Culture of *Tropheryma whipplei* from Human Samples: a 3-Year Experience (1999 to 2002)

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Whipple’s disease is a systemic disease caused by a bacterium, *Tropheryma whipplei* (17). Described by Whipple in 1907, this disease is known mainly as a chronic pathology involving the intestine. Malabsorption, diarrhea, and weight loss eventually associated with adenopathies and polyarthritides correspond to the classical symptoms (5, 8, 24, 28). In the absence of specific antibiotic therapy, the disease is always fatal (8, 24, 28). The reference method for diagnosis of the disease is the histological examination of small-bowel biopsy specimens where periodic acid-Schiff (PAS)-positive, diastase-resistant organisms are observed in macrophages (5, 8, 24, 28). However, gastrointestinal symptoms are minimal or absent for approximately 15% of patients (5, 7, 8, 24, 28). Neurologic Whipple’s disease without digestive symptoms has been reported (12, 23). The bacterium was isolated more frequently from sterile specimens (5 of 8) than from duodenal biopsy specimens (2 of 10), but the difference (P = 0.14) was not significant. Decontamination of digestive samples containing colistin, amphotericin B, and cephalexin or ciprofloxacin did not impair the isolation of *T. whipplei*. The use of vancomycin precluded the primary isolation (7 of 12 versus 0 of 6; P = 0.08) and the establishment of *T. whipplei* (3 of 12 versus 0 of 6; P = 0.5). Omitting samples cultured with vancomycin, the establishment of the strain was significantly higher when antibiotics were prescribed for no more than 7 days (3 of 4 versus 0 of 8; P = 0.03). Our results demonstrate that samples must be collected within 1 week of an antibiotic regimen’s initiation for the successful establishment of the bacterium.

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**MATERIALS AND METHODS**

In our laboratory, we routinely used a JNSP protocol on various clinical samples, and more than 5,000 JNSP protocols have been performed to date. To detect the growth of bacteria, we used 16S rDNA amplification followed by sequencing, and we also stained the cells with either the Gimenez stain or an immunofluorescence stain using the patient serum diluted to 1/100 or specific
antibodies. PAS staining is not useful, because false positives may be observed. We have adapted this JNSP protocol for the growth of T. whippelii. Since 1999, we have tried to culture the Whipple's disease bacterium from clinical samples. We have tested 18 samples from 15 patients with Whipple's disease. Eight noncontaminated samples consisting of duodenal biopsy specimens were naturally contaminated by intestinal flora, were obtained from 10 patients with digestive Whipple's disease. Eight noncontaminated samples consisting of five cardiac valves and three blood samples were also obtained from five patients with Whipple's disease endocarditis.

The several steps of T. whippelii cell culture are summarized in Fig. 1. Culture was performed by the centrifugation-shell vial technique with a human fibroblast cell line (HEL). These cells are routinely used, and more than 6,000 attempts to culture intracellular organisms have been performed in our laboratory using this cell line (unpublished data). For cardiac valves, frozen tissues were placed in minimal essential medium (MEM) and crushed. Duodenal biopsy specimens were incubated for 30 min in 2 ml of Rinaldini medium (6.8 g of NaCl, 0.4 g of KCl, 0.15 g of NaH2PO4, 1.0 g of glucose, 2.2 g of NaHCO3, and 0.002 g of phenol red in 1.0 liter of distilled water) containing a mix of antibiotics. This protocol was established and used and sequenced using primers TW-III of the 23S rRNA gene was amplified and sequenced using primers bw3 and bw4, domain III of the 23S rRNA gene was amplified and sequenced using primers TW-23SmF and TW-InsR2, and the rpoB gene was amplified and sequenced using primers TWRPOB.F and TWRPOB.R, as previously described (6, 14, 16). PCR products were detected by 1% agarose gel electrophoresis analysis and ethidium bromide staining. Amplicons were purified using Qiagen columns (QIAquick Spin PCR purification kit).

For sequencing, a commercially available sequencing kit (dRhodamine Terminator Cycle sequencing kit; Perkin-Elmer Applied Biosystems, Warrington, England) was used according to the manufacturer’s recommendations, as previously reported. All the sequences from the isolated strains were aligned with the sequences in the GenBank DNA sequence database with the BLAST program (version 2.0, National Center for Biotechnology Information).

We compared the number of strains isolated from the valves with the number of strains isolated from the duodenal biopsy specimens by using Fisher's exact test.

