JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2009, p. 2647–2650 Vol. 47, No. 8
0095-1137/09/$08.00+0 doi:10.1128/JCM.00721-09

Duplex PCR Assay Simultaneously Detecting and Differentiating *Bartonella quintana*, *B. henselae*, and *Coxiella burnetii* in Surgical Heart Valve Specimens[▽]

Yi-Wei Tang*

Departments of Pathology and Medicine, Vanderbilt University Medical Center, Nashville, Tennessee

Received 8 April 2009/Returned for modification 24 May 2009/Accepted 16 June 2009

A duplex PCR (dPCR) assay was developed to simultaneously detect and differentiate *Bartonella quintana*, *Bartonella henselae*, and *Coxiella burnetii* from surgical heart valve tissue specimens with an analytic sensitivity of 10 copies/reaction. Among 17 specimens collected from patients with a clinical diagnosis of culture-negative endocarditis, 2, 4, and 2 were positive for *B. quintana*, *B. henselae*, and *C. burnetii*, respectively, by the dPCR assay, which matched the results obtained by universal bacterial 16S rRNA gene amplification and sequencing.

Infective endocarditis (IE) remains a major medical concern because of its associated mortality rate and expense. Fastidious and unculturable organisms represent approximately half of culture-negative endocarditis (4). With enriched nutrients in culture media and prolonged culture time, the recovery of fastidious microorganisms has been enhanced significantly. Identification of *Coxiella burnetii* and *Bartonella* spp., however, remains a diagnostic challenge (21). Routine serologic testing provides results retrospectively with difficulty in distinguishing the three organisms due to reciprocal cross-reactions of organism-specific antibodies (8, 16, 20).

Currently, surgery is required in 20% to 40% of patients with IE (13), but cultures of valvular tissue specimens are generally unreliable (5). However, with the use of modern diagnostic techniques led by PCR of infected valves, the number of cases without a detectable etiology dropped from 27% to 9% and 1.4% in the last published series (9). Molecular genetic screening for bacteria, especially in cases of heart valve replacement, is a beneficial additional diagnostic strategy. Several recent reports have demonstrated the utility of culture-independent universal 16S rRNA gene PCR, combined with sequencing, in the diagnosis of IE and in the recognition of new IE pathogens (1, 3, 6, 12, 14, 15, 18). Alternatively, monoplex PCR procedures that amplify and detect organism-specific gene targets have been described (19, 20, 25). A duplex PCR (dPCR) assay was developed to simultaneously detect and differentiate three bacterial pathogens, *Bartonella quintana*, *Bartonella henselae*, and *C. burnetii*. An organism-specific citrate synthase gene (*gltA*) and an insertion sequence gene (IS*111*) were used as the target sequences for amplification of the *Bartonella* species and *C. burnetii*, respectively. Three species-specific probes were used to detect and differentiate between *B. quintana*, *B. henselae*, and *C. burnetii* in a colorimetric microtiter plate. Such an approach may facilitate the diagnosis of IE by decreasing the reaction mixture and specimen volumes needed to make a diagnosis.

(This study was presented in part at the 109th General Meeting of the American Society for Microbiology, Philadelphia, PA, 17 to 21 May 2009.)

Clinical specimens and standards. A total of 17 cases diagnosed as IE collected between 2000 and 2008 were included in the study. Patient charts were reviewed by members of the infectious disease team to confirm the clinical diagnosis of IE. No institutional research board approval was sought, and other laboratory data were unavailable. The *B. quintana*-, *B. henselae*-, and *C. burnetii*-specific fragments were generated by PCR amplification (see below) and were subsequently cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA). The DNA concentration of the three recombinant plasmid standards (pCT-Bq for *B. quintana*, pCT-Bh for *B. henselae*, and pcT-Cb for *C. burnetii*) was calibrated by spectrophotometry at 260 nm (17). The inserted plasmids were adjusted to 10,000 copies/μl and stored at −70°C prior to testing.

Specimen processing and DNA extraction. Approximately 20 mg from surgical heart valve tissue specimens was used for processing and extraction. Nucleic acids were extracted using the QIAquick blood/tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Formalin-fixed and paraffin-embedded (FFPE) heart valve tissue blocks were marked to localize the areas most suspected to be infected according to the corresponding slides stained with hematoxylin and eosin. The FFPE specimens were treated with xylene in order to remove the paraffin before tissue lysis and DNA extraction were performed (12). The total extracted nucleic acids were eluted into 200 μl of water.

