JOURNAL OF CLINICAL MICROBIOLOGY, July 2004, p. 3164–3168 Vol. 42, No. 7
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.7.3164–3168.2004

Multiplex Real-Time PCR for Detection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi*

Joshua W. Courtney,[1] Leah M. Kostelnik,[1] Nordin S. Zeidner,[2] and Robert F. Massung[1]*

Viral and Rickettsial Zoonoses Branch, Division of Viral and Rickettsial Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia,[1] and Bacterial Zoonoses Branch, Division of Vector-Borne Diseases, Centers for Disease Control and Prevention, Fort Collins, Colorado[2]

Received 21 January 2004/Returned for modification 15 February 2004/Accepted 20 March 2004

A multiplex real-time PCR assay was developed for the simultaneous detection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi*. The assay was tested on various *Anaplasma*, *Borrelia*, *Erhlichia*, and *Rickettsia* species, as well as on *Bartonella henselae* and *Escherichia coli*, and the assay was found to be highly specific for *A. phagocytophilum* and the *Borrelia* species tested (*B. burgdorferi*, *B. parkeri*, *B. andersonii*, and *B. bissettii*). The analytical sensitivity of the assay is comparable to that of previously described nested PCR assays (*A. phagocytophilum*, 16S rRNA; *B. burgdorferi*, *fla* gene), amplifying the equivalent of one-eighth of an *A. phagocytophilum*-infected cell and 50 borrelia spirochetes. The dynamic range of the assay for both *A. phagocytophilum* and *B. burgdorferi* was ≥4 logs of magnitude. Purified DNA from *A. phagocytophilum* and *B. burgdorferi* was spiked into DNA extracted from uninfected ticks and from negative control mouse and human bloods, and these background DNAs were shown to have no significant effect on sensitivity or specificity of the assay. The assay was tested on field-collected *Ixodes scapularis* ticks and shown to have 100% concordance compared to previously described non-probe-based PCR assays. To our knowledge, this is the first report of a real-time multiplex PCR assay that can be used for the simultaneous and rapid screening of samples for *A. phagocytophilum* and *Borrelia* species, two of the most common tick-borne infectious agents in the United States.

Lyme disease and human anaplasmosis (formerly human granulocytic ehrlichiosis) have emerged as two of the most common vector-borne bacterial illnesses in the United States, where *Borrelia burgdorferi* and *Anaplasma phagocytophilum* have been identified as their respective etiologic agents (3–5, 20). Both *B. burgdorferi* and *A. phagocytophilum* share common tick vectors and rodent reservoirs, with *Ixodes scapularis* characterized as the principal vector species and the white-footed mouse, *Peromyscus leucopus*, as a major reservoir species (3, 14, 19, 22, 25). Lyme disease and human anaplasmosis are found in overlapping regions of the United States, with most cases occurring in the northeast and upper midwest regions, areas that have large populations of both rodents and *I. scapularis* ticks.

The screening of tick and rodent samples for *B. burgdorferi* and *A. phagocytophilum* has been used for studies designed to assess the prevalence of these agents in nature and the risk they pose to the human population. Numerous methods for the detection of *B. burgdorferi* and *A. phagocytophilum* have been described and include antibody and antigen detection assays (immunofluorescence assay, enzyme-linked immunosorbent assay, and Western blotting), culture isolation, and PCR-based assays. PCR assays have been shown to be both sensitive and specific for the detection of *B. burgdorferi* and *A. phagocytophilum* in clinical and field-collected samples (5, 11, 17, 18, 23, 26). However, PCR assays can be time-consuming and labor-intensive, particularly when testing for multiple pathogens in a large number of samples. Multiplex PCR assays pose an advantage to single detection systems because they allow for the simultaneous detection of multiple organisms. Real-time PCR assays, including TaqMan assays using fluorogenic 5′-nuclease methods, eliminate post-PCR processing and the need for agarose gel electrophoresis of amplification products. The combination of multiplex capabilities with real-time PCR provides a high-throughput method for the detection of multiple pathogens in a single-tube assay format. The present study describes the development and validation of a real-time, multiplex PCR assay for the rapid, sensitive, and specific detection of *B. burgdorferi* and *A. phagocytophilum* in experimental samples.

* Corresponding author. Mailing address: Centers for Disease Control and Prevention, 1600 Clifton Rd., MS G-13, Atlanta, GA 30333. Phone: (404) 639-1082. Fax: (404) 639-4436. E-mail: rfm2@cdc.gov.

