VECTOR-BORNE AND ZOONOTIC DISEASES
Volume 9, Number 2, 2009
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DOI: 10.1089/vbz.2008.0088

Detection of Tick-Borne Pathogens by MassTag Polymerase Chain Reaction

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Abstract

MassTag polymerase chain reaction (PCR) is a platform that enables microbe detection using primers labeled through a photocleavable link with tags that vary in molecular weight. After multiplex PCR, tags are released by ultraviolet irradiation and analyzed by mass spectroscopy. The identification of a microbe in a sample is determined by its cognate tags. Here we describe establishment and implementation of a MassTag PCR panel for surveillance of microbes implicated in tick-vectored infectious diseases. **Key Words:** Aedes—Arbovirus(es)—Birds—Culex—Diagnostics—Mosquito(es)—Vector-borne—Zoonosis.

Introduction

Ticks can harbor many pathogens; thus, a single tick bite may result in polymicrobial infections (Benach et al. 1985, Magnarelli et al. 1995, Krause et al. 1996, Mitchell et al. 1996). Common human-biting ticks associated with pathogen transmission in the United States include *Ixodes scapularis*, *Amblyomma americanum*, *Dermacentor variabilis*, and *Dermacentor andersoni* (Bratton and Corey 2005). Lyme disease, the most common vector-borne disease in the United States, is caused by the spirochete *Borrelia burgdorferi* and transmitted by *I. scapularis* ticks (Burgdorfer et al. 1982). In addition, *I. scapularis* can transmit *Anaplasma phagocytophilum* bacteria, the etiologic agent of human granulocytic anaplasmosis, and the protozoan *Babesia microti*, the agent of babesiosis (Spielman et al. 1979, Pancholi et al. 1995). Other microorganisms detected in *I. scapularis*, notably *Bartonella* species (*spp*.), may have a role in tick-borne infections; however, whether they are transmitted by ticks remains to be determined (Chang et al. 2001, Adelson et al. 2004, Holden et al. 2006). *Dermacentor* ticks (both *D. variabilis* and *D. andersoni*) are vectors of *Rickettsia rickettsii*, the etiologic agent of Rocky Mountain spotted fever, and *Francisella tularensis*, the etiologic agent of tularemia (McDade and Newhouse 1986, Goethert et al. 2004). *Amblyomma americanum* can transmit *Ehrlichia chaffeensis*, the etiologic agent of human monocytic ehrlichiosis, as well as *F. tularensis* (Childs and Paddock 2003). *A. americanum* ticks can also harbor *Borrelia lonestari*, a *Borrelia* species of unclear pathogenicity related to relapsing fever *Borreliae* (Fukunaga et al. 1996, James et al. 2001). *Coxiella burnetii* has been detected in a wide variety of ticks, including *Dermacentor* and *Amblyomma*, and natural tick transmission to humans documented, although this is considered to contribute only a minor component of human acute Q fever (Maurin and Raoult 1999).

We recently described the application of a multiplex polymerase chain reaction (PCR) method for microbial surveillance wherein primers are attached to tags of varying mass that serve as digital signatures for their genetic targets (Briese et al. 2005). Tags are cleaved from primers and recorded by mass spectroscopy enabling sensitive, multiplex microbial detection. The method, MassTag PCR, has been implemented for differential diagnosis of respiratory infection and hemorrhagic fevers (Briese et al. 2005, Lamson et al. 2006, Palacios et al. 2006, Renwick et al. 2007, Briese et al. 2008). In this report, we describe a MassTag PCR assay adapted for rapid screening of field-collected ticks for multiple pathogens. The ease and efficiency of the assay allows rapid analysis of large sample numbers.