PCR amplification. Primer sets were designed to target the citrate synthase gene (*gltA*) for the *Bartonella* species (1240F and 1497Rm) (19) and an insertion sequence IS*111* gene (10) for *C. burnetii* (Cox-F [5′-ACT CAA CGC ACT GGA ACC-3′] and Cox-R [5′-TAG CTG AAG CCA ATT CGC-3′]). PCR amplification was performed in an ABI 9700 thermal cycler (Applied Biosystems, Foster City, CA) programmed for a three-step PCR procedure as previously described (23). In brief, 50 μl of the PCR mixture contained the following: 1× buffer; 1.5 mM $MgCl_2$; 10% glycerol; 200 μM (each) dATP, dCTP, and dGTP; 100 μM dTTP; 90 μM dUTP; 10 μM digoxigenin-11-dUTP (Roche Biochemicals, Indianapolis, IN); 1 μM

* Mailing address: Molecular Infectious Disease Laboratory, Vanderbilt University Hospital, 4605 TVC, Nashville, TN 37232-5310. Phone: (615) 322-0126. Fax: (615) 343-8420. E-mail: yiwei.tang@vanderbilt.edu.
▽ Published ahead of print on 24 June 2009.

TABLE 1. Analytical sensitivities of the dPCR assay for detection of *B. quintana*, *B. henselae*, and *C. burnetii*

Test format	Organism	No. of copies/reaction in pooled pathogen-free FFPE tissue samples ($n = 3$) with the following no. of copies of plasmids/reaction:						Analytical sensitivity	
		0	1	10	100	1,000	10,000	No. of copies/reaction	No. of copies/g tissue
Monoplex	*B. quintana*	0	2	3	3	3	3	10	80
	B. henselae	0	2	3	3	3	3	10	80
	C. burnetii	0	3	3	3	3	3	1	8
Duplex	*B. quintana*	0	0	3	3	3	3	10	80
	B. henselae	0	1	3	3	3	3	10	80
	C. burnetii	0	0	3	3	3	3	10	80

of each primer; 0.01 unit/μl uracil *N*-glycosylase (Epicentre Technologies, Madison, WI); 0.025 unit/μl AmpliTaq gold DNA polymerase (Applied Biosystems); and 25 μl of nucleic acid extract. Two monoplex PCRs were used to separately amplify the *Bartonella* species and *C. burnetii*. For the dPCR procedure, the two primer sets were added to one single tube to simultaneously amplify the three bacterial pathogens.

Enzyme immunoassay (EIA) detection and differentiation. Amplification products were then identified by detecting digoxigenin-labeled PCR products with a PCR enzyme-linked immunosorbent assay kit (Roche Biochemicals) in a 96-well microtiter plate as previously described (23). Three species-specific 5′-biotinylated capture probes (5′-ACG CTT GAA AAT ATT GCT CTA-3′ for *B. henselae*, 5′-GCA CTT GAA AAT ACT GCT CTG-3′ for *B. quintana*, and 5′-ATC ACC ACG GAA AAC ACC-3′ for *C. burnetii*) were applied to detect and differentiate the amplification product. Output signals were measured at an optical density of 450 nm (OD_{450}) and 490 (OD_{490}). A positive result was defined as an $OD_{450} - OD_{490}$ value greater than or equal to 0.1 as previously described (23).

Human β-actin gene real-time TaqMan PCR. The human β-actin gene was amplified as a "housekeeping" gene for each sample extract as an internal amplification control. The real-time TaqMan PCR assay was performed on the 7700 ABI Prism sequence detector (Applied Biosystems, Foster City, CA) as described previously (17). The primers and fluorophore hydrolysis probes for human β-actin gene and real-time PCR protocol were published previously (17).

Evaluation standard. Results from the 16S rRNA gene amplification and sequencing of extracted DNA from the FFPE blocks were used as the evaluation standard. PCR amplification of the first 500 bp of the 16S rRNA gene was performed using the MicroSeq 500 16S bacterial sequencing kit according to procedures described previously (22, 24). Bidirectional sequences of the PCR amplification product were determined, and a phylogenetic analysis was performed by online analysis at the Ribosomal Database Project II site (http://wdem.nig.ac.jp/RDP/html/index.html) and the MicroSeq Database Library (Applied Biosystems, Foster City, CA).

The dPCR assay was designed to amplify two targets, a 258-bp *Bartonella* species-specific *gltA* gene and a 256-bp *C. burnetii*-specific IS*111* gene, in one PCR tube. Three microwells were used in the subsequent EIA detection procedure to detect and differentiate the three pathogens, *B. quintana*, *B. henselae*, and *C. burnetii*. The entire procedure, from specimen processing to result reporting, can be completed within 6 hours.