MATERIALS AND METHODS

Bacterial strains and DNA extraction. *A. phagocytophilum* strains (USG3, MRK, and Webster) were grown in HL-60 cells, and *B. burgdorferi* strains (B31 and JD-1) were grown in BSK-H medium as previously described (6, 27). DNA was extracted from culture grown stocks of each agent and from uninfected EDTA-treated blood from humans (200 μl) and mice (50 μl) by using the QIAamp DNA blood kit (Qiagen, Inc., Valencia, Calif.) according to the manufacturer's recommended protocol. DNA was extracted from uninfected laboratory-reared questing nymphal *I. scapularis* ticks and from questing female *I. scapularis* collected in Connecticut by using either the DNeasy tissue kit (Qiagen) or the IsoQuick nucleic acid extraction kit (Orca Research, Bothell, Wash.). The DNAs from uninfected sources (human, mouse, or tick) were used as diluents to make the serial dilutions for background sensitivity testing. The specificity of the TaqMan assay was tested with DNA extracted from the following organisms: *Rickettsia rickettsii* Sheila Smith strain, *Anaplasma marginale* Florida strain, *Rickettsia prowazekii* Brienl strain, *Escherichia coli* JM109 strain, *Bartonella henselae* Houston-1 strain, *Ehrlichia canis* Oklahoma strain, *Ehrlichia chaffeensis* Arkansas strain, *Neorickettsia sennetsu* Miyayama strain, *Borrelia bissettii* strain CO-504, *Borrelia andersonii* strain 21038, and *Borrelia parkeri* (9).

***B. burgdorferi*-specific and *A. phagocytophilum*-specific probe and primer design.** Oligonucleotide probe and primer sequences were designed to be specific for *B. burgdorferi* 23S rRNA and *A. phagocytophilum msp2* genes by using Primer Express software (Perkin-Elmer/Applied Biosystems, Foster City, Calif.). Primer sequences specific for the *B. burgdorferi* 23S rRNA genes were identified as Bb23Sf (5′-CGAGTCTTAAAAGGGCGATTTAGT) and Bb23Sr (5′-GCTTC AGCCTGGCCATAAATAG) to generate a 75-bp fragment with a TaqMan

probe identified as Bb23Sp-FAM (5′-AGATGTGGTAGACCCGAAGCCGAGTG) and labeled at the 5′ and 3′ ends with 6-carboxy-fluorescein (6-FAM) and 6-carboxyl-tetramethyl-rhodamine (TAMRA), respectively. Primer sequences specific for the *A. phagocytophilum msp2* gene were identified as ApMSP2f (5′-ATGGAAGGTAGTGTTGGTTATGGTATT) and ApMSP2r (5′-TTGGTCTTGAAGCGCTCGTA) to generate a 77-bp fragment by using a TaqMan probe identified as ApMSP2p-HEX (5′-TGGTGCCAGGGTTGAGCTTGAGATTG) and labeled at the 5′ and 3′ ends with hexachloro-6-carboxy-fluorescein (HEX) and TAMRA, respectively. Sequences were evaluated on the basis of the following criteria: predicted cross-reactivity with closely related organisms, internal primer binding properties for hairpin and primer-dimer potential, length of the desired amplicon, G-C content, and melting temperatures (T_ms) of probes and primers.

Optimized conditions for multiplex PCR. PCR was performed in a multiplex format with a reaction volume of 25.0 μl, by using the Brilliant Quantitative PCR core reagent kit with SureStart *Taq* DNA polymerase (Stratagene, La Jolla, Calif.) in a Bio-Rad iCycler quantitative thermal cycler (Bio-Rad, Hercules, Calif.). Optimal reaction conditions used 6 mM MgCl, primers Bb23Sf and Bb23Sr at 700 nM each, probe Bb23Sp-FAM at 175 nM, primers ApMSP2f and ApMSP2r at 900 nM each, probe ApMSP2p-HEX at 125 nM, and 2 μl of template DNA. Cycling conditions included an initial activation of the *Taq* DNA polymerase at 95°C for 10 min, followed by 40 cycles of a 15-s denaturation at 95°C followed by a 1-min annealing-extension step at 60°C.

Sensitivity and specificity testing. Previously described non-TaqMan-based, nested PCR assays that amplify the 16S rRNA gene from *A. phagocytophilum* (17) and the flagellin gene from *B. burgdorferi* (11) were used to evaluate the relative analytical sensitivity of the multiplex TaqMan assay. Reactions were performed as previously described and stored at 4°C until analysis by electrophoresis in a 2% agarose gel and staining with ethidium bromide. The analytical sensitivity standards for the *A. phagocytophilum* assays were samples for which the number of infected HL-60 cells per milliliter previously had been determined. The analytical sensitivity standards for the *B. burgdorferi* assays were samples for which the number of spirochetes had previously been determined.