Materials and Methods

Species- and genus-specific PCR primer sets were designed from multiple nucleotide sequence alignments of target pathogens using Greene SCPrimer, a program based on set cover theory (Jabado et al. 2006; Tables 1 and 2). Prior to committing to synthesis and conjugation of mass tagged primers, primer set performance was assessed using unmodified primers in singleplex and multiplex PCR assays wherein products were detected by electrophoresis in ethidium bromide-stained agarose gels. Primer pairs were first

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Table 1. MassTag Primer Sequences (5′ to 3′ Direction)

Pathogen/gene target	*Genus/species specific*	*Primer pair*	*Sensitivity (copies/rxn)*
Anaplasma/16S rRNA	Genus	Fwd: GGGCATGTAGGCGGTTCGGT Rev: TCAGCGTCAGTACCGGACCA	<20
Borrelia burgdorferi/flaB	Species	Fwd: AATGACAAAACATATTGRGGAASTTGA Rev: YACAATGACMGATGAGGTTGTRGC	<20
Bartonella spp./pap31	Genus	Fwd: CTTCTGCRGCACAAGCTGCTGAT Rev: CCACCAATATARAAACCTGTCCAAGA	<20
Borrelia lonestari and *miyamotoi/flaB*	Genus	Fwd: AGCACAAGCTTCATGGACATTGA Rev: GAGCCGCTTGAACACCTTCTC	<20
Babesia microti/18S rRNA	Species	Fwd: CTGCCTTGTCATTAATCTCGCTTC Rev: TGCTGTAGTATTCAAGGCRAATGC	<20
Coxiella burnetti/IS1111	Species	Fwd: GCTCCTCCACACGCTTCCAT Rev: GGTTCAACTGTGTGGAATTGATGAGT	<20
Ehrlichia spp./16S rRNA	Genus	Fwd: CGTAAAGGGCACGTAGGTGGACTA Rev: CACCTCAGTGTCAGTATCGAACCA	<200
Francisella tularensis/fopA	Species	Fwd: ATGTTTCGGCATGTGAATAGTTAA Rev: ACCACTGCTTTGTGTAGTAGCTGAA	<20
Rickettsia rickettsii/ompB	Species	Fwd: ATACAAAGTGCTAATGCAACTGGG Rev: GTAAAATTACCGGTAAGGGTTATAGC	<200

Fwd, forward; Rev, reverse; rxn, reaction.

Table 2. Primers Used for Singleplex Confirmatory PCR Assays (5′ to 3′ Direction)

Pathogen/gene target (PCR product length)	*Genus/species specific*	*Primer pair*
A. phagocytophilum/mspA (112 bp)	Species	Fwd: TGTGGGCTTGGGATATGGAC Rev: TTCCTCTCTGTGCACTCGCTC
B. microti/18S rRNA (170 bp)	Species	Fwd: GGGACTTTGCGTTCATAAAACGC Rev: GCAATAATCTATCCCCATCACGAT
Bartonella/16S rRNA (460 bp)	Genus	Fwd: TAGGCGGATATTTAAGTCAGAGGTG Rev: GATCCAGCCTAACTGAAGGAG
B. miyamotoi and *lonestari/flaB* (965 bp)	Genus	Fwd: GGGATTATMAATCATAATACRTCAGC Rev: TTGCTTGTGCAATCATAGCCATTGC
B. burgdorferi/ospA (676 bp)	Species	Fwd: GCGTTTCAGTAGATTTGCCT Rev: TTGGTGCCATTTGAGTCGTA

PCR, polymerase chain reaction; Fwd, forward; Rev, reverse.

