Validation of an rpoB Gene PCR Assay for Detection of Tropheryma whipplei: 10 Years’ Experience in a National Reference Laboratory

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The performance of a real-time PCR assay targeting the Tropheryma whipplei rpoB gene was evaluated using test strains and 1,236 clinical specimens in a national reference laboratory. The novel rpoB-PCR assay proved to be specific, revealed improved analytical sensitivity, and substantially accelerated detection of T. whipplei DNA in clinical specimens.

Laboratory diagnosis of Whipple’s disease is currently based on a combination of PCR and histopathological methods (1). The emergence of real-time PCR technology has substantially improved and facilitated the molecular detection of Tropheryma whipplei, the causative agent of Whipple’s disease (2, 3). In search of a specific and sensitive PCR protocol, several target genes have already been evaluated, including the 16S rRNA gene (4), the 16S-23S rRNA intergenic spacer region (5), the hsp65 gene (6), and certain repeated sequences (7). Targeting a T. whipplei-specific segment within the rpoB gene, encoding the β subunit of the RNA polymerase, has shown promising results (8, 9), but a comprehensive study, including melting curve analysis, with respect to its robustness and suitability in routine diagnostics has not been conducted. Over an 8-year period, we evaluated a novel real-time PCR assay targeting T. whipplei-specific segments within the rpoB gene on a considerable number of clinical specimens and compared the results to a conventional PCR protocol. Starting from 2012, the rpoB-PCR was used as a screening PCR.

Total genomic DNA from the various clinical specimens was isolated using either the Amplicor respiratory specimen preparation kit or the MagNA Pure compact nucleic acid isolation kit (both from Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. Both extraction methods use alkaline lysis that successfully disintegrated T. whipplei in cerebrospinal fluid, blood, gut biopsy specimens, and histological sections from methacrylate- or paraffin-embedded tissues (e.g., heart valves and skin samples).

Conventional PCR assay. Based on a slightly modified protocol published by von Herbay et al. (10), a 267-bp fragment of the 16S rRNA gene of T. whipplei was amplified in a seminested protocol using primers TPU5 (AAACTYAAAKGAATTGACGG) (11) and whip1 and whip2 (10). Successful amplification was confirmed by agarose gel electrophoresis. The identity of PCR products of the expected size was verified by DNA sequencing on an automated capillary DNA sequencer and comparison with all currently available sequences from public databases (EMBL and GenBank) as published elsewhere (12).

Real-time PCR assay. Primers TwrpoB-F4 (CTCGGTGTGTA TGTTGATCCAA) and TwrpoB-R (GCACCGCAACCTCGGGAG AAA) (8) were selected to amplify a 109-bp segment of the T. whipplei rpoB gene. Real-time detection of the amplicons was achieved by hybridization probes TwrpoB-HP1 (ACGAGGTCGG ATATTATCGC-FL) (5′-3′) and TwrpoB-HP2 (Red 640-AACAAT TCGTTATCTCGCGGCC) (5′-3′). The in silico specificity of primer and probe sequences was verified by EMBL and GenBank sequence alignments.

The 20-μl amplification mixture contained 2 μl LightCycler FastStart DNA Master HybProbe (Roche Diagnostics, Mannheim, Germany), 5 mM MgCl2, 0.25 μM primer TwrpoB-F4, 0.5 μM primer TwrpoB-R, 0.2 μM (each) hybridization probe, and 5 μl of DNA. Primer and hybridization probe oligonucleotides were synthesized by TIB Molbiol, Berlin, Germany. Real-time PCR was performed on a LightCycler instrument, version 2.0 (Roche Diagnostics). Thermocycling parameters were as follows: initial denaturation at 95°C for 10 min and 50 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 20 s, and extension at 72°C for 30 s. Following the amplification phase, a melting curve analysis (starting at 40°C) was performed with a temperature transition rate of 0.2°C/s to determine the melting temperature (Tm) values for the sequences targeted by the hybridization probes.

Samples were deemed positive when (i) the fluorescence attributable to the target sequence exceeded the background signal and (ii) a characteristic Tm value of 63°C and biphasic curve progression were observed in melting curve analysis.

Evaluation of the PCR assays. For both PCR assays, several no-template controls, a diagnostic negative control (containing human background DNA), a positive control extracted...
from T. whipplei strain Twist ATCC VR-1528, and an inhibition control spiked with DNA from the positive control were included.

The specificity of the real-time PCR was evaluated by testing DNA from 48 species phylogenetically closely related to T. whipplei (Table 1) with negative results.