Two other nonnested, non-probe-based PCR assays were used to evaluate the relative diagnostic sensitivity and specificity of the multiplex TaqMan assay. These assays amplified the *fla* gene of *B. burgdorferi* (12) and the *msp2* gene of *A. phagocytophilum* (18, 28). Reactions were performed as previously described and stored at 4°C until analyzed by agarose gel electrophoresis as described above.

Statistical analysis. The statistical significance of differences in the threshold cycle (C_t) values found when testing the multiplex assay in various backgrounds (water, human, tick, and mouse) was evaluated by using the Wilcoxon rank-sum test and the median test. Initially, the water results were compared to the natural backgrounds of field-collected and clinical samples (human, tick, and mouse) by using either one-sided or two-sided *P* values depending on whether the distributions overlapped. Subsequently, similar analyses were performed to compare the human, tick, and mouse distributions. On the basis of the relatively small sample size and, to avoid possible statistical significance due to chance alone, the significance level of 0.05 was adjusted by using the Bonferroni adjustment ($P = 0.05/3 = 0.017$).

RESULTS

Multiplex assay specificity. The real-time multiplex assay amplified all *Borrelia* species and strains tested (*B. burgdorferi* strains JD-1 and B31, *B. bissettii*, *B. andersonii*, and *B. parkeri*) and the *A. phagocytophilum* strains USG3, MRK, and Webster. Genomic DNAs from *A. marginale*, *R. rickettsii*, *R. prowazekii*, *E. coli*, *Bartonella henselae*, *E. canis*, *E. chaffeensis*, and *N. sennetsu* were not amplified by the multiplex PCR assay (data not shown).

Multiplex assay analytical sensitivity. The limit of detection of the multiplex PCR was comparable to that of traditional nested PCR assays amplifying a dynamic range of ≥4 logs of magnitude, with the ability to detect the equivalent of 50 borrelial spirochetes and one-eighth of an *A. phagocytophilum*-infected cell and at respective C_t values of 33.9 and 32.6 cycles (Fig. 1 and 2). Reaction efficiencies were determined to be consistent among strains of *B. burgdorferi* and *A. phagocytophilum* and consistently yielded efficiencies of >90.0%.

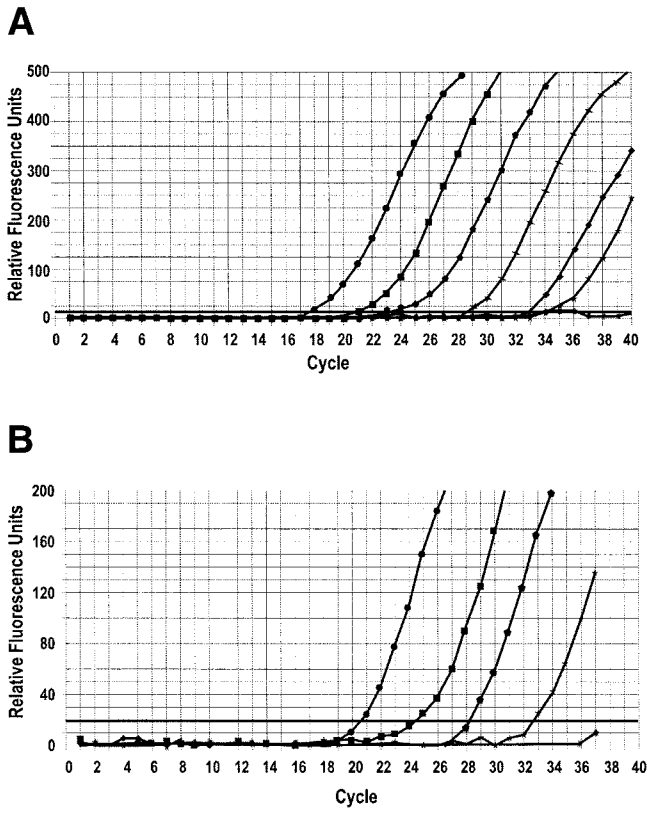

FIG. 1. Amplification plots showing the analytical sensitivity of the real-time multiplex PCR assay for *B. burgdorferi* (A) and *A. phagocytophilum* (B). The results from the amplification of 10-fold serial dilutions ranging from 5×10^6 to 50 *B. burgdorferi* spirochetes (C_t values 17.3, 21.0, 23.1, 28.5, 32.8, and 33.9) and from 125 to 0.125 *A. phagocytophilum*-infected HL-60 cells (C_t values 20.6, 24.4, 28.2, and 32.6) are shown. C_t values represent the cycle (indicated on the *x* axis) at which the fluorescence intensity (indicated by relative fluorescence units on the *y* axis) reached the threshold level.

