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.
from *T. whipplei* strain Twist ATCC VR-1528, and an inhibitory 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 node, 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

| Source/strain | 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). 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The superior diagnostic performance and robustness were observed. When applying the real-time PCR assay to clinical samples, a 20-fold-higher analytical sensitivity along with excellent specificity. The presented real-time PCR assay is clearly distinguished by its independence from clinical sample. Regarding inconclusive results in follow-up 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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REFERENCES

10. von Herbay A, Ditton HJ, Maiwald M. 1996. Diagnostic application of a