Culture and Immunological Detection of *Tropheryma whippelii* From the Duodenum of a Patient With Whipple Disease

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**Context** Culture of *Tropheryma whippelii* has been established only once, in human fibroblast cell lines from a heart valve inoculum. Molecular-based diagnostic techniques, although highly sensitive, may be less specific. New diagnostic tools involving isolation of bacteria from contaminated intestinal biopsies and immunohistological detection need to be developed.

**Objective** To describe a novel method for detection and culture of *T whippelii* strains.

**Design, Setting, and Subjects** Laboratory analysis of duodenal biopsy specimens from a patient with typical relapsing Whipple disease with intestinal involvement, performed Marseille, France, in March 2000. Biopsy specimens were decontaminated with antimicrobial agents and inoculated onto cell cultures. Mouse anti-*T whippelii* polyclonal antibodies were used to detect *T whippelii* in fixed specimens taken from the patient before and after relapse, compared with specimens from 10 controls. The genotype of the isolate was determined by amplification and sequencing of 2 DNA fragments (ITS and 23S rRNA).

**Main Outcome Measure** Isolation and genotyping of a new strain(s) of *T whippelii* from the case patient’s biopsy specimens.

**Results** A strain was grown from the case patient’s intestinal specimen that has a genotype different from the first strain isolated. During 2 episodes of Whipple disease, *T whippelii* bacteria were detected by immunohistology in the patient’s duodenal biopsy specimens, but not in controls.

**Conclusions** A second strain of *T whippelii* has been isolated and a protocol for isolation from the intestine has been proven to be efficient. Immunodetection of *T whippelii* in intestinal biopsy specimens may provide a useful tool for the diagnosis and follow-up of patients with Whipple disease. Both techniques need further evaluation and confirmation.

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based on sequence variation within the inter 16S-23S rDNA spacer (ITS)\textsuperscript{12,19} and 2 genotypes (named A and B) based on that of the 23S rRNA gene.\textsuperscript{10} The report of the isolation of the Whipple disease bacterium in 1997 using human macrophages inactivated with interleukin 4 raised interest in this microorganism,\textsuperscript{20} but this work has unfortunately not been pursued or reproduced. We have since reported the stable isolation of \textit{T whippelii} from a patient with endocarditis without evidence of typical Whipple disease.\textsuperscript{21} Herein we report the isolation of the bacterium from the intestine and the immunohistochemical demonstration of the bacterium in a duodenal biopsy specimen from a patient with typical relapsing Whipple disease who had received cotrimoxazole treatment.

### METHODS

A 33-year-old woman presented in March 2000 with suspected Whipple disease. This patient had been hospitalized in 1995 with fever, weight loss of 10 kg, fatigue, and vomiting. A diagnosis of Whipple disease was based on lymph node and duodenal biopsies, which were periodic acid–Schiff (PAS)– and PCR-positive.\textsuperscript{22} She responded well to cotrimoxazole and was treated for 1 year. She was clinically cured, but no duodenal control biopsy was performed at the end of therapy.

The patient complained 4 years later that she had experienced vomiting, abdominal pain, and a weight loss of 8 kg over the preceding 3 months. On examination, a palpable subclavicular lymph node was found. The patient had a normal white blood cell count, anemia (hemoglobin, 93 g/L), hyperglobulinemia, hypoalbuminemia, and an increased erythrocyte sedimentation rate (76 mm/h). A duodenal biopsy was performed to confirm the diagnosis and to isolate the causative bacterium.

Since the sample was contaminated by the intestinal flora, we used decontaminating antibiotics. Culture was performed by the centrifugation shell-vial technique with human fibroblast cell line (HEL) cells.\textsuperscript{21} Frozen duodenal tissue was incubated for 45 minutes in 2 mL of Rinaldini medium (NaCl, 6.8 g; KCl, 0.4 g; NaHPO\textsubscript{4}, 0.15 g; glucose, 1.0 g; NaHCO\textsubscript{3}, 2.2 g; phenol red, 0.002 g; in 1.0 L of distilled water) containing colistin sulfate (10 µg/mL), cephalothin sodium (2 µg/mL), and amphotericin B (1 µg/mL). The specimen was then rinsed by immersion for 5 minutes in 5 mL of minimum essential medium (MEM) (Gibco, Gaithersburg, Md). Rinsing was repeated twice and the biopsy was then removed and crushed in MEM. The suspension was used to inoculate 3 shell vials for decontamination control, the biopsy specimen was also inoculated onto Columbia sheep blood agar and polyvitex chocolate agar plates (Biomérieux, Marcy l’Etoile, France) incubated at 37°C for 10 days under a 5% CO\textsubscript{2} atmosphere and onto Shaedler broth incubated at 37°C for 10 days without CO\textsubscript{2}. Inoculated vials were processed as previously described.\textsuperscript{21} The culture medium was changed on day 15.