Effect of background DNA on PCR amplification. DNA purified from *B. burgdorferi* or *A. phagocytophilum* was spiked into reaction mixtures containing no background template DNA (water), DNA extracted from human or white-footed mouse blood samples, or DNA extracted from *I. scapularis* ticks that tested PCR negative for *B. burgdorferi* and *A. phagocytophilum*. Fivefold serial dilutions for each agent were tested in triplicate by using the multiplex assay, and the resultant C_t values are shown in Tables 1 and 2. The C_t values remained relatively consistent for each background that was tested, with no statistically significant differences based on the Wilcoxon rank-sum and median tests.

Comparison of multiplex assay to traditional PCR assays with field-collected ticks. The utility of the TaqMan assay for the rapid screening of field-collected ticks for *B. burgdorferi* and *A. phagocytophilum* was assessed by testing 41 host-seeking female ticks collected in Connecticut. DNA extracted from each tick was tested by using both direct and nested non-probe-based PCR assays (*fla* gene for *B. burgdorferi*; *msp2* gene for *A. phagocytophilum*) and the multiplex TaqMan assay (11, 12, 18, 28). This testing showed 100% concordance between the

TABLE 1. Effect of DNA background on the amplification of *B. burgdorferi*

Dilution[a]	Threshold cycles (C_t) in various DNA backgrounds[b]			
	Water	Human	Tick	Mouse
10^{-1}	20.6, 20.7, 20.9	20.6, 20.3, 20.2	20.7, 20.6, 20.4	20.1, 20.4, 20.5
10^{-2}	23.9, 23.7, 23.8	23.6, 23.8, 23.7	23.9, 23.7, 23.8	23.6, 23.7, 23.7
10^{-3}	27.5, 27.7, 27.6	27.8, 27.2, 27.6	27.5, 27.2, 27.8	27.0, 27.3, 27.2
10^{-4}	31.1, 31.1, 31.7	31.3, 31.8, 30.9	30.4, 30.6, 30.6	30.7, 31.4, 30.9
10^{-5}	35.9, 36.3, 35.2	34.7, 34.6, 35.5	34.7, 34.4, 34.5	34.1, 34.0, 33.5

[a] Values represent the dilution factors using DNA extracted from *B. burgdorferi* strain B31 grown in BSK-H medium. The diluent for each dilution series consisted of undiluted DNA extracted from 200 μl of EDTA-treated whole blood (human), a single adult *I. scapularis* tick (tick), or 50 μl of EDTA-treated whole blood (mouse).

[b] Each value represents the threshold cycle from one of three separate real-time multiplex PCR amplifications.

standard and multiplex real-time assays. Of the 41 ticks examined, 15 were positive only for *B. burgdorferi*, 7 were positive only for *A. phagocytophilum*, and 11 ticks tested positive for both *B. burgdorferi* and *A. phagocytophilum*. Eight ticks were negative for both pathogens.

DISCUSSION

Lyme disease and human anaplasmosis are two of the most common vector-borne zoonotic diseases in the United States, and their respective agents, *B. burgdorferi* and *A. phagocytophilum*, share many of the same vectors and reservoirs and have a similar geographic distribution (3–5, 19, 20, 22, 25). In tick-endemic areas and at the time of the year when ticks are actively seeking hosts, a group of several workers may collect upwards of 1,000 ticks per day. Therefore, a real-time, multiplex assay would be extremely beneficial for screening such large numbers of samples. We describe here a novel fluorogenic multiplex assay for the simultaneous detection of *B. burgdorferi* and *A. phagocytophilum*, with the 23S rRNA and *msp2* genes, respectively, as amplification targets.

