARIETY of pathogens ranging from viruses and bacteria to protozoa (de la Fuente et al. 2008). Tick-borne pathogens are widespread and prevalent throughout Europe. *Ixodes ricinus*, the most abundant tick species in Germany (Cornely and Schultz 1992), harbors numerous pathogens including several Lyme borreliosis-causing *Borrelia* genospecies, spotted fever group (SFG) *Rickettsia* species, *Borrelia miyamotoi*, *Anaplasma phagocytophilum*, *Babesia divergens*, and *Babesia microti*. One study showed that infestation rates in wooded recreational areas was as great as 7.4 ticks per person per hour (Faulde and Robbins 2008), resulting in relatively high risks of pathogen exposure. Often these ticks carry multiple pathogens, raising the possibility of a co-infection with a *Borrelia* species and another pathogen (Loebermann et al. 2006, Pichon et al. 2006, Hildebrandt et al. 2010b, Franke et al. 2011). In Eur-

ope, studies indicate a potentially higher prevalence of *Babesia* species infections than previously believed with higher levels of seroreactivity to *Babesia* antigens in 11% and 4.4% of people exposed to ticks in Germany and Poland, respectively (Hunfeld et al. 2002, Pancewicz et al. 2011). As of 2007, at least 39 cases of babesiosis had been reported in Europe (Hildebrandt et al. 2007). In the United States, there have been instances of babesiosis following blood transfusion since the 1980s, but a sharp increase in the number of reported cases has been observed in recent years, with at least 12 documented fatalities due to transfusion-transmitted babesiosis (Asad et al. 2009, Gubernot et al. 2009a, Gubernot et al. 2009b, Tonnetti et al. 2009).

Examining a single tick for each of the likely tick-borne pathogens (*Borrelia burgdorferi* sensu lato (s.l.), *A. phagocytophilum*, SFG *Rickettsiae* incl. *R. helvetica*, *Babesia* species) is laborious and requires a number of different endpoint and/or real-time PCR assays with sequencing to

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identify the pathogen to the species level. This single pathogen testing approach also requires prior knowledge of likely pathogens. In contrast, the complex ecology of potentially pathogenic microorganisms within a single tick can be resolved easily from one DNA extraction followed by a single analysis on a broad-range PCR and electrospray ionization mass spectrometry system (PCR/ESI-MS). Using the PCR/ESI-MS technology, identification of relevant genospecies, discrimination from clinically irrelevant genospecies, and genotyping of pathogens carried by a single tick are possible (Crowder et al. 2010, Eshoo et al. 2010, Grant-Klein et al. 2010, Eshoo et al. 2012).

In this study, we examined 226 field-collected *I. ricinus* ticks from the 2009 tick season from southern Germany together with engorged ticks removed from humans and used broad-range PCR/ESI-MS to determine the breadth of tick-borne pathogens and potential pathogens in this region. Results were confirmed using pathogen-specific nested PCR or PCR and DNA sequencing on a subset of the specimens.

Materials and Methods

Tick collection and DNA extraction

I. ricinus Linné ticks were collected from April through June of 2009 in the area of Constance (Lake Constance, southern Germany) by flagging or were obtained from patients after a tick bite. Patients who sent ticks for pathogen detection to the laboratory lived in the southwestern parts of Germany, covering the area from Stuttgart (in the north of Baden Württemberg) to Constance in the south, Freiburg in the west, and Friedrichshafen in the east. All ticks were preidentified visually by a trained entomologist as *I. ricinus*, the predominant species and the species almost exclusively found after tick bites in humans in Germany. Although the life stage of the tick was not recorded, on the basis of measurements of the tick length, it was estimated that the sample set was a mixture of nymphs and adults.

DNA was extracted from the ticks at the laboratory of Dr. Brunner (Constance, Germany) as described previously (Crowder et al. 2010). Portions of the DNA extracts were sent to Ibis Biosciences (Carlsbad, CA) for analysis by international overnight delivery at room temperature, and portions were retained in Constance for microbe-specific PCR analysis.