On day 30, detection of growing bacteria was carried out directly inside 1 shell vial by immunofluorescence. After fixation with methanol, the vial was washed twice with phosphate-buffered saline (PBS). One hundred microliters of a locally produced mouse \textit{T whippelii} antisera diluted 1:200 in PBS with 3% nonfat dry milk was added, and the vial incubated at 37°C for 30 minutes. After 3 washes with PBS, 100 µL of fluorescein isothiocyanate-conjugated goat anti–mouse IgG (Jackson Immunoresearch Laboratories, West Grove, Pa) diluted 1:200 in PBS with 3% nonfat dry milk was added, and the vial incubated at 37°C for 30 minutes. After 3 washes with PBS, the coverslip was mounted (cells face down) in phosphate-buffered glycerol medium (pH 8.0) and examined at \times 400 with an epifluorescence microscope.

One month after inoculation, cells harvested from another shell vial were inoculated in 3 shell vials. After 30 days of incubation, 1 of these 3 second-passage shell vials was prepared as described above, and the coverslip was examined using a laser confocal fluorescence microscope equipped with a \times 100 (numeric aperture, 1.4) oil immersion lens. The supernatant fluids of the vials from the first inoculation and the second passage were used for PCR-based identification. Cells from the remaining vials were inoculated in a 25-cm\textsuperscript{2} cell culture flask to establish the isolate.

Histological analysis was performed on intestinal biopsy specimens from the first and second episodes of disease (1995 and 2000). These were fixed in formalin together with intestinal specimens from 10 patients with a diagnosis other than Whipple disease, which acted as negative controls. Paraffin-embedded tissue samples were cut to 5-µm thickness and stained with hematoxylin-cosin. Special stains used for detection of bacteria and fungi included PAS, Giemsa, Brown-Hopps Tissue Gram, Grocott-Gomori methamine silver (GMS), and Warthin-Starry.

In order to specifically detect the presence of \textit{T whippelii} in tissues, immunohistochemistry was performed on 3-µm-thick formalin-fixed and paraffin-embedded intestinal sections. After deparaffinization, each tissue section was incubated with an anti–\textit{T whippelii} mouse polyclonal antibody prepared as described above, and diluted 1:500 in PBS. After incubation with the primary antibody, immunodetection was performed with biotinylated anti–mouse immunoglobulins,\textsuperscript{21} followed by peroxidase-labeled streptavidin (Histostain-Plus Kit, Zymed, Montrouge, France) with aminoethylcarbazole as the substrate. The excess chromogen was removed and the slides were counterstained with Mayer hematoxylin for 5 minutes. For each tissue section, a negative control was performed using normal mouse serum. In order to test the specificity of the anti–\textit{T whippelii} mouse polyclonal antibody, blind testing by microimmunofluorescence assay with this antibody was carried out on 5 bacterial strains isolated in our laboratory from clinical samples: \textit{Mycobacterium avium}, \textit{Actinomyces meyeri}, \textit{Corynebacterium ANF}, \textit{Listeria monocytogenes}, and \textit{Streptococcus bovis}. 