Many real-time PCR assays have been developed as a means for quantifying the number of viral or bacterial pathogens within a given sample. For these quantitative applications, the number of copies of the gene that is targeted must be known, and most quantitative assays are designed to amplify single-copy genes. In contrast to these quantitative assays, our multiplex assay purposely targets multicopy genes in order to enhance the sensitivity of the assay. The genome sequence of *B. burgdorferi* has been determined, and there are two highly conserved copies of the 23S rRNA gene that our assay targets (8). Although the 23S rRNA gene is highly conserved among *Borrelia* species, the borrelia primers and probe were designed to amplify a region of the gene that is either not present in the 23S gene of other bacteria (*Anaplasma*, *Rickettsia*, and *Ehrlichia* species) or is present but highly divergent (*E. coli*, *Vibrio cholerae*, and gram-positive bacteria). Phylogenetic diversity among *Borrelia* isolates has provided evidence of at least 10 unique spirochetal Lyme agents that have been characterized, on the basis of molecular analyses (*ospC*, rRNA genes, intergenic spacers, and *fla* genes), as *B. burgdorferi* sensu lato (2, 8). Given the genotypic heterogeneity among the *Borrelia* spp., the high conservation of the 23S rRNA gene provides a prime target for detection of these organisms (13). The 23S rRNA primers and probe that are used in the multiplex assay were designed to detect *Borrelia* spp. closely related to *B. burgdorferi*, and this was confirmed by our results.

The *A. phagocytophilum msp2* gene encodes a 44-kDa immunodominant outer membrane protein that has been suggested to be unique to *Anaplasma* species (10, 21, 30). Supporting this theory, the real-time multiplex assay was very specific for *A. phagocytophilum* and did not amplify the DNA of any other bacterial species that was tested. Recent studies have shown that the genome of *A. phagocytophilum* may contain >80 copies of *msp2* paralogs, including full-length genes and truncated pseudogenes (1, 7). The *msp2* genes that have been sequenced show a highly variable central region flanked by conserved amino- and carboxy-terminal regions (21, 29). Our primers were designed specifically to amplify the conserved amino-terminal coding region of the *msp2* gene and to amplify the multiple copies of the gene that are in the *A. phagocytophilum* genome, thereby increasing the sensitivity of the assay. Despite the location of the primers and probe in a conserved region of the *msp2* gene the assay did not detect *A. marginale*, the closely related agent of bovine anaplasmosis. The genome of *A. marginale* also encodes an *msp2* gene family with 60 to 66% similarity to the *A. phagocytophilum msp2* paralogs (1). Examination of the *A. phagocytophilum* primer and probe sequences used in the multiplex assay revealed that the reverse primer has ≤50% identity to the *msp2* genes of *A. marginale* and is probably the reason the assay did not detect this agent. The limited homology of the *A. phagocytophilum* reverse primer to the sequences of *msp2* homologues from *Anaplasma ovis* (GenBank accession numbers AF299052 to AF299058) and *Anaplasma centrale* (GenBank accession numbers AY040556 to AY040563) suggests that the assay will also not amplify the DNA of either *A. ovis* or *A. centrale*, although this has not been demonstrated experimentally. However, it should be noted that the multiplex assay will detect but not differentiate strains and closely related genetic variants of *A. phagocytophilum*. These variants have not been associated with human infections, and at least one variant strain has demonstrated reser-

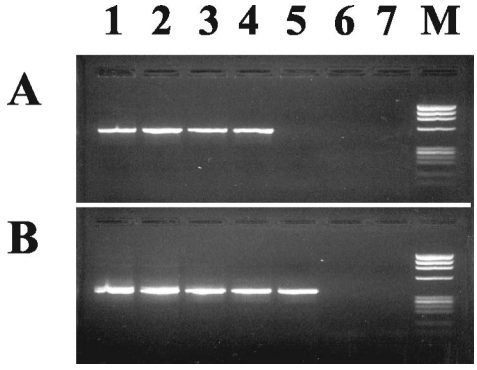

FIG. 2. Determination of the analytical sensitivity of previously published nested PCR assays for *B. burgdorferi* (11) and *A. phagocytophilum* (17). The results from the amplification of 10-fold serial dilutions ranging from 125 to 1.25×10^{-3} *A. phagocytophilum*-infected HL-60 cells (upper lanes 1 to 6) and from 5×10^{5} to 5 *B. burgdorferi* spirochetes (lower lanes 1 to 6) are shown on a 2% agarose gel stained with ethidium bromide. Lanes 7 are negative controls, and lanes M contain phage ϕX174 DNA digested with HaeIII.

