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

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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 diagnosing IE (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 (IS111) 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.

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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 PRC 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 CTG GAA AAT ACT GCT CTA-3′ for B. henselae, 5′-GCA CTG GAA AAT ACT GCT CTG-3′ for B. quintana, and 5′-ATT 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 (OD450) and 490 (OD490). A positive result was defined as an OD450 − OD490 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://rdp.yahoo.com/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 IS111 gene, in one PCR tube. Three micro-wells 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
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.

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REFERENCES