JOURNAL OF CLINICAL MICROBIOLOGY, July 2005, p. 3516–3518 Vol. 43, No. 7
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.7.3516–3518.2005

Detection of *Tropheryma whipplei* DNA in Clinical Specimens by LightCycler Real-Time PCR

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Received 18 October 2004/Returned for modification 6 December 2004/Accepted 24 February 2005

A real-time PCR method using the LightCycler (Roche Applied Science, Indianapolis, IN) was compared to a conventional PCR assay for the detection of *Tropheryma whipplei* in 321 clinical specimens. The LightCycler method had sensitivity and specificity comparable to the conventional method but required considerably less labor and time (3 h versus 3 to 5 days).

The diagnosis of Whipple's disease, caused by the bacillus *Tropheryma whipplei*, is supported by the observation of periodic acid-Schiff-positive bacilli in infected tissue (3). Recent reports have demonstrated that *T. whipplei* can be cultured in a variety of human cell lines (1, 2, 5, 6). However, due to technical difficulties with culturing, PCR is the preferred method for confirming the presence of *T. whipplei* in human specimens. We report an easy-to-perform and rapid PCR method for detecting *T. whipplei* in a variety of human specimens.

Specimens. This study was approved by the Institutional Review Board of the Mayo Foundation. Clinical specimens from 321 patients (132 whole blood, 82 tissue, 62 cerebrospinal fluid [CSF], and 45 synovial fluid) were collected from February 1996 to October 2002. The great majority of specimens were received through our referral laboratory practice from health care institutions outside of the Mayo Clinic. Therefore, clinical histories and additional specimens for testing were generally only available for those patients evaluated at the Mayo Clinic.

Specimens were tested by the conventional PCR assay as part of the routine clinical work flow in our laboratory. Conventional PCR was performed on relatively fresh samples, depending on when the assay was set up. Because the conventional PCR assay is only set up once weekly due to low specimen numbers, fresh tissue samples were frozen at −70°C, paraffin blocks were stored at room temperature, and blood samples were refrigerated at 4°C until the assay was performed. The LightCycler assay was performed on specimens which were frozen, refrigerated, or at room temperature and stored for longer periods of time than the specimens tested by the conventional PCR method.

Isolation of DNA. For the LightCycler PCR assay, a 200-μl aliquot of each blood, CSF, and synovial fluid specimen was extracted using the automated MagNA Pure LC instrument and the MagNA Pure total nucleic acid isolation kit (Roche Applied Science). For the conventional PCR assay, 200 μl of blood or CSF was extracted using the manual IsoQuick (Orca Research, Inc., Bothell, WA) nucleic acid extraction kit and 1 ml of synovial fluid was extracted with the QIAamp DNA Blood Midi Kit (QIAGEN, Valencia, CA). Extractions were performed according to the manufacturer's instructions. Biopsy samples of tissue fixed in paraffin blocks were cut into sections and dewaxed with xylene and 95% ethanol washes. The tissue was digested overnight at 55°C and 500 rpm in 1× Tris-EDTA (0.01 M Tris-HCl [pH 8.0], 0.001 M EDTA; Sigma, St. Louis, MO)–10% sodium dodecyl sulfate–proteinase K (Sigma, St. Louis, MO) at a final concentration of 80 to 100 U/ml. A 200-μl aliquot of the tissue digest was extracted on the MagNA Pure instrument for the LightCycler assay, and 200 μl was extracted using the IsoQuick extraction kit for the conventional PCR assay.

Conventional PCR assay. A 284-bp target sequence from a region of the 16S rRNA gene sequence of *T. whipplei* was amplified (polyacrylamide gel electrophoresis-purified primers pW3FE [5′ GGAATTCCAGAGATACGCCCCCCGCAA 3′] and pW2RB [5′ CGGGATCCCATTCGCTCCACCTTGCGA 3′]), electrophoresed in a 2% agarose gel, and Southern blotted using the ECL DNA hybridization probe for *T. whipplei* (101 bp, positions 243 to 344) (4).

LightCycler assay. The heat shock protein (hsp65) gene of *T. whipplei* was selected as the target sequence. A 213-bp target sequence of *hsp65* was amplified using the forward primer TW704 (5′ AAAGAGGTTGAGACTG 3′) and the reverse primer TW899 (5′ ATCGGTTACAAAATAAGC 3′). The sequence of the anchor probe (3′ fluorescein labeled), TW795, was 5′ AGAAGGTTGGCAAGGAAGGC 3′, and the sequence of the donor probe (5′ LC RED-640 labeled), TW817, was 5′ TGTCACTGTCGAGGAGTCAAATACT 3′.

The reaction mixture consisted of 15 μl of the PCR master mix plus 5 μl of the DNA extracts from the clinical specimens per cuvette. The PCR master mix included 3 mM $MgCl_2$, 1× LightCycler FastStart DNA master hybridization probes (Roche Applied Science, Indianapolis, IN), 0.35 μM each primer, and 0.2 μM each fluorescein- and LC RED-640-labeled probe. Cycling parameters consisted of 1 cycle of 95°C for 10 min, followed by amplification for 45 cycles of 95°C for 10 s, 55°C for 15 s, and 72°C for 15 s. A melting curve was generated by measuring the fluorescent signal generated with

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TABLE 1. Bacterial strains used to test the specificity of the primers and probes of the LightCycler assay