TABLE 2. Effect of DNA background on the amplification of *A. phagocytophilum*

Dilution[a]	Threshold cycles (C_t) in various DNA backgrounds[b]			
	Water	Human	Tick	Mouse
10^{-3}	19.2, 19.2, 19.6	18.8, 18.8, 18.8	18.3, 18.6, 18.9	18.8, 18.7, 18.9
10^{-4}	22.1, 22.4, 22.5	22.1, 22.0, 21.9	21.4, 21.4, 21.7	21.7, 21.6, 21.7
10^{-5}	26.9, 26.9, 27.2	25.8, 25.7, 25.3	25.1, 25.0, 26.1	25.0, 24.8, 24.7
10^{-6}	30.1, 30.2, 30.5	29.4, 29.0, 29.1	29.3, 29.0, 29.4	28.6, 29.1, 28.5
10^{-7}	34.1, 34.1, 33.5	33.8, 32.7, 33.3	32.8, 32.4, 33.1	34.0, 33.8, 32.8

[a] Values represent the dilution factor using DNA extracted from *A. phagocytophilum* strain USG3 grown in HL-60 cells. The diluent for each dilution series consisted of undiluted DNA extracted from 200 μl of EDTA-treated whole blood (human), a single adult *I. scapularis* tick (tick), or 50 μl of EDTA-treated whole blood (mouse).

[b] Each value represents the threshold cycle from one of three separate real-time multiplex PCR amplifications.

voir host tropism distinct from the *A. phagocytophilum* strains that have been associated with human infections (15–17). Amplification and sequencing of the 16S rRNA gene or another conserved genetic marker remains necessary to differentiate closely related strains and will be needed to identify any novel strains or species of *Anaplasma* that the assay may detect.

This analytical sensitivity of the real-time multiplex assay (*B. burgdorferi*, 50 spirochetes; *A. phagocytophilum*, 0.125 infected cells) was comparable to previously described nested PCR assays for both agents (*B. burgdorferi*, *fla* gene; *A. phagocytophilum*, 16S rRNA) (11, 17). The number of *A. phagocytophilum* bacteria that the assay can detect is difficult to determine because the bacteria are obligately intracellular and form large clusters, referred to as morulae, in infected cells. However, the sensitivity of the multiplex assay for *A. phagocytophilum* was equivalent to a previously published nested assay that was shown to detect <2 copies of the single-copy 16S rRNA gene, suggesting that the multiplex assay also can detect <2 copies of genomic DNA (17).

The multiplex assay showed 100% concordance compared to previously described nonmultiplex assays by using a cohort of host-seeking female *I. scapularis* ticks. Recent studies have demonstrated that inhibitors of PCR amplification may be present in both engorged and unengorged ticks, possibly preventing accurate determination of *B. burgdorferi* and *A. phagocytophilum* infection rates (24). However, we found that DNA extracted from *I. scapularis* ticks, human EDTA-treated whole blood, or EDTA-treated blood from *P. leucopus* mice had no significant affect on the sensitivity and specificity of the multiplex assay.

In summary, we have developed a real-time multiplex assay for the detection of *B. burgdorferi* and *A. phagocytophilum* that shows sensitivity and specificity comparable to that of traditional nested PCR assays but offers the advantage of simultaneous detection of both agents and eliminates the need for a second-round of amplification or for agarose gel electrophoretic analysis of amplified products. The combination of multiplex capabilities with fluorescence-based detection provides the most efficient means of identifying samples harboring *B. burgdorferi* and *A. phagocytophilum*. For example, this assay will enable researchers to detect both agents in up to 96 DNA-extracted samples (using a 96-well plate) by setting up 96 reactions compared to the traditional nested assays, which would require 384 reactions ([96 primary reactions × 2 agents] + [96 nested reactions × 2 agents]) and subsequent postamplification detection by gel analysis. Therefore, the multiplex assay results in significant savings in time, reagents, thermal cycler usage, and overall cost and should facilitate molecular epidemiologic studies that require the screening of large numbers of potential vector and reservoir populations for *B. burgdorferi* and *A. phagocytophilum*.

ACKNOWLEDGMENTS

This study was supported in part by the APHL-CDC Emerging Infectious Diseases Fellowship Program.

We are grateful to Michael Levin for supplying the field-collected ticks; Marc Dolan and Joseph Piesman for contributing the *Borrelia* controls; Tony Barbet for the *A. marginale* DNA control; Eric Mandel for assisting with the statistical analyses; and Danielle Ross, Emily Lapkowitz, and Brooke Helfer for excellent technical assistance. We thank the CDC Scientific Resources Program for the synthesis of oligonucleotides and TaqMan probe and Michael Levin and Gregory Dasch for review of the manuscript and useful suggestions.