Broad-range PCR/ESI-MS detection of vector-borne pathogens

The PCR/ESI-MS vector-borne pathogen detection assay was performed at Ibis Biosciences. The assay employed nine PCR primer pairs targeting various groups of vector-borne pathogens and was performed in eight PCR reactions with primer pairs BCT3517 and BCT2328 multiplexed together in a single PCR reaction (Table 1). The primers target conserved regions of the target clade genomes and were used to amplify variable regions. The primer design was such that primers would only amplify the target group and not any other group. PCR/ESI-MS and base composition analysis was carried out as described previously (Ecker et al. 2005, Ecker et al. 2006, Ecker et al. 2008, Crowder et al. 2010, Eshoo et al. 2010, Crowder et al. 2012, Rounds et al. 2012). Specimens were considered positive for *Borrelia* if any of the three *Borrelia*-

specific primer pairs detected *Borrelia*-specific amplicons. In most instances, species identification was made with a single base count signature.

Microbe-specific PCR detection assays

These assays were performed in the laboratory of Dr. Brunner (Constance, Germany). Particular care was taken to avoid carryover and/or contamination events. The laboratory itself is organized following a four-room concept. Sufficient controls were implemented to allow detection of carryover and/or contamination events. In particular, negative controls (sterile water, processed in the QIAcube instrument at the beginning of each working day) were run in each PCR experiment. Nested PCR results yielding a weak band were repeated. Assays were also repeated if two consecutive specimens were found positive for *Borrelia* DNA.

End point PCR for the detection of *B. burgdorferi* s.l. DNA in tick extracts was performed on 178 of the 226 ticks studied as follows: A 218-bp fragment of the *rrlA/rrlB* (23S–5S) ribosomal protein gene region was amplified employing nested PCR. A first round of PCR was carried out with 2 μ L of DNA extract in a final reaction volume of 25 μ L (using the premixed mastermix including Taq polymerase, supplied by Fermentas, St. Leon-Rot, Germany) and primer pair Bor-N/Bor-F (identical to JS1/JS2 described in Schwartz et al. 1992, yielding a 264-bp fragment). Following 20 cycles of °C (60 s), 50°C (45 s), and 72°C (30 s), 2 μ L of the amplicons were transferred to a subsequent PCR of 40 cycles. The annealing temperature in this second PCR was 56°C (45 s) with primer pair BorNest (Table 1).

Tick extracts were screened for the presence of *A. phagocytophilum* and SFG *Rickettsia* DNA using previously described the PCR/primer systems targeting *msp2* of *A. phagocytophilum* (primers Msp2-3F/Msp2-3R) and *ompA* (primers Rr190.70p/Rr190.602n) of SFG *Rickettsia* (Barandika et al. 2007). *Babesia*-specific DNA was screened using primers targeting the 18S rRNA gene, as previously described (Blaschitz et al. 2008), and amplicons were visualized by 8% acrylamide gel electrophoresis and ethidium bromide stain.

Sequence confirmation of *Borrelia*, *Rickettsia*, and *Babesia* species

For *Borrelia* and *Rickettsia* signatures that were not in our PCR/ESI-MS base count signature database, the detections were confirmed by Sanger DNA sequencing of a portion of the 16S rRNA gene from representative positive specimens. PCR primer pair B16SF and B16SR targeted the *Borrelia* 16S rRNA gene, and 16SF and 16SR targeted all bacteria including the *Rickettsia* 16S rRNA gene. All sequencing primer pairs contained M13 primer sequence tagged to their 5' ends to facilitate Sanger DNA sequencing and are shown in Table 1. The amplification of the 16S rRNA genes was performed in a 50- μ L reaction containing 1 μ L of nucleic acid extract, 1 unit of Platinum Taq High Fidelity polymerase (Invitrogen, Carlsbad, CA) or Immolase Taq (Bioline, Randolph, MA), the manufacturer's PCR buffer, 2.0 mM MgSO₄, 200 μ M dATP, 200 μ M dCTP, 200 μ M dTTP, 200 μ M dGTP (Bioline, Randolph, MA), and 250 nM of each primer. The following PCR cycling conditions were used on an MJ Dyad 96-well