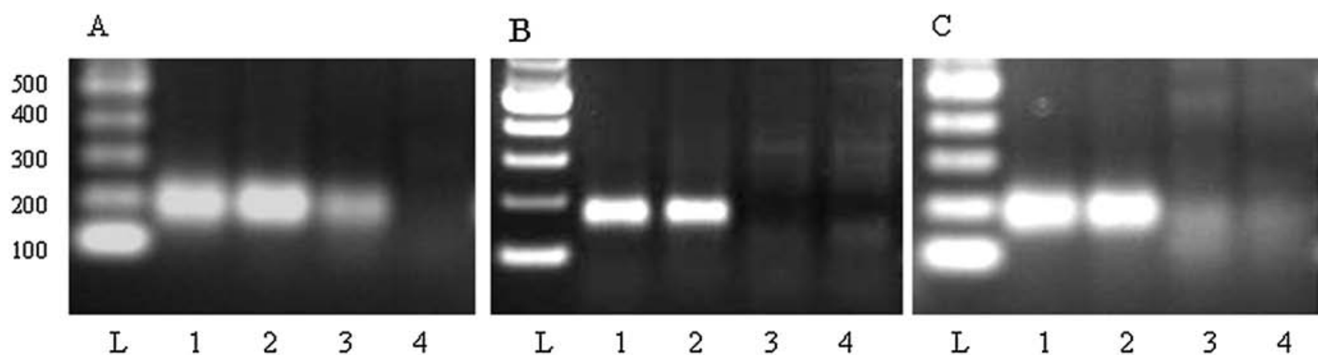

FIG. 1. MassTag PCR primer optimization. Agarose gels representative of polymerase chain reaction assays utilized for *B. microti* primer optimization. Linearized DNA standards were spiked into a background of 25 ng/μL DNA from *Ixodes scapularis* (**A**), *Dermacentor variabilis* (**B**), and *Amblyomma americanum* (**C**) and subjected to singleplex PCR. Lanes 1–4 represent 10^3, 10^2, 10^1, and 0 copies, respectively. Similar assays were performed for all primer pairs in the MassTag panel. L, 100-bp DNA ladder.

tested for specificity in singleplex PCR reactions containing their cognate and irrelevant targets. Thereafter, all primer sets were combined in multiplex reactions to assess for interference in performance. Primer sets that passed quality control tests were conjugated to mass tags (Operon) and incorporated into the panel. DNA standards used for assay development were cloned by PCR from pathogen DNA and ligated into pGEM-T Easy vector (Promega). In our laboratory singleplex PCR assays are typically pursued using 25 ng of tick DNA. Thus, sensitivity assays were performed with 10-fold dilutions of linearized plasmid in a background of 25 ng/μL of tick DNA. For tick assays, live adult ticks were collected in 2006 and 2007 from Suffolk County, New York. Individual ticks were homogenized in sterile H_2O, and total DNA was isolated using Qiaprep DNA kit (Qiagen, Valencia, CA). DNA was resuspended in 20 μL of water, of which 2 μL was used in the MassTag PCR reaction. The specificity of pathogen detection in ticks by MassTag PCR