REFERENCES

1. **Barbet, A. F., P. F. M. Meeus, M. Belanger, M. V. Bowie, J. Yi, A. M. Lundgren, A. R. Alleman, S. J. Wong, F. K. Chu, U. G. Munderloh, and S. D. Jauron.** 2003. Expression of multiple outer membrane protein sequence variants from a single genomic locus of *Anaplasma phagocytophilum.* Infect. Immun. **71:**1706–1718.
2. **Barbour, A. G., and B. I. Restrepo.** 2000. Antigenic variation in vector-borne pathogens. Emerg. Infect. Dis. **6:**449–457.
3. **Burgdorfer, W., A. G. Barbour, S. F. Hayes, J. L. Benach, E. Grunwaldt, and J. P. Davis.** 1982. Lyme disease: a tick-borne spirochetosis. Science **216:** 1317–1319.
4. **Centers for Disease Control and Prevention.** 2002. Lyme disease—United States, 2000. Morb. Mortal. Wkly. Rep. **51:**29–31.
5. **Chen, S.-M., J. S. Dumler, J. S. Bakken, and D. H. Walker.** 1994. Identification of a granulocytotropic *Ehrlichia* species as the etiologic agent of human disease. J. Clin. Microbiol. **32:**589–595.
6. **Dolan, M. C., J. Piesman, M. L. Mbow, G. O. Maupin, O. Peter, M. Brossard, and W. T. Golde.** 1998. Vector competence of *Ixodes scapularis* and *Ixodes ricinus* (Acari: Ixodidae) for three genospecies of *Borrelia burgdorferi.* J. Med. Entomol. **35:**465–470.
7. **Felek, S., S. Telford III, R. C. Falco, and Y. Rikihisa.** 2004. Sequence analysis of *p44* homologs expressed by *Anaplasma phagocytophilum* in infected ticks feeding on naive hosts and in mice infected by tick attachment. Infect. Immun. **72:**659–666.
8. **Fraser, C. M., S. Casjens, W. M. Huang, G. G. Sutton, R. Clayton, R. Lathigra, O. White, K. A. Ketchum, R. Dodson, E. K. Hickey, M. Gwinn, B. Dougherty, J. F. Tomb, R. D. Fleischmann, D. Richardson, J. Peterson, A. R. Kerlavage, J. Quackenbush, S. Salzberg, M. Hanson, R. van Vugt, N. Palmer, M. D. Adams, J. Gocayne, J. C. Venter, et al.** 1997. Genomic sequence of a Lyme disease spirochete, *Borrelia burgdorferi.* Nature **390:**580–586.
9. **Gage, K. L., M. E. Eggleston, R. D. Gilmore, Jr., M. C. Dolan, J. A. Montenieri, D. T. Tanda, and J. Piesman.** 2001. Isolation and characterization of *Borrelia parkeri* in *Ornithodoros parkeri* (Ixodida: Argasidae) collected in Colorado. J. Med. Entomol. **38:**665–674.
10. **Ijdo, J. W., W. Sun, Y. Zhang, L. A. Magnarelli, and E. Fikrig.** 1998. Cloning of the gene encoding the 44-kilodalton antigen of the agent of human granulocytic ehrlichiosis and characterization of the humoral response. Infect. Immun. **66:**3264–3269.
11. **Johnson, B. J. B., C. M. Happ, L. W. Mayer, and J. Piesman.** 1992. Detection of *Borrelia burgdorferi* in ticks by species-specific amplification of the flagellin gene. Am. J. Trop. Med. Hyg. **47:**730–741.
12. **Levin, M. L., and D. Fish.** 2000. Acquisition of coinfection and simultaneous transmission of *Borrelia burgdorferi* and *Ehrlichia phagocytophila* by *Ixodes scapularis* ticks. Infect. Immun. **68:**2183–2186.
13. **Liveris, D., S. Varde, R. Iyer, S. Koenig, S. Bittker, D. Cooper, D. McKenna, J. Nowakowski, R. B. Nadelman, G. P. Wormser, and I. Schwartz.** 1999. Genetic diversity of *Borrelia burgdorferi* in Lyme disease patients as determined by culture versus direct PCR with clinical specimens. J. Clin. Microbiol. **37:**565–569.
14. **Magnarelli, L. A., J. F. Anderson, K. C. Stafford III, and J. S. Dumler.** 1997. Antibodies to multiple tick-borne pathogens of babesiosis, ehrlichiosis, and Lyme borreliosis in white-footed mice. J. Wildl. Dis. **33:**466–473.
15. **Massung, R. F., M. J. Mauel, J. H. Owens, N. Allan, J. W. Courtney, K. C. Stafford III, and T. N. Mather.** 2002. Genetic variants of *Ehrlichia phagocytophila*, Rhode Island and Connecticut. Emerg. Infect. Dis. **8:**467–472.