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Polymerase chain reaction amplifications and gene sequencing of the 16S rRNA gene, the intergenic spacer region, and domain III of the 23S rDNA gene were performed on both a fresh duodenal biopsy sample and on the isolate. The biopsy sample was ground mechanically and resuspended in 500 µL of sterile deionized water in a sterile PCR tube. Five hundred microliters of cell culture supernatant was centrifuged at 12000g for 5 minutes. The resulting pellet was resuspended in 90 µL of Tris-EDTA buffer. The suspension was frozen in liquid nitrogen and immediately thawed at 100°C in a dry bath. This procedure was repeated 3 times. Ten microliters of a stock solution of lysozyme (10 g/L [Research Organics Inc., Cleveland, Ohio]) was added and both tubes were incubated for 1 hour at 37°C. The DNA was extracted using Qiagen columns (QIAamp tissue kit, Qiagen, Hilden, Germany) as described by the manufacturer. Using the broad-range primer pair ID1 and rP2, the 16S rRNA gene was PCR-amplified. This fragment was sequenced as previously described. The 16S-23S rDNA intergenic spacer region was amplified and sequenced using the primers TW3F and TW4R, and domain III of the 23S rRNA gene was amplified using the primers TW-23InsF and TW-23InsR2 as previously described. For sequencing, a commercially available sequencing kit (dRhodamine Terminator Cycle Sequencing, PE Applied Biosystem, Warrington, England) was used as previously reported. Sequencing products were purified by ethanol-magnesium precipitation and resolved on a 5% polyacrylamide gel (Long Ranger Singel pack, 377-36 cm, BioWhittaker Molecular Applications, Rockland, Me) by electrophoresis with an ABI PRISM 377 DNA sequencer (Perkin Elmer, Wellesley, Mass).

RESULTS

The inoculated duodenal biopsy specimen was decontaminated according to the antibiotic protocol. No contaminant was grown on control media. Immunofluorescence staining performed on the vial examined on day 30 after inoculation resulted in the detection of numerous fluorescent bacteria. On confocal fluorescent microscopy of the second-passage shell vial, numerous fluorescent bacteria mostly aggregated within intracellular vacuoles were observed (FIGURE 1). The appearance was comparable to that of the Twist-Marseille T whipelli strain. The supernatant of positive vials, as well as of the remaining 2 vials, was inoculated onto confluent layers of HEL cells allowing the isolate to establish itself. This isolate was named the Slow-Marseille strain.

Amplification and sequencing of the 16S rRNA gene of both the isolate and the biopsy specimen generated a 1484-base pair (bp) sequence, which was compared with DNA sequences accessible in public databases. The sequence was identical to 16S rRNA gene sequences of T whipelli (GenBank accession numbers X996361 and M874841). The 472-bp sequence derived from the ITS of the isolate was identical to the ITS sequence of T whipelli (GenBank accession number X99636) designated by Hinrikson et al as type 1. The 250-bp sequence derived from domain III of the 23S rDNA sequence from the isolate was identical to the sequence available in GenBank under the accession number AF 148136, designated by Hinrikson et al as type A. Thus, the genotype of the T whipelli Slow-Marseille strain corresponded to molecular variant 1A as defined by Hinrikson et al.

Histological examination of the intestinal biopsy specimen showed typical Whipple disease in the samples from 1995 and 2000. Both revealed large, foamy macrophages. These macrophages were seen in the lamina propria of small intestinal mucosa (FIGURE 2A). They contained numerous intracytoplasmic granules that were positive on PAS staining and resistant to diastase (Figure 2B). These PAS-positive granules represent intact or partially degraded bacteria. Giemsa, Brown-Hopps, GMS, and Warthin-Starry stains revealed no microorganisms. By using polyclonal antibody raised against the bacterium for immunohistology, detection of T whipelli antigenic material was performed in small intestinal tissues. Tropheryma whipelli antigen was packed as coarse granular immunopositive material in the cytoplasm of foamy macrophages (Figure 2C). None of the 10 control duodenal specimens were positive. The polyclonal antibody did not react with any of the 5 bacteria tested by microimmunofluorescence.

COMMENT

Whipple disease was first described in 1907 by Whipple, and its bacterial etiology was confirmed in 1961 by electron microscopy. However, no isolate was successfully established until 1999. We have been able to isolate a second strain that was obtained from a duodenal biopsy. A specific protocol has to be designed because these samples are usually contaminated with intestinal flora. This antibiotic protocol was based on preliminary results derived from studying the in vitro susceptibility of our first isolate (D.R., J. M. Rolain, unpublished data, January 2000). We used colistin, a compound active against gram-negative bacteria, cephalothin, a broad-spectrum antibiotic, and amphotericin B, a potent antifungal agent. This procedure did not prevent
further isolation of the bacterium, showing that our culture technique is efficient. The new strain, type 1A, is genotypically different from the first isolate, which was type 2A.26 Further studies should reveal other genotypic and phenotypic (serologic) differences between these isolates. Due to the duration of incubation needed to obtain isolates, culture may not be useful as a routine diagnostic tool in cases of Whipple disease. Nevertheless, isolation of more T. whippelii strains will lead to better understanding of the epidemiology, pathophysiology, and antibiotic susceptibility of this microorganism. In the patient we studied, the disease relapsed after 1 year of treatment with cotrimoxazole. Cultivation of the recrudescent strain will allow further antibiotic susceptibility testing.