Experiments were performed to determine the assay analytical sensitivity by testing three recombinant plasmid standards (pCT-Bq, pCT-Bh, and pcT-Cb) spiked with pooled pathogen-free FFPE tissues. Plasmids from 0 to 10,000 copies/reaction were included in the experiment, and each dilution was tested three times. The analytic sensitivities of the dPCR assay for all plasmids into which a *B. quintana*, *B. henselae*, or *C. burnetii* amplification fragment was inserted were the same at 10 copies per reaction, which was equivalent to 80 copies/gram of tissue (Table 1). The analytical sensitivities of the dPCR assay were equivalent to the test run in a monoplex PCR format (Table 1).

A total of 17 surgical specimens collected from patients (10 males and 7 females, ages 11 to 81 years) with a clinical diagnosis of culture-negative endocarditis who received valve replacements were included in the study. The specimens included samples from three prosthetic, eight FFPE, and six fresh/frozen heart valves. The human β-actin gene was amplified in each nucleic acid extract, indicating that total inhibition had not occurred in the nucleic acid amplification reaction.

Among 17 specimens collected from patients with a clinical diagnosis of culture-negative endocarditis, 2 (11.8%), 4 (23.5%), and 2 (11.8%) were positive for *B. quintana*, *B. henselae*, and *C. burnetii*, respectively, by the dPCR assay. The results matched those obtained by bacterial 16S rRNA gene amplification and sequencing, which was used as the validation standard. In nine dPCR-negative specimens, 16S rRNA gene amplification and sequencing identified four additional bacterial pathogens, including *Haemophilus parainfluenzae*, *Streptococcus dysgalactiae*, *Streptococcus gordonii*, and *Streptococcus sanguinis* (Table 2). The dPCR assay provided 100% sensitivities/specificities in the detection of *B. quintana*, *B. henselae*, and *C. burnetii* in surgical heart valve specimens.

In this study, a dPCR assay was developed to simultaneously detect and differentiate between *B. quintana*, *B. henselae* and *C. burnetii*. The assay possesses the following characteristics: (i) involvement of microscopic findings to localize the most suspicious specimens for processing; (ii) amplification of two targets simultaneously in a single reaction; (iii) incorporation of an additional signal amplification in the EIA detection procedure, providing higher sensitivities than other test formats, such as real-time TaqMan PCR; (iv) maintenance of species-level specificity with an internal probe incorporated into the EIA detection procedure; (v) nonbinding of the reporter molecule to the microwell with the signal-to-noise ratio of the

TABLE 2. Detection and identification of *B. quintana*, *B. henselae*, and *C. burnetii* from surgical FFPE heart valve specimens

Specimen	Patient		Tissue type	dPCR result	16S rRNA result[a]	Reference
	Gender	Age (yr)				
1	Female	11	FFPE, aortic	*B. henselae*	*B. henselae*	This study
2	Female	41	Fresh, mitral	Negative	Negative	This study
3	Female	81	Fresh, aortic	Negative	Negative	This study
4	Female	34	FFPE, aortic	*B. quintana*	*B. quintana*	This study
5	Male	58	Fresh, mitral	Negative	Negative	This study
6	Male	17	FFPE, aortic	*B. henselae*	*B. henselae*	This study
7	Male	49	Fresh, aortic	*C. burnetii*	*C. burnetii*	This study
8	Male	60	FFPE, mitral	*C. burnetii*	*C. burnetii*	This study
9	Male	43	FFPE, aortic	*B. henselae*	*B. henselae*	25
10	Male	51	FFPE, aortic	*B. quintana*	*B. quintana*	20
11	Female	56	Fresh, aortic	*B. henselae*	*B. henselae*	This study
12	Female	27	Fresh, mitral	Negative	*H. parainfluenzae*	This study
13	Male	72	Fresh, prosthetic	Negative	*S. sanguinis*	This study
14	Male	46	Fresh, prosthetic	Negative	Negative	This study
15	Male	64	Fresh, prosthetic	Negative	Negative	This study
16	Male	54	FFPE, aortic	Negative	*S. dysgalactiae*	14
17	Female	21	FFPE, aortic	Negative	*S. gordonii*	14

[a] 16S rRNA gene amplification and sequencing result.

assay up to 1,000, providing an objective qualitative cutoff value; and (vi) avoidance of carryover contamination by uracil *N*-glycosylase-based chemical modification.