TABLE 1. PRIMERS USED FOR PCR/ESI-MS AND SEQUENCING

Application	Target	Target clade/genus	Primer pair name	Orientation	Primer sequence
Broad-range PCR	<i>gyrB</i>	All spirochetes	BCT3511 ^a	F	TGCATTTTGAAAGCTTGGCATTGCC
	<i>rpoC</i>	All spirochetes	BCT3514 ^a	R	TCATTTTAGCACTTCTCCAGCAATC
	<i>flagellin</i>	All <i>Borrelia</i> spp.	BCT3517 ^a	F	TTGGTACCACAAAAGGAATGGA
	<i>asd</i>	<i>Francisella tularensis</i>	BCT2328 ^b	R	TGCGAGCTCTATATGCCCCAT
	<i>RNaseP</i>	All <i>Rickettsia</i> spp.	BCT1083 ^b	F	TGCTGAAGAGCTTGGAATGCA
	<i>gltA</i>	Alphaproteobacteria	BCT3570 ^b	R	TACAGCAATTGCTTCATCTTGATTGC
	<i>rpoB</i>	Alphaproteobacteria	BCT3575 ^b	F	TGAGGGTTTTATGCTTAAAGTTGGTTTATTGGTT
	<i>18S rRNA</i>	All <i>Babesia</i> spp.	INV4443 ^b	R	TGATTCGATCATACGAGACATTAACAACTGAG
	<i>β-tubulin</i>	All <i>Babesia</i> spp.	INV4855 ^b	F	TAAAGCGCACCGTAAAGTTGG
	23S–5S spacer	<i>Bo. burgdorferi</i> s.l.	BorNest	R	TCAAGCGATCTACCCGCATTACAA
	16S rRNA	All <i>Borrelia</i> spp.	B16S	F	TGCATGCAGATCATGAACAGAATGC
	16S rRNA	All bacteria	16S	R	TCCACCATGAGCTGGTCCCA
				R	TGCATCATTGGTTGATGATAAGATACATGC
Nested PCR – inner				R	TCACCAAAACGCTGACCACCAAA
				F	TGCGCAATTAACCAATCCTGACAC
				R	TCCAGACTTGCCTCCCAATTGGTA
				F	TGAGAGAAATCGTACACATTCAGCGGG
Organism sequencing				R	TCCATGTTCTCGGAGATGACTTCCCA
				R	TGCTCCCAGCCACATGTACCA
				F	ACTAAATAAGGCTGAACTTAAAT
				R	AAGCGAGTCTTAAAGGGCGAT
				F	M13F/TGGTGTAAAGGTGGAATCTGTGTATATCAG
				R	M13R/CTTGTACGACTTCACCCCTCACTAAAC
				F	M13F/TTGGAGAGTTTGATCCTGGCTC
				R	M13R/GGCGTGGACTTCCAGGGTATCT

The forward and reverse sequencing primers have the following sequences at their 5' end to facilitate the sequencing of the PCR amplicons: M13F, CCCAGTCACGACGTTGTAAAAACG; M13R, AGCGGATAACAATTTCACACAGG.

^aCrowder et al. (2010).

^bEshoo et al. (2010).

PCR/ESI-MS, PCR and electrospray ionization mass spectrometry; F, forward; R, reverse.

thermocycler (Bio-Rad Inc., Hercules, CA): 95°C for 2 min, followed by eight cycles of 95°C for 15 s, 50°C for 45 s, and 68°C for 90 s, with the 50°C annealing temperature increasing 0.6°C for each cycle. The PCR was then continued for 37 additional cycles of 95°C for 15 s, 60°C for 15 s, and 68°C for 60 s. The PCR cycle ended with a final extension of 4 min at 72°C. Reactions were visualized on 1% agarose gels to ensure the presence of appropriately-sized products before being sent to SeqWright (Houston, TX) for purification and sequencing with M13 primers.

Sequence confirmation of the *B. microti* detections was performed by sequencing part of the 18S rRNA gene. The primers used were specific for the *Babesia* 18S rRNA gene and have been described previously (Blaschitz et al. 2008). Specimens positive for an approximately 620-bp amplicon in an 8% polyacrylamide gel were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's recommendations. Amplicons were sequenced at a commercial service provider (GATC, Constance, Germany) using the amplification primers. Sequences were compared against the GenBank database using the online National Center for Biotechnology Information (NCBI) algorithm.

When more than one organism was present that was not in our PCR/ESI-MS database, plasmid cloning and sequencing were employed. Specifically, the specimens were amplified with Platinum Taq as described above, and the enzyme was inactivated at 99°C for 20 min. Products were subsequently adenylated with Immolase Taq (Bioline, Randolph, MA) at 60°C for 1 h to facilitate cloning. Reactions were ligated into pGEM-T (Promega, Madison, WI) and transformed into JM109 cells, both according to the manufacturer's instructions. Minipreps were performed with the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA), and samples were then sent to SeqWright (Houston, TX) for sequencing with SP6 and T7 promoter primers. Sequences were analyzed using BioEdit software (www.mbio.ncsu.edu/BioEdit/bioedit.html).