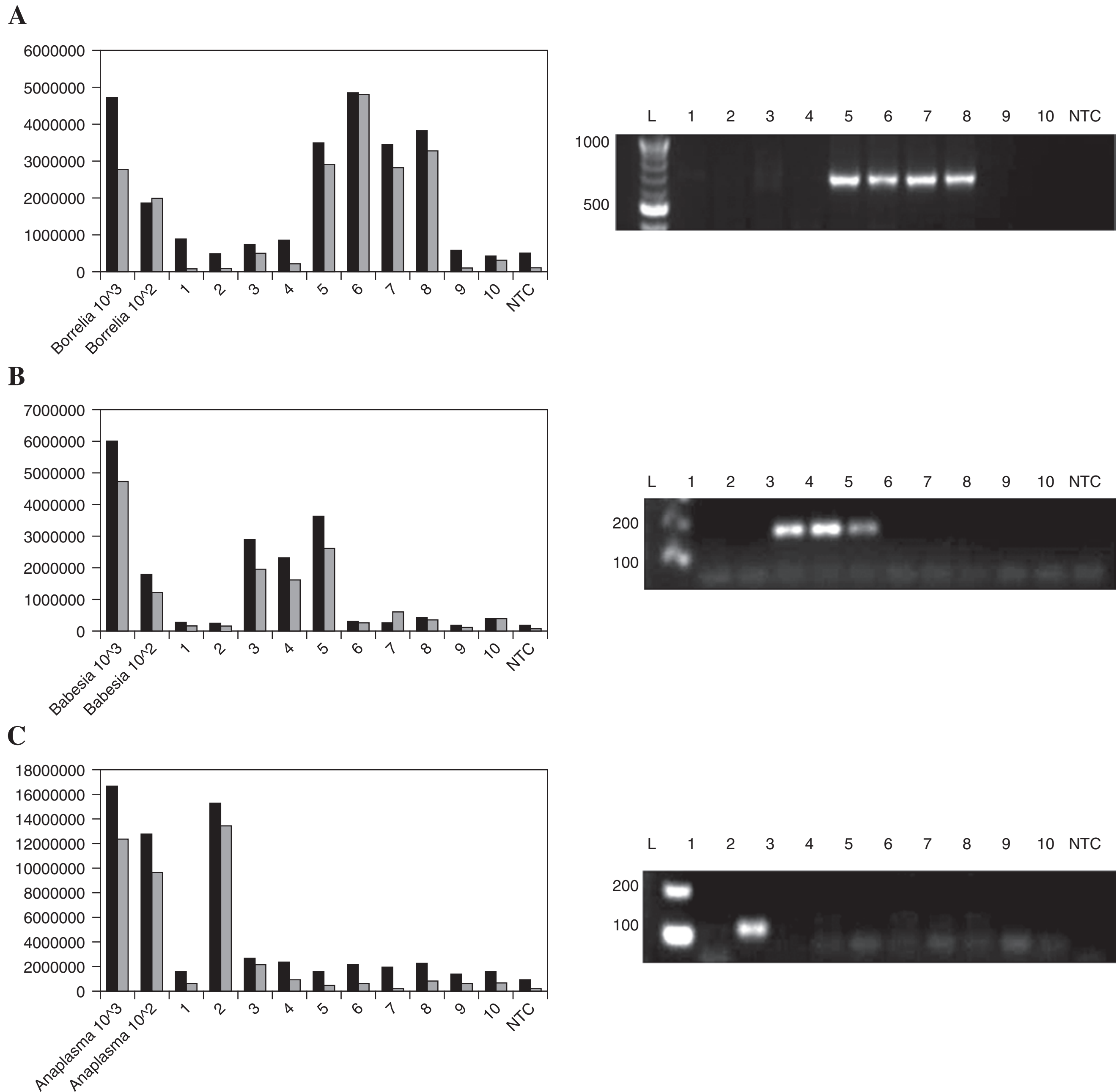

FIG. 2. MassTag polymerase chain reaction (PCR) validation. Signal abundance data (**left**) obtained by MassTag PCR for 10 *Ixodes scapularis* samples (labeled 1–10) and corresponding confirmatory singleplex PCR (**right**) using alternative primer pairs. In MassTag PCR, pathogens are detected by the signal from two tags (indicated by black and gray bars), one attached to each primer (forward and reverse). Both tags must be detected to register as a confirmed signal. (**A**) *Borrelia burgdorferi*; (**B**) *Babesia microti*; (**C**) *Anaplasma phagocytophilum*. Standards correspond to 10^3 and 10^2, respectively. L, 100-bp DNA ladder; NTC, no template control.

TABLE 3. MASSTAG POLYMERASE CHAIN REACTION RESULTS ON FIELD-COLLECTED TICKS

Pathogen	*Ixodes scapularis*	*Dermacentor variabilis*	*Amblyomma americanum*
Anaplasma phagocytophilum	14	0	0
Borrelia burgdorferi	51	0	0
Bartonella henselae	2	0	0
Borrelia lonestari/miyamotoi	4	0	3
Babesia microti	5	0	0
Coxiella burnetti	0	0	0
Ehrlichia chaffensis	0	0	2
Francisella tularensis	0	0	0
Rickettsia rickettsii	0	0	0
Total No. of ticks screened	88	40	55

was confirmed by singleplex PCR followed by sequencing of amplification products.