16. **Massung, R. F., R. A. Priestley, N. J. Miller, T. N. Mather, and M. L. Levin.** 2003. Inability of a variant strain of *Anaplasma phagocytophilum* to infect mice. J. Infect. Dis. **188:**1757–1763.
17. **Massung, R. F., K. Slater, J. H. Owens, W. L. Nicholson, T. N. Mather, V. B. Solberg, and J. G. Olson.** 1998. Nested PCR assay for detection of granulocytic ehrlichiae. J. Clin. Microbiol. **36:**1090–1095.
18. **Massung, R. F., and K. G. Slater.** 2003. Comparison of PCR assays for detection of the agent of human granulocytic ehrlichiosis, *Anaplasma phagocytophilum.* J. Clin. Microbiol. **41:**717–722.
19. **Mather, T. N., M. L. Wilson, S. I. Moore, J. C. M. Ribeiro, and A. Spielman.** 1989. Comparing the relative potential of rodents as reservoirs of the Lyme disease spirochete (*Borrelia burgdorferi*). Am. J. Epidemiol. **130:**143–150.
20. **McQuiston, J. H., C. D. Paddock, R. C. Holman, and J. E. Childs.** 1999. The human ehrlichioses in the Untied States. Emerg. Infect. Dis. **5:**635–642.
21. **Murphy, C. I., J. R. Storey, J. Recchia, L. A. Doros-Richert, C. Gingrich-Baker, K. Munroe, J. S. Bakken, R. T. Coughlin, and G. A. Beltz.** 1998. Major antigenic proteins of the agent of human granulocytic ehrlichiosis are encoded by members of a multigene family. Infect. Immun. **66:**3711–3718.
22. **Pancholi, P., C. P. Kolbert, P. D. Mitchell, K. D. Reed, J. S. Dumler, J. S. Bakken, S. R. Telford III, and D. H. Persing.** 1995. *Ixodes dammini* as a potential vector of human granulocytic ehrlichiosis. J. Infect. Dis. **172:**1007–1012.
23. **Schmidt, B. L.** 1997. PCR in laboratory diagnosis of human Borrelia burgdorferi infections. Clin. Microbiol. Rev. **10:**185–201.
24. **Schwartz, I., S. Varde, R. B. Nadelman, G. P. Wormser, and D. Fish.** 1997. Inhibition of efficient polymerase chain reaction amplification of *Borrelia burgdorferi* DNA in blood-fed ticks. Am. J. Trop. Med. Hyg. **56:**339–342.
25. **Telford, S. R., III, J. E. Dawson, P. Katavolos, C. K. Warner, C. P. Kolbert, and D. H. Persing.** 1996. Perpetuation of the agent of human granulocytic ehrlichiosis in a deer tick-rodent cycle. Proc. Natl. Acad. Sci. USA **93:**6209–6214.
26. **Walls, J. J., P. Caturegli, J. S. Bakken, K. M. Asanovich, and J. S. Dumler.** 2000. Improved sensitivity of PCR for diagnosis of human granulocytic ehrlichiosis using *epank1* genes of *Ehrlichia phagocytophila*-group ehrlichiae. J. Clin. Microbiol. **38:**354–356.
27. **Yeh, M.-T., T. N. Mather, R. T. Coughlin, C. Gingrich-Baker, J. W. Sumner, and R. F. Massung.** 1997. Serologic and molecular detection of granulocytic ehrlichiosis in Rhode Island. J. Clin. Microbiol. **35:**944–947.
28. **Zeidner, N. S., T. R. Burkot, R. Massung, W. L. Nicholson, M. C. Dolan, J. S. Rutherford, B. J. Biggerstaff, and G. O. Maupin.** 2000. Transmission of the agent of human granulocytic ehrlichiosis by *Ixodes spinipalpis* ticks: evidence of an enzootic cycle of dual infection with *Borrelia burgdorferi* in Northern Colorado. J. Infect. Dis. **182:**616–619.
29. **Zhi, N., N. Ohashi, and Y. Rikihisa.** 1999. Multiple *p44* genes encoding major outer membrane proteins are expressed in the human granulocytic ehrlichiosis agent. J. Biol. Chem. **274:**17828–17836.
30. **Zhi, N., Y. Rikihisa, H. Y. Kim, G. P. Wormser, and H. W. Horowitz.** 1998. Cloning and expression of the 44-kilodalton major outer membrane protein gene of the human granulocytic ehrlichiosis agent and application of the recombinant protein to serodiagnosis. J. Clin. Microbiol. **36:**1666–1673.