Identical dilution series derived from a suspension of T. whipplei strain Twist ATCC VR-1528 were tested to compare the real-time PCR assay and the conventional PCR assay with respect to sensitivity. A reliable quantification of T. whipplei cells in the corresponding serial dilutions was problematic because T. whipplei cells tend to clump (Fig. 1) and cell counts by eye were rather inconsistent. Higher accuracy was achieved by fluorescence microscopy followed by digital image analysis (13). The number of bacteria was estimated by calculating the total area covered by microorganisms in 1 μl of dilutions 1 to 3 (Fig. 1). Cell counts of further dilutions were extrapolated. Bacteria were stained with the nucleic acid stain SYTO 9, and the area occupied was quantified using automatic two-dimensional (2D) segmentation and manual thresholding features of the daime digital image analysis program, version 1.2 (14).

The novel real-time PCR protocol showed a sensitivity of approximately 17 T. whipplei target organisms per 5-μl suspension used for DNA extraction (3,480 microorganisms/ml) (Fig. 1). The sensitivity of the conventional PCR assay was 20-fold less, corresponding to about 350 microorganisms/5 μl.

From June 2003 to December 2011, all specimens with clinical suspicion for Whipple’s disease received by the German National Reference Laboratory for T. whipplei (Konsiliarlabor appointed by the Robert Koch Institute) were compared by the two PCR protocols. During this period, 970 specimens from 693 patients from numerous German hospitals and health care institutions were investigated. Inhibition of PCR was observed with 57 DNA preparations. Of the remaining 913 samples, 102 (11.2%) corresponding to 67 patients (7.3%) tested positive by both assays. The surprisingly overall high number of positive samples is due to the status of the laboratory as a German reference laboratory and not to be expected in routine diagnostic laboratories. Among 22 samples with inconsistent results, which either tested positive in conventional PCR but remained negative in real-time PCR (10 samples, 1.1%) or vice versa (12 samples, 1.3%), 13 samples (1.4%) were taken after antimicrobial therapy from patients with known Whipple’s disease and were therefore at the limit of detection. However, six samples (0.7%) of the remaining inconsistent samples were missed by the conventional PCR (corresponding histopathology of one sample was periodic acid-Schiff [PAS] stain positive; for the remaining 5 samples, no PAS stain result was available, but the patients had PAS stain-positive biopsy specimens from other tissues or sites). Only three samples (0.3%) were false negative in the real-time PCR (one sample with negative PAS stain and two without information regarding histopathology). Thus, the rpoB-PCR can be regarded as more sensitive in clinical specimens than conventional PCR.

A variety of different sample materials proved to be positive, including gastrointestinal biopsy specimens, knee and wrist joint fluid, cerebrospinal fluid, skin, lymph nodes, heart valves, bone marrow, and bronchoalveolar lavage.

The real-time PCR can be accomplished in about 90 min (including 30 min of hands-on time) without the need for any post-PCR manipulations, whereas the conventional PCR followed by electrophoresis and sequencing may take at least 2 working days with approximately 3.5 h of hands-on time.

Therefore, from January 2012 we used the rpoB-PCR as a first-line routine diagnostic PCR. With respect to the high number of uncultured species with unknown rpoB gene sequences in the human gut, any initial diagnosis of Whipple’s disease was routinely verified by 16S rRNA gene sequencing. Among 266 samples, 31 (11.7%) tested positive in rpoB-PCR. Seventeen of the positive
samples were follow-up specimens from confirmed Whipple’s disease patients. All 14 newly diagnosed patients were successfully confirmed by 16S rRNA gene sequencing. All 14 new cases showed respective clinical signs, and the diagnosis was confirmed by PAS stain and/or immunostain.

Because of variation in sample size and type, we are cautious with quantification of T. whipplei in biopsy samples. Still, we saw high concentrations of T. whipplei cells in untreated patients followed by a clear decrease of T. whipplei during therapy which correlated with clinical improvement (15). Nevertheless, the diagnostic value for staging of Whipple’s disease patients is yet to be determined and needs a thorough workup of all diagnostic measures and clinical outcome, which was beyond the scope of this paper.

We would like to emphasize that PCR results should always be interpreted together with clinical and histopathological findings. In the case of discrepant results in initial diagnosis of Whipple’s disease, definite diagnosis should be urged by analysis of an independent clinical sample. Regarding inconclusive results in follow-up of a Whipple’s disease patient, one should keep in mind that PAS stain and immunostain usually remain positive longer than PCR analysis. In these cases, we recommend a shorter recall interval of 6 months’ maximum to ensure timely therapeutic intervention in relapsing cases.

Compared to a well-established conventional PCR protocol, the presented real-time PCR assay is clearly distinguished by its 20-fold-higher analytical sensitivity along with excellent specificity. When applying the real-time PCR assay to clinical samples, a superior diagnostic performance and robustness were observed. The rpoB gene seems to be a suitable target providing a highly sensitive, rapid, and reliable detection of T. whipplei DNA in clinical specimens. In initial diagnosis of Whipple’s disease and inconclusive cases, a second PCR targeting a different gene is advisable.

In our study groups, we still use the 16S rRNA gene for this purpose.

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