Results

A MassTag PCR panel was designed to detect known or suspected pathogens transmitted by ticks endemic on the east coast of the United States, including *A. phagocytophilum*, *B. microti*, *Bartonella spp.*, *B. burgdorferi sensu lato*, *B. lonestari*, *Coxiella burnetti*, *Ehrlichia spp.*, *F. tularensis*, and *R. rickettsi*. Specificity was tested using relevant and irrelevant targets. All primer sets amplified only their cognate targets. To model conditions encountered in field samples, sensitivity was tested using serially diluted linearized DNA standards in a background of *I. scapularis*, *D. variabilis*, and *A. americanum* DNA (Fig. 1). Sensitivity for detection of *Ehrlichia* and *R. rickettsii* was <200 copies/reaction; sensitivity for other targets was <20 copies/reaction. To test assay performance in environmental samples, we isolated DNA from 88 individual adult *I. scapularis* ticks collected in Suffolk County, New York in 2006 (Fig. 2). MassTag PCR detected *B. burgdorferi* in 51 (58%) of ticks analyzed (Table 3). For confirmation of assay fidelity, we elected 25 MassTag positive samples for singleplex PCR amplification and sequencing of a 676-bp fragment of the *B. burgdorferi ospA* gene (Fig. 2A). All 25 MassTag *B. burgdorferi* positive samples were positive by singleplex PCR assays. We also selected 15 MassTag negative samples, all of which were negative in singleplex PCR assays. Other agents detected by MassTag PCR included *A. phagocytophilum* (14 *I. scapularis* ticks, four of which also contained *B. burgdorferi*) and *B. microti* (five *I. scapularis* ticks, one of which also contained *B. burgdorferi*; Tables 3 and 4). To test fidelity of these assays, a 112-bp portion of *Anaplasma*-specific *mspA* gene sequence was cloned from 10 *I. scapularis* ticks positive for *A. phagocytophilum* in MassTag PCR. Sequence analysis confirmed the presence of *A. phagocytophilum*. Ten samples negative for *A. phagocytophilum* in MassTag PCR were also negative by singleplex PCR (Fig. 2C). Similar fidelity assays were performed for *B. microti* positive (five samples) and negative samples (10 samples). Singleplex PCR confirmed MassTag results (Fig. 2B). In one *I. scapularis* tick, we detected a triple infection with *B. microti*, *B. burgdorferi*, and *A. phagocytophilum* (Fig. 3).

TABLE 4. MIXED INFECTIONS IN *I. SCAPULARIS* DETECTED BY MASSTAG POLYMERASE CHAIN REACTION

Pathogens detected	*Number of ticks*
Anaplasma phagocytophilum, Borrelia burgdorferi	4
B. burgdorferi, Babesia microti	1
B. burgdorferi, Borrelia miyamotoi	1
A. phagocytophilum, B. burgdorferi, B. microti	1
A. phagocytophilum, B. burgdorferi, B. miyamotoi	1

MassTag PCR assays employ both species- and genus-specific primers (Table 1). Two *I. scapularis* ticks were positive in MassTag PCR assays with genus-specific *Bartonella* primers. A 460-bp informative region of the 16S rRNA was amplified by PCR (Table 2) and sequenced to enable speciation. Both contained *Bartonella henselae*. *B. lonestari* was not detected in any specimen from *I. scapularis*; however, four ticks contained a closely related species, *Borrelia miyamotoi*. For confirmation, a 965-bp region of the *flaB* gene was amplified by PCR (Table 2) and sequenced to enable speciation. All four sequences were *B. miyamotoi*. Like *B. lonestari*, *B. miyamotoi* is a species grouped to the relapsing fever *Borreliae*. It has been previously reported in *I. scapularis* (Scoles et al. 2001). One of the *I. scapularis* ticks infected with *B. miyamotoi* was also coinfected with *B. burgdorferi*. In another sample, we detected a mixed infection with *B. burgdorferi*, *A. phagocytophilum*, and *B. miyamotoi* (Table 4).