Organism	Strain	Organism	Strain
Propionibacterium acnes	QC[a] strain	*Mycobacterium simiae*	
Rhodococcus equi	ATCC 6939	*Tsukamurella tyrosinosolvens*	
Helicobacter pylori	QC strain	*Mycobacterium triviale*	ATCC 23291
Propionibacterium granulosum[b]		*Mycobacterium bovis*	ATCC 35735
Tsukamurella paurometabola	ATCC 8368	*Mycobacterium marinum*	
Propionibacterium acnes	ATCC 11827	*Mycobacterium szulgai*	ATCC 35799
Mycobacterium chelonae		*Mycobacterium mucogenicum*	
Mycobacterium abscessus	ATCC 19977	*Mycobacterium intracellulare*	
Mycobacterium avium	ATCC 7000897	*Streptomyces griseus*	ATCC 23345
Actinomyces odontolyticus		*Actinomyces odontolyticus*	ATCC 17929
Actinomyces sp.		*Nocardia farcinica*	ATCC 3318
Actinomyces urogenitalis		*Mycobacterium tuberculosis*	ATCCH37RV
Helicobacter pylori[b]		*Tsukamurella inchonensis*	ATCC 700082

[a] QC, quality control.
[b] Two strains.

the following profile: 95°C for 0 s, 59°C for 20 s, 45°C for 20 s with a 0.2°C/s transition, and 85°C for 0 s with a 0.2°C/s transition. Version 3.5 of the LightCycler software was used for most experiments. Software version 3.5.17 was installed and used on all samples after September 2004. Verification of the software showed no significant difference between software versions 3.5 and 3.5.17. Sterile water was used as a negative control and a plasmid clone constructed from the 213-bp amplicon was used as a positive control with each run. A specimen with a melting curve at the same location as the positive control (65°C ± 2°C) was interpreted as positive.

The analytical sensitivity of the LightCycler assay was evaluated by (i) a 10-fold dilution series (10^4 to 10^0 targets/μl) of a *T. whipplei*-positive plasmid clone in triplicate and (ii) spiking of dilutions of the positive control plasmid into the various specimen matrices (blood, CSF, synovial fluid) and extraction with the MagNA Pure extraction system. The analytical specificity was determined by evaluation of DNA extracted from pure cultures of a variety of organisms genetically similar to *T. whipplei* (Table 1). Two hundred thirty-three nucleic acid extracts of samples (51 blood, 66 CSF, 60 synovial fluid, 56 tissue) negative for *T. whipplei* were spiked with 50 target sequences per reaction mixture and tested by the LightCycler assay to test for the presence of PCR inhibitors.

The results of the LightCycler assay were compared to the conventional PCR assay to determine sensitivity, specificity, and positive and negative predictive values. Discordant results between the two PCR assays were resolved by repeat testing of a recent extract of the original specimen. The approximate time required for specimen processing and assay performance was recorded for each PCR method.

Results. The LightCycler PCR method demonstrated an analytical sensitivity of less than 25 target sequences per 5-μl reaction mixture (5,000 target sequences/ml) for both the plasmid clone dilutions and the positive plasmid control spiked into the different specimen matrices. All non-*T. whipplei* bacteria (Table 1) tested were negative by the LightCycler assay. Complete inhibition of LightCycler assay amplification occurred in 1 of 60 (2%) synovial fluid samples and 1 of 56 (2%) tissue samples. The overall inhibition rate for the LightCycler assay for blood, CSF, synovial fluid, and tissue was 2 of 233 samples or less than 1%.

Of the 321 clinical specimens, 47 (15%) were identified as positive and 270 (84%) were negative by both the LightCycler and conventional PCR assays. Three specimens were positive only by LightCycler PCR, and one specimen was positive only by conventional PCR (Table 2). The medical history was available for only one of these discrepant specimens (conventional PCR positive, LightCycler assay negative), and the diagnosis was not felt to be Whipple's disease. The sensitivity, specificity, and positive and negative predictive values of the LightCycler assay compared to the conventional PCR assay were 98%, 99%, 94%, and 100%, respectively.

Approximately 2.5 h was required for MagNA Pure extraction of nucleic acid and 1 h for analysis and reporting of results (total time requirement for LightCycler assay = 3.5 h). The actual hands-on time was approximately 20 to 25 min for 32 samples. For the conventional PCR, manual extraction (IsoQuick) required approximately 65 to 70 min of hands-on time for 32 samples. Amplification on a conventional thermocycler required up to 5 h. Adding these times to the times required for electrophoreses on an agarose gel and transfer by overnight Southern blotting resulted in a total time of 2 to 3 days.

Discussion. The results of our study indicate that automated extraction of DNA using the MagNA Pure instrument combined with the LightCycler PCR is a reliable, easy-to-perform method for detection of *T. whipplei* DNA in a variety of human specimens. The sensitivity, specificity, and positive and negative predictive values of the LightCycler PCR were comparable to the conventional PCR. Importantly, the hands-on labor time and time for completing the LightCycler assay versus the con-

TABLE 2. Comparison of LightCycler PCR versus conventional PCR for detection of *T. whipplei* in clinical samples

LightCycler assay	No. of samples with a conventional PCR assay result of:		Total
	Positive	Negative	
Positive	47	3[a]	50
Negative	1	270	271
Total	48	273	321

[a] Crossing points of these specimens, 32, 38, and 43 cycles.

ventional PCR assay were appreciably less (20 min versus 70 min and 3.5 h versus 2 to 3 days, respectively).

We thank JoAnn Brunette for assistance in preparing the manuscript.

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