We were able to observe the presence of T. whippelii antigenic material in the duodenal specimens by immunochemistry. To date, no specific morphologic method exists to detect T. whippelii in tissues except electron microscopy. Pathologists usually use PAS staining on sectioned specimens because it shows an intense staining of foamy macrophages.27 It is most likely that PAS-positive granules are phagolysosomes that contain bacilliform bodies at various states of degeneration. However, the detection of numerous PAS-positive macrophages is nonspecific, since they may also be seen in other gut or lymph node disorders such as histiocytosis, melanosis coli, and pneumatosis cystoides intestinalis.28 Mycobacterium avium complex infection of the intestine in patients with acquired immunodeficiency syndrome may be confused with Whipple disease, although mycobacteria are also Ziehl-Nielsen positive, allowing the differentiation of mycobacterial infection from Whipple disease. As phagocytic cells, foamy macrophages may contain numerous degraded PAS-positive products. Lymph nodes and other tissues may also present diagnostic problems, since the changes in routinely stained sections may mimic those of sarcoidosis, with well-formed granulomas and sometimes foreign-body giant cells surrounding fat globules.29

The appearance of T. whippelii on electron microscopy is highly characteristic and appears specific, with a trilamellar membrane appearance.30 However, electron microscopy is time consuming and is not routinely available. Biopsy material contains large numbers of bacilli, so sufficient bacteria should be present in tissue samples for detection by immunohistological techniques using specific antibodies. In this study, we have used the immunohistological method for diagnosis of Whipple disease during active infection.13-15 Discrepancies were noted between PCR and PAS staining of biopsy

DETECTION OF TROPHERYMA WHIPPELII

Figure 2. Sections of a Villus Tip From a Patient With Whipple Disease

A, Inflammatory infiltrate seen in the lamina propria is made up of numerous foamy macrophages with a clear distended cytoplasm (hematoxylin-eosin, original magnification ×250). B, High-power magnification of foamy macrophages containing the characteristic rod-shaped inclusion bodies in the lamina propria. Mucous cells in the epithelium are also periodic acid–Schiff (PAS) positive (original magnification ×400). C, Demonstration of T. whippelii antigenic material by immunohistochemistry in the lamina propria of the villus tips. Tropheryma whippelii antigenic material is revealed in foamy macrophage cytoplasm as red-brown deposits. No immunopositive material is detected within epithelial cells, especially mucous cells (polyclonal mouse anti–T. whippelii, 1:500 dilution, Mayer hematoxylin counterstain, original magnification ×400).
specimens because bacterial DNA disappeared more rapidly than PAS-positive structures. This finding was explained as a persistence of dead bacterial structures. Moreover, since the description of the 16S rRNA specific sequence,\textsuperscript{3,7} PCR has been widely used for the diagnosis of Whipple disease, with good sensitivity\textsuperscript{9-15} despite false-negative results probably due to PCR inhibitors in the samples.\textsuperscript{11,15}

Specificity of intestinal PCR for Whipple disease has been shown to vary with different investigators and PCR procedures. Several investigators have reported nearly 100% specificity,\textsuperscript{9-15} whereas more recent works have demonstrated a lower specificity in part because 4.8% of patients without Whipple disease have a PCR-positive duodenal biopsy sample, 11.4% have PCR-positive gastric fluid,\textsuperscript{17} and 35% have PCR-positive saliva.\textsuperscript{18} These results are discrepant with previously reported results. Contamination of DNA could therefore have been responsible, since such contaminations are well-known problems encountered with diagnostic PCR. Moreover, the observation of bacterial immunopositive material within macrophages of patients may be more confirmative than the detection of microbial DNA within a crushed and non-naturally sterile sample containing what is supposed to be an environmental bacterium.\textsuperscript{31} Given these data, immunohistology may be a helpful diagnostic tool.

**Author Contributions:** Dr Raoult participated in study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, obtained funding, provided administrative, technical, or material support, and supervised conduct of the study. Dr La Scola participated in study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, provided administrative, technical, or material support, and supervised conduct of the study. Dr Leecoq participated in acquisition of data, critical revision of the manuscript for important intellectual content, and provided a record of the patient case. Dr Lepidi participated in acquisition of data and critical revision of the manuscript for important intellectual content.

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**REFERENCES**