Results

Survey of tick-borne pathogens by broad-range PCR/ESI-MS

Using an assay designed to detect and identify vector-borne pathogens, one or more pathogens, or putative pathogens, were detected in 92 of the 226 ticks (40.7%). The results of the overall prevalence for individual pathogens are shown in Table 2. The majority of pathogen-positive ticks were infected with *B. burgdorferi* s.l. complex: 48/226 (21.2%). *Borrelia afzelii* comprised the majority of Lyme *B. burgdorferi* s.l. complex detections (12% of total ticks tested) with other species such as *Borrelia garinii* (4.9%), *B. burgdorferi* sensu stricto (s.s.) (2.2%), *Borrelia spielmanii* (0.4%), *Borrelia valaisiana* (1.3%), and *Borrelia lusitaniae* (0.4%) making up the rest of the Lyme *Borrelia* species observed. *Rickettsia helvetica* was the second most prevalent organism found in the ticks surveyed (38/226, 16.8%). The relapsing fever group *Borrelia*, *B. miyamotoi* was present in four of the 226 ticks tested (1.8%). Other organisms detected were *B. microti* (8/226, 3.5%), *A. phagocytophilum* (5/226, 2.2%), and *Rickettsia monacensis* (2/226, 0.9%). Additionally, four

TABLE 2. INDIVIDUAL PATHOGEN/MICROBE PREVALENCE IN TICKS BY PCR/ESI-MS (N=226)

Pathogen	n ^a	%
<i>Anaplasma phagocytophilum</i>	5	2.2%
<i>Babesia microti</i>	8	3.5%
Total <i>Borrelia burgdorferi</i> s.l.	48	21.2%
<i>B. afzelii</i>	27	12.0%
<i>B. garinii</i>	11	4.9%
<i>B. burgdorferi</i> s.s.	5	2.2%
<i>B. valaisiana</i>	3	1.3%
<i>B. lusitaniae</i>	1	0.4%
<i>B. spielmanii</i>	1	0.4%
<i>Borrelia miyamotoi</i>	4	1.8%
<i>Rickettsia helvetica</i>	38	16.8%
<i>Rickettsia monacensis</i>	2	0.9%
Putative SFGR ^b	4	1.8%
Total organisms detected ^c	109	NA

^aCo-infecting pathogen/microbes are counted separately.

^bPutative SFGR (spotted fever group *Rickettsia*).

^cA total of 109 organisms were detected out of 92 positive ticks (76 ticks had one organism and 16 ticks were co-infected; see Table 3).

PCR/ESI-MS, PCR and electrospray ionization mass spectrometry; NA, not applicable.

different ticks were found to have a putative SFG *Rickettsia* by PCR/ESI-MS, but we were not able to confirm their identities by 16S sequencing. Of the 226 ticks analyzed, 119 (52.7%) were removed from humans, 102 ticks were field-collected ticks (45.1%), and five ticks were removed from dogs (2.2%). The only significant difference (*p* value of 0.03 using test of two proportions using the Fisher exact test) in organism prevalence between the ticks removed from humans and field-collected ticks was in the prevalence of *Babesia*. One tick removed from a human was found to be infected with *Babesia*, whereas seven field-collected ticks were infected with *Babesia*. The test of two proportions for *Borrelia*, *Anaplasma*, and *Rickettsia* prevalences were not significantly different (*p* values >0.7).

Detection sensitivity and specificity of PCR/ESI-MS compared to nested end point PCR for *Borrelia* and *Rickettsia*

Of the 226 ticks in this study, 178 ticks were tested for *Borrelia* by nested PCR. In this subset of 178 ticks, a total of 42 tick specimens were found to test positive for a *Borrelia* species (*B. burgdorferi* s.l. complex and *B. miyamotoi*) by the nested end point PCR assay and 40 results were confirmed by the PCR/ESI-MS assay. For the two discordant specimens, retesting with the nested end point PCR assay yielded one negative and one weakly positive, suggesting that these extracts may have had low levels of *Borrelia* DNA or may have experienced DNA degradation. These results demonstrate that the broad-range PCR/ESI-MS assay is as sensitive as nested end point PCR and can also resolve the Lyme *Borrelia* genospecies and *B. miyamotoi*.