The previous molecular protocols that detected bacterial pathogens in surgical heart valves have been based mainly on amplification and sequencing, if detected, of the universal bacterial 16S rRNA gene (1, 3, 6, 12, 14, 15, 18). However, this broad-range procedure suffered from high numbers of false-positive results and indeterminate results due to indigenous and exogenous bacterial genome contamination, limiting its use in clinical diagnostic services. Besides airborne and carryover contamination during the specimen collection/processing and reaction setup, the assays may be severely compromised by DNA backgrounds in PCR reagents, including the polymerizing enzymes, nucleotides, primers, buffer, and water (2, 7, 11). This time-consuming, multistep amplification and sequencing procedure makes quality assurance and quality control extremely difficult when used in routine diagnostic services. In addition, the coexistence of multiple bacterial pathogens in heart valve specimens may result in indeterminate results (mixed sequencing) by the universal process. In this study, I focused on targeting organism-specific genomes (10, 19) and developed a dPCR assay to simultaneously detect *B. quintana*, *B. henselae*, and *C. burnetii*, the three most encountered bacterial organisms that cause culture-negative endocarditis (4, 21).

Serologic testing remains the test of choice for laboratory confirmation of culture-negative endocarditis, including those caused by *B. quintana*, *B. henselae*, and *C. burnetii*. While clinical presentations of *Bartonella*- and *Coxiella*-caused endocarditis may be similar, distinguishing between these organisms is of importance, because optimal therapy and duration of treatment are different for these organisms. Serological cross-reactions between *B. quintana*, *B. henselae*, and *C. burnetii* have been reported (8, 16). We reported a case of *B. quintana* endocarditis with positive serology for *C. burnetii*, demonstrating the difficulty in using routine serology alone to make a diagnosis of *Bartonella*- or *Coxiella*-caused endocarditis (20). The dPCR assay described here detects and differentiates *Bartonella* or *Coxiella* species simultaneously, providing a useful distinguishing/confirmatory test, especially for anatomic pathology consultation services.

I thank Haijing Li and Shufang Meng for technical assistance and Geraldine Miller, David Haas, Joyce Johnson, Robert Latham, Bruce Hanna, Mark Bochan, and Carol Quinter for providing specimens for study.

REFERENCES

1. **Bosshard, P. P., A. Kronenberg, R. Zbinden, C. Ruef, E. C. Bottger, and M. Altwegg.** 2003. Etiologic diagnosis of infective endocarditis by broad-range polymerase chain reaction: a 3-year experience. Clin. Infect. Dis. **37:**167–172.
2. **Bottger, E. C.** 1990. Frequent contamination of *Taq* polymerase with DNA. Clin. Chem. **36:**1258–1259.
3. **Breitkopf, C., D. Hammel, H. H. Scheld, G. Peters, and K. Becker.** 2005. Impact of a molecular approach to improve the microbiological diagnosis of infective heart valve endocarditis. Circulation **111:**1415–1421.
4. **Brouqui, P., and D. Raoult.** 2001. Endocarditis due to rare and fastidious bacteria. Clin. Microbiol. Rev. **14:**177–207.
5. **Giladi, M., O. Szold, A. Elami, D. Bruckner, and B. L. Johnson, Jr.** 1997. Microbiological cultures of heart valves and valve tags are not valuable for patients without infective endocarditis who are undergoing valve replacement. Clin. Infect. Dis. **24:**884–888.
6. **Goldenberger, D., A. Kunzli, P. Vogt, R. Zbinden, and M. Altwegg.** 1997. Molecular diagnosis of bacterial endocarditis by broad-range PCR amplification and direct sequencing. J. Clin. Microbiol. **35:**2733–2739.
7. **Goto, M., S. Ando, Y. Hachisuka, and T. Yoneyama.** 2005. Contamination of diverse nifH and nifH-like DNA into commercial PCR primers. FEMS Microbiol. Lett. **246:**33–38.
8. **Graham, J. V., L. Baden, S. Tsiodras, and A. W. Karchmer.** 2000. Q fever endocarditis associated with extensive serological cross-reactivity. Clin. Infect. Dis. **30:**609–610.
9. **Hoen, B., F. Alla, C. Selton-Suty, I. Beguinot, A. Bouvet, S. Briancon, J. P. Casalta, N. Danchin, F. Delahaye, J. Etienne, V. Le Moing, C. Leport, J. L. Mainardi, R. Ruimy, and F. Vandenesch.** 2002. Changing profile of infective endocarditis: results of a 1-year survey in France. JAMA **288:**75–81.
10. **Hoover, T. A., M. H. Vodkin, and J. C. Williams.** 1992. A *Coxiella burnetti* repeated DNA element resembling a bacterial insertion sequence. J. Bacteriol. **174:**5540–5548.
11. **Hughes, M. S., L. A. Beck, and R. A. Skuce.** 1994. Identification and elimination of DNA sequences in *Taq* DNA polymerase. J. Clin. Microbiol. **32:**2007–2008.
12. **Imrit, K., M. Goldfischer, J. Wang, J. Green, J. Levine, J. Lombardo, and T. Hong.** 2006. Identification of bacteria in formalin-fixed, paraffin-embedded heart valve tissue via 16S rRNA gene nucleotide sequencing. J. Clin. Microbiol. **44:**2609–2611.
13. **Jenkins, N. P., G. Habib, and B. D. Prendergast.** 2005. Aorto-cavitary fistu-