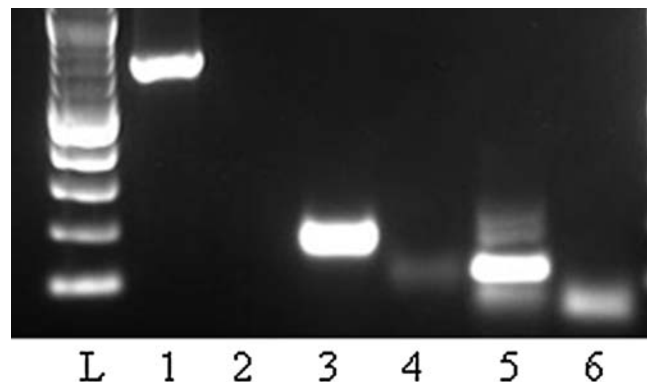

FIG. 3. Singleplex polymerase chain reaction confirmation of polymicrobial infection of a single *Ixodes scapularis* tick. Lane 1, *Borrelia burgdorferi ospA*; lane 3, *Babesia microti 18S rRNA*; lane 5, *Anaplasma phagocytophilum mspA*; lanes 2, 4, and 6 represent *ospA*, *18S rRNA*, and *mspA* no template controls. L, 100-bp DNA ladder.

In additional experiments, we screened *Dermacentor* and *Amblyomma* ticks collected in Suffolk County, in the spring of 2007. DNA from a total of 40 *D. variabilis* and 55 *A. americanum* ticks was isolated and screened by MassTag PCR. *B. lonestari* was detected in three *A. americanum* ticks and *Ehrlichia chaffensis* in two other *Amblyomma* ticks. The presence of these pathogens was confirmed by sequencing.

Discussion

Our results indicate that MassTag PCR is an efficient tool for surveillance of tick microflora. Each assay, comprising up to 20 primer pairs (20 different microbial genetic targets), costs $15 and allows detection of coinfections as well as single infection with sensitivity similar to that obtained with singleplex assays. We typically use a 96-well plate format to simultaneously run 1920 tests. Tagged primers are available from commercial vendors; primers and protocols are freely available; costs for mass spectrometry instruments are approximately $75,000. Although we have not tested for the presence of tick-borne pathogens in human materials, based on previous work in differential diagnosis of respiratory diseases (Lamson et al. 2006, Renwick et al. 2007, Briese et al. 2008) and hemorrhagic fevers (Palacios et al. 2006), the assays reported here may also be of utility in clinical microbiology.

Acknowledgments

We thank Courtney Bolger for *Francisella tularensis fopA* DNA and Brian Fallon and Gustavo Palacios for helpful comments.

Disclosure Statement

Work reported here was supported by NIH awards AI070411, HL83850, NS047537, and U54AI5758 (Northeast Biodefense Center-Lipkin).

References

Adelson, ME, Rao, RV, Tilton, RC, Cabets, K, et al. Prevalence of Borrelia burgdorferi, Bartonella spp., Babesia microti, and Anaplasma phagocytophila in Ixodes scapularis ticks collected in Northern New Jersey. J Clin Microbiol 2004; 42:2799–801.

Benach, JL, Coleman, JL, Habicht, GS, MacDonald, A, et al. Serological evidence for simultaneous occurrences of Lyme disease and babesiosis. J Infect Dis 1985; 152:473–477.

Bratton, RL, Corey R. Tick-borne disease. Am Fam Physician 2005; 71:2323–2330.

Briese, T, Palacios, G, Kokoris, M, Jabado, O, et al. Diagnostic system for rapid and sensitive differential detection of pathogens. Emerg Infect Dis 2005; 11:310–313.

Briese, T, Renwick, N, et al. Global distribution of novel rhinovirus genotype. Emerg Infect Dis 2008; 14:944–947.

Burgdorfer, W, Barbour, AG, Hayes, SF, Benach, JL, et al. Lyme disease: A tick-borne spirochetosis? Science 1982; 216:1317–1319.

Chang, CC, Chomel, BB, Kasten, RW, Romano, V et al. Molecular evidence of Bartonella spp. in questing adult Ixodes pacificus ticks in California. J Clin Microbiol 2001; 39:1221–1226.