Of the total 226 tick specimens tested by PCR/ESI-MS, 150 were also examined for the presence of *Rickettsia* species by a nested end point PCR assay. In this subset of 150 ticks, *R. monacensis* was detected in two of the specimens; these detections were also confirmed by the PCR/ESI-MS assay.

The PCR/ESI-MS assay also detected *R. helvetica* in 23 ticks and SFG *Rickettsia* in four ticks in this subset of 150 ticks; these detections were not confirmed by the nested end point PCR assay.

Co-infection of ticks

Of the 226 ticks tested, 76 (33.6%) were infected with a single microorganism and 16 (7.1%) contained more than one (Table 3). For co-infections with two organisms, co-infection with a *Borrelia* species and *R. helvetica* was the most common (10/226, 4.4%). Co-infections with *Borrelia* species and *Ba. microti* (2/226, 0.9%), *Ba. microti*, and *R. helvetica* (1 of 226, 0.4%) and *A. phagocytophilum* and *R. helvetica* (2/226, 0.9%) were also detected. One tick was found to harbor three different micro-organisms: *B. afzelii*, *Ba. microti*, and *R. helvetica* (1/226, 0.4%). With a total of 16 ticks with various combinations of co-infecting organisms, our sample size was too small to determine significance, although the observed prevalence of co-infections was similar to the expected prevalence. The combination of micro-organisms most often observed was co-infection of *Borrelia* and *R. helvetica*; this was not unexpected because these two organisms are the two most common infecting organisms identified. No co-infection with different *Borrelia* species was observed.

Sequence conformation of Babesia, Borrelia, and Rickettsia PCR/ESI-MS detections

Certain specimens were sequenced to verify PCR/ESI-MS results. A *R. helvetica* detection was confirmed by Sanger sequencing a 721-bp fragment of the 16S rRNA gene and was found to be 100% identical to *R. helvetica* (GI accession number L36212.1). The specimen positive for *R. monacensis* by PCR/ESI-MS was found to have 100% identity with *R. monacensis* strain IRR/Munich (GI accession number DQ100164.1).

We sequenced an approximately 760-bp fragment of the *Borrelia* 16S rRNA gene of nine representative *Borrelia*-positive specimens. The 16S rRNA gene sequence from the *B. garinii*-positive specimen was identical to that of the *B. garinii* strain BgVir (CP003151.1), and the *B. afzelii*-

positive specimen was identical to *B. afzelii* strain PKo (CP000395.1). The two *B. valaisiana*-positive specimens sequenced were identical to *B. valaisiana* VS116 (X98232.1). The *B. spielmanii* specimen matched the *B. spielmanii* strain PMai (AM182229.1) with 99.74% sequence identity. The three *B. miyamotoi*-positive specimens sequenced all yielded identical sequences with 99.87% homology to the 16S rRNA gene of the Russian *B. miyamotoi* strain (JF951378). One specimen appeared to be a mixture of *B. lusitanae* isolates, and the 16S rRNA gene amplicons were cloned and 14 colonies sequenced. All clones had 98.4–100% identity to the *B. lusitanae* Poti B2 strain (NR036806).

The *Babesia* sequences obtained from the tick extracts matched deposited sequences of *Ba. microti*. In addition, in a phylogenetic analysis, the sequences clustered in a distinct cluster with the published *Ba. microti* sequences GenBank accession numbers AY789075 and EF413181 but clearly separated from a cluster with *Ba. divergens* (AY098643), *Ba. venatorum* (JX042323), *Babesia* species EU1 (GQ888709), and *Ba. capreoli* (GQ304526) (data not shown).