lae in infective endocarditis: understanding a rare complication through collaboration. Eur. Heart J. **26:**213–214.

14. **Khulordava, I., G. Miller, D. Haas, H. Li, J. McKinsey, D. Vanderende, and Y. W. Tang.** 2003. Identification of the bacterial etiology of culture-negative endocarditis by amplification and sequencing of a small ribosomal RNA gene. Diagn. Microbiol. Infect. Dis. **46:**9–11.
15. **Lang, S., R. W. Watkin, P. A. Lambert, R. S. Bonser, W. A. Littler, and T. S. Elliott.** 2004. Evaluation of PCR in the molecular diagnosis of endocarditis. J. Infect. **48:**269–275.
16. **La Scola, B., and D. Raoult.** 1996. Serological cross-reactions between *Bartonella quintana*, *Bartonella henselae*, and *Coxiella burnetii*. J. Clin. Microbiol. **34:**2270–2274.
17. **Li, H., J. S. Dummer, W. R. Estes, S. Meng, P. F. Wright, and Y. W. Tang.** 2003. Measurement of human cytomegalovirus loads by quantitative real-time PCR for monitoring clinical intervention in transplant recipients. J. Clin. Microbiol. **41:**187–191.
18. **Marin, M., P. Munoz, M. Sanchez, M. del Rosal, L. Alcala, M. Rodriguez-Creixems, and E. Bouza.** 2007. Molecular diagnosis of infective endocarditis by real-time broad-range polymerase chain reaction (PCR) and sequencing directly from heart valve tissue. Medicine (Baltimore) **86:**195–202.
19. **Patel, R., J. O. Newell, G. W. Procop, and D. H. Persing.** 1999. Use of polymerase chain reaction for citrate synthase gene to diagnose *Bartonella quintana* endocarditis. Am. J. Clin. Pathol. **112:**36–40.
20. **Rahimian, J., D. Raoult, Y. W. Tang, and B. A. Hanna.** 2006. *Bartonella quintana* endocarditis with positive serology for *Coxiella burnetii*. J. Infect. **53:**e151–e153.
21. **Raoult, D., P. E. Fournier, F. Vandenesch, J. L. Mainardi, S. J. Eykyn, J. Nash, E. James, C. Benoit-Lemercier, and T. J. Marrie.** 2003. Outcome and treatment of *Bartonella* endocarditis. Arch. Intern. Med. **163:**226–230.
22. **Tang, Y. W., M. K. Hopkins, C. P. Kolbert, P. A. Hartley, P. J. Severance, and D. H. Persing.** 1998. *Bordetella holmesii*-like organisms associated with septicemia, endocarditis, and respiratory failure. Clin. Infect. Dis. **26:**389–392.
23. **Tang, Y. W., P. N. Rys, B. J. Rutledge, P. S. Mitchell, T. F. Smith, and D. H. Persing.** 1998. Comparative evaluation of colorimetric microtiter plate systems for detection of herpes simplex virus in cerebrospinal fluid. J. Clin. Microbiol. **36:**2714–2717.
24. **Tang, Y. W., A. Von Graevenitz, M. G. Waddington, M. K. Hopkins, D. H. Smith, H. Li, C. P. Kolbert, S. O. Montgomery, and D. H. Persing.** 2000. Identification of coryneform bacterial isolates by ribosomal DNA sequence analysis. J. Clin. Microbiol. **38:**1676–1678.
25. **Walker, V. A., S. W. Palmer, Y. W. Tang, and M. R. Bochan.** 2006. *Bartonella henselae* endocarditis associated with profound anemia. Infect. Med. **23:**321–324.