Childs, JE, Paddock, CD. The ascendancy of Amblyomma americanum as a vector of pathogens affecting humans in the United States. Annu Rev Entomol 2003; 48:307–337.

Fukunaga, M, Okada, K, Nakao, M, Konishi, T, et al. Phylogenetic analysis of Borrelia species based on flagellin gene sequences and its application for molecular typing of Lyme disease borreliae. Int J Syst Bacteriol 1996; 46:898–905.

Goethert, HK, Shani, I, Telford, SR III. Genotypic diversity of Francisella tularensis infecting Dermacentor variabilis ticks on Martha's Vineyard, Massachusetts. J Clin Microbiol 2004; 42:4968–4973.

Holden, K, Boothby, JT, Kasten, RW, Chomel, BB, et al. Co-detection of Bartonella henselae, Borrelia burgdorferi, and Anaplasma phagocytophilum in Ixodes pacificus ticks from California, USA. Vector Borne Zoonotic Dis 2006; 6:99–102.

Jabado, OJ, Palacios, G, Kapoor, V, Hui, J, et al. Greene SCPrimer: a rapid comprehensive tool for designing degenerate primers from multiple sequence alignments. Nucleic Acids Res 2006; 34:6605–6611.

James, AM, Liveris, D, Wormser, GP, Schwartz, I, et al. Borrelia lonestari infection after a bite by an Amblyomma americanum tick. J Infect Dis 2001; 183:1810–1814.

Krause, PJ, Telford, SR 3rd, Spielman, A, Sikand, V, et al. Concurrent Lyme disease and babesiosis. Evidence for increased severity and duration of illness. JAMA 1996; 275:1657–1660.

Lamson, D, Renwick, N, Kapoor, V, Liu, Z, et al. MassTag polymerase-chain-reaction detection of respiratory pathogens, including a new rhinovirus genotype, that caused influenza-like illness in New York State during 2004-2005. J Infect Dis 2006; 194:1398–1402.

Magnarelli, LA, Dumler, JS, Anderson, JF, Johnson, RC, et al. Coexistence of antibodies to tick-borne pathogens of babesiosis, ehrlichiosis, and Lyme borreliosis in human sera. J Clin Microbiol 1995; 33:3054–3057.

Maurin, M, Raoult, D. Q fever. Clin Microbiol Rev 1999; 12:518–553.

McDade, JE, Newhouse, VF. Natural history of Rickettsia rickettsii. Annu Rev Microbiol 1986; 40:287–309.

Mitchell, PD, Reed, KD, Hofkes, JM. Immunoserologic evidence of coinfection with Borrelia burgdorferi, Babesia microti, and human granulocytic Ehrlichia species in residents of Wisconsin and Minnesota. J Clin Microbiol 1996; 34:724–727.

Palacios, G, Briese, T, Kapoor, V, Jabado, O, et al. MassTag polymerase chain reaction for differential diagnosis of viral hemorrhagic fever. Emerg Infect Dis 2006; 12:692–695.

Pancholi, P, Kolbert, CP, Mitchell, PD, Reed, KD, Jr. et al. Ixodes dammini as a potential vector of human granulocytic ehrlichiosis. J Infect Dis 1995; 172:1007–1012.

Renwick, N, Schweiger, B, Kapoor, V, Liu, Z, et al. A recently identified rhinovirus genotype is associated with severe respiratory-tract infection in children in Germany. J Infect Dis 2007; 196:1754–1760.

Scoles, GA, Papero, M, Beati, L, Fish, D. A relapsing fever group spirochete transmitted by Ixodes scapularis ticks. Vector Borne Zoonotic Dis 2001; 1:21–34.

Spielman, A, Clifford, CM, Piesman, J, Corwin, MD. Human babesiosis on Nantucket Island, USA: description of the vector, Ixodes (Ixodes) dammini, n. sp. (Acarina: Ixodidae). J Med Entomol 1979; 15:218–234.

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