Discussion

In this study, we tested using a broad-range PCR/ESI-MS assay 226 field-collected *I. ricinus* ticks or engorged ticks removed from humans from the 2009 tick season in southern Germany to characterize the breadth of tick-borne pathogens and potential pathogens in this region. The most frequently detected pathogens were those belonging to the *B. burgdorferi* s.l. group. This is consistent with at least nine previous studies (Schaarschmidt et al. 2001, Rauter et al. 2002, Pichon et al. 2006, Dietrich et al. 2010, Franke et al. 2010, Gern et al. 2010, Hildebrandt et al. 2010b, Franke et al. 2011, Strube et al. 2011) that analyzed the prevalence of Lyme disease spirochetes, SFG *Rickettsia*, *A. phagocytophilum*, and the parasitic *Babesia* spp. in ticks collected in Germany or neighboring countries. The lowest prevalence was reported from a region in Thuringia with 2.6% *Borrelia*-positive ticks, collected from mammals (Franke et al. 2010), and the highest rate was 35.2% in the southwestern parts of Germany (Rauter et al. 2002) (mean 20.1% $\pm$ 9.2% standard error [SE] with a range of 3.1–35.2% for all nine reports). A previous study, conducted with 7373 ticks also from the Lake of Constance area reported that 15% of ticks carried at least one genospecies of Lyme *Borrelia*, which is consistent with our findings (Schaarschmidt et al. 2001). Differences in prevalence rates may reflect seasonal or local geographic variations. Strube et al. (2011) reported that the percentage of ticks carrying *Borrelia* spp. decreased from 24.4% in 2007 to 12.8% in 2010, a phenomenon that needs further attention. The prevalence of *R. helvetica* (16.8%) observed in this study was similar to previously reported prevalences for various regions of Germany which ranged from 0.5% to 18.8% (Hartelt et al. 2004, Dobler and Wölfel 2009, Hildebrandt et al. 2010a, Schorn et al. 2011, Silaghi et al. 2011). The low prevalence of *R. monacensis* observed in our study (0.9%) was also consistent with previous studies of other regions of Germany (Hildebrandt et al. 2010b, Schorn et al. 2011). The putative SFG *Rickettsia* signatures observed in four ticks could be a strain variant of a single *Rickettsia* species or could be due to different *Rickettsia* species. Other studies examining the prevalence of various *Rickettsia*

TABLE 3. MIXED INFECTIONS DETECTED IN TICKS (N=226)

Pathogens detected	n	Observed %	Expected ^a %
<i>B. afzelii</i> , <i>R. helvetica</i>	3	1.3%	2.0%
<i>B. garinii</i> , <i>R. helvetica</i>	3	1.3%	0.8%
<i>B. burgdorferi</i> , <i>R. helvetica</i>	2	0.9%	0.4%
<i>R. helvetica</i> , <i>A. phagocytophilum</i>	2	0.9%	0.4%
<i>B. afzelii</i> , <i>Ba. microti</i>	1	0.4%	0.4%
<i>B. miyamotoi</i> , <i>Ba. microti</i>	1	0.4%	0.1%
<i>B. miyamotoi</i> , <i>R. helvetica</i>	1	0.4%	0.3%
<i>R. helvetica</i> , <i>Ba. microti</i>	1	0.4%	0.6%
<i>B. valaisiana</i> , <i>R. helvetica</i>	1	0.4%	0.2%
<i>B. afzelii</i> , <i>Ba. microti</i> , <i>R. helvetica</i>	1	0.4%	0.1%
Total number of co-infected ticks	16	7.1%	NA

^aExpected percent calculated by multiplying prevalence of co-infecting organisms.

NA, not applicable.

species from ticks also often found previously unreported *Rickettsiae* sequence signatures, suggesting that the genus may be diverse. Further studies of these *Rickettsia* using gold standard 16S sequencing is needed to characterize them.

In our study, 7.1% of ticks were infected with more than one pathogen. As expected from the infection rates of individual pathogens, co-infection with *B. burgdorferi* s.l. and *R. helvetica* was the most common combination in our tick collection. The actual medical importance of *R. helvetica* is still not clear. Nilsson et al. (1999) reported detection of *R. helvetica* DNA in heart tissue of two previously healthy males who had died from sudden cardiac failure. From Slovakia, a seroprevalence rate in humans of 32% for *R. helvetica* was reported recently (Sekeyova et al. 2012). Although the presence of microorganism-specific DNA in ticks and a corresponding seroprevalence do not indicate virulence of the pathogen *per se*, our finding raises the question as to whether nontypical clinical cases of Lyme disease might be influenced by a second transmissible and potentially pathogenic species. The finding of *Ba. microti* in our ticks was unexpected, although this species has been reported from central Europe (Sytykiewicz et al. 2012). A recent clinical case of human babesiosis, caused by *Ba. divergens* rather than *Ba. microti*, was reported by Martinot et al. (2011).

The majority of studies published so far have focused on ticks collected by flagging from the environment. Prevalence data obtained with such datasets are likely to reflect population-based prevalence rates (i.e., the *B. burgdorferi* s.l. prevalence in the tick population might be a result of the specific epidemiology of *Borrelia* and other microorganisms in the host populations). In wildlife, dozens to hundreds of ticks may feed from a single individual, and therefore flagging measures the entomological risk posed by ticks at a specific location under examination regardless of human traffic. In contrast, human-attached ticks sent to a diagnostic laboratory for the purpose of testing for the presence of *B. burgdorferi* s.l. may cover a broader geographic area and represent both entomological risk combined with human exposure risk. The prevalence data obtained from ticks sent to a diagnostic lab, however, are not useful to define a region of particular risk. Rather, the data allow the estimation of a mean risk for the population, in the present study the population of southwestern Germany. Because the ticks we tested were not aged, many of the ticks may have had more than one blood meal, which in theory may have increased the prevalence of pathogens in those ticks.

In addition to *B. burgdorferi* s.l., we have detected DNA of a number of potentially pathogenic microorganisms in our tick collection. However, no conclusion on the actual risk for the population of developing disease can be drawn because the likelihood of transmission of these pathogens has not been determined. We know that pathogenic *Borrelia* are well adapted to the arachnid host and that infectious *Borrelia* species are transmissible at some point in time during attachment and tick bite, but this is not certain for the other pathogens detected. Confirmed infections and/or co-infections in humans might serve as sentinels for successful transmission of pathogens in populations at occupational risk of tick bites. Krause et al. (1996) reported that 10% of 1,156 patients with Lyme disease were co-infected with *A. phagocytophilum* and/or *Ba. microti*. Serological surveys done very recently in Poland indicate that those microorganisms we have

detected by PCR in our tick collection do frequently infect humans (Chmielewska-Badora et al. 2012). Dobler and Wölfel (2009) reported that up to 5% of persons bitten by a tick and undergoing fever (tick-bite associated fever) were positive for *Rickettsia* immunoglobulin M (IgM) antibodies or had an increase in IgG, indicating that the *Rickettsiae* detected by our PCR assays are indeed infectious. The same holds true for the other pathogens: Seroprevalence data for *Anaplasma* indicate transmission of viable bacteria by a vector and the recent report of human babesiosis following tick bite in the Vosges region (Martinot et al. 2011) demonstrate that these protozoan microorganisms are transmissible by tick vectors. Whereas the rate of ticks testing positive for at least one of the pathogens was generally in line with the results of previous reports, the superior breadth of coverage and resolution of the PCR/ESI-MS technique over conventional PCR techniques is documented here.

No data are available about how long the ticks sent to laboratory by 'patients' had been attached to their host. This raises the possibility that at least a few ticks might have been attached for periods of time long enough to allow *Borrelia* to be transmitted. Some patients sent their ticks alive in small plastic containers, whereas others preferred to take revenge for being attacked by the arachnids, sending only debris sticking to adhesive strips. If there was doubt that this residue actually had been a tick, an additional PCR, targeting the 18S rRNA gene of *I. ricinus*, was performed (Schwaiger et al. 2003). Finally, the sample itself is limited. From the DNA extracted from a single tick, a number of PCR assays were performed. The volume used for PCR/ESI-MS assay was therefore limited and below the actual recommendations (at least 80 μ L of extract). Nevertheless, the data for *Borrelia* prevalence in the tick extracts were in good accordance with published results, indicating that the lower volume did not decrease sensitivity of the PCR/ESI-MS assay significantly.

With climates around the world changing, it may be more important than ever to be vigilant for pathogens appearing beyond their typical ranges. The PCR/ESI-MS assay is a single sensitive test that can screen for a wide range of vector-borne pathogens and provide results down to the species level, even in co-infected ticks. The assay has broad applications to pathogen surveillance where specific pathogens that may not be expected.

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Author Disclosure Statement

M.W.E., C.D.C., H.E.M., M.A.R., and D.J.E. are all employees of Ibis Biosciences and Abbott Company, which developed the PCR/ESI-MS assays and instrumentation used in these studies. Assays described are for research use only. H.H., B.M., and O.N. are all employees of the Laboratory of Dr. Brunner, who was contracted to perform analyses.

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