**TABLE 1. Data for 18 inoculated samples from 15 patients with diagnosed Whipple’s disease**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex/age (yr)</th>
<th>Localization</th>
<th>Sample</th>
<th>PAS resulta</th>
<th>Immu-nohistology resulta</th>
<th>PCR result/ type</th>
<th>Antibiotic therapy before biopsy/length</th>
<th>Medium with vancomycin</th>
<th>Culture/day of primary detection</th>
<th>Passage no/strain name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F/33</td>
<td>Digestive</td>
<td>Duodenal biopsy</td>
<td>+</td>
<td>+</td>
<td>+/1A</td>
<td>No</td>
<td>No</td>
<td>Yes/30 days</td>
<td>3/Slow 1</td>
</tr>
<tr>
<td>2</td>
<td>M/70</td>
<td>Digestive</td>
<td>Duodenal biopsy</td>
<td>+</td>
<td>+</td>
<td>+/1A</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>M/63</td>
<td>Digestive</td>
<td>Duodenal biopsy</td>
<td>+</td>
<td>+</td>
<td>+/1A</td>
<td>Ofloxacin and cotrimoxazole/2 wk</td>
<td>No</td>
<td>Yes/30 days</td>
<td>10/Slow 2</td>
</tr>
<tr>
<td>4</td>
<td>M/47</td>
<td>Digestive</td>
<td>Duodenal biopsy</td>
<td>+</td>
<td>+</td>
<td>+/1A</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>M/58</td>
<td>Digestive</td>
<td>Duodenal biopsy</td>
<td>+</td>
<td>+</td>
<td>+/2A</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>F/70</td>
<td>Digestive, arthralgia</td>
<td>Duodenal biopsy</td>
<td>–</td>
<td>+</td>
<td>+/1A</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>M/60</td>
<td>Digestive</td>
<td>Duodenal biopsy</td>
<td>+</td>
<td>+</td>
<td>+/2A</td>
<td>Cotrimoxazole/3 wk</td>
<td>Yes</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>F/74</td>
<td>Digestive</td>
<td>Duodenal biopsy</td>
<td>+</td>
<td>NAb</td>
<td>+/1A</td>
<td>Cotrimoxazole/3 wk</td>
<td>No</td>
<td>Yes/30 days</td>
<td>10/Slow 2</td>
</tr>
<tr>
<td>9</td>
<td>M/52</td>
<td>Digestive</td>
<td>Duodenal biopsy</td>
<td>+</td>
<td>NA</td>
<td>+/1A</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>M/66</td>
<td>Digestive, uveitis</td>
<td>Duodenal biopsy</td>
<td>+</td>
<td>+</td>
<td>+/1A</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>M/42</td>
<td>Endocarditis</td>
<td>Cardiac valve</td>
<td>+</td>
<td>+</td>
<td>+/2A</td>
<td>Penicillin G and gentamicin/1 wk</td>
<td>No</td>
<td>Yes/72 days</td>
<td>36/Twist</td>
</tr>
<tr>
<td>12</td>
<td>M/63</td>
<td>Endocarditis</td>
<td>Cardiac valve</td>
<td>+</td>
<td>+</td>
<td>+/2A</td>
<td>Amoxicillin and gentamicin/3 wk</td>
<td>No</td>
<td>Yes/15 days</td>
<td>3/Endo 2</td>
</tr>
<tr>
<td>13</td>
<td>M/61</td>
<td>Endocarditis</td>
<td>Cardiac valve</td>
<td>+</td>
<td>+</td>
<td>+/2A</td>
<td>Ofloxacin and vibramycin/3 wk</td>
<td>No</td>
<td>Yes/30 days</td>
<td>2/Endo 4</td>
</tr>
<tr>
<td>14</td>
<td>M/59</td>
<td>Endocarditis</td>
<td>Cardiac valve</td>
<td>+</td>
<td>+</td>
<td>+/1A</td>
<td>Amoxicillin/6 wk</td>
<td>No</td>
<td>Yes/30 days</td>
<td>2/Endo 4</td>
</tr>
<tr>
<td>15</td>
<td>M/61</td>
<td>Endocarditis</td>
<td>Cardiac valve</td>
<td>+</td>
<td>+</td>
<td>+/1A</td>
<td>Amoxicillin and gentamicin/5 wk</td>
<td>No</td>
<td>No</td>
<td>6/Endo 5</td>
</tr>
</tbody>
</table>

* M, male; F, female.

b NA, not available.

c NP, not performed.

d +, positive; –, negative.

methanol, 100 μl of a homemade rabbit anti-T. whippelii antiserum diluted 1:200 in phosphate-buffered saline (PBS) with 3% nonfat dry milk was added, and the slides were incubated at 37°C for 30 min (1). The specificity of this antibody was established against 34 different bacterial strains (22). After three washes with PBS, 100 μl of fluorescein isothiocyanate-conjugated goat-anti rabbit immuno-globulin G (Jackson Immunoresearch Laboratories, West Grove, Pa.) was diluted 1:200 in PBS with 3% nonfat dry milk was added, and the slides were incubated at 37°C for 30 min. After three washes with PBS, a coverslip was mounted in phosphate-buffered glycerol medium (pH 8.0) and examined at a magnification of ×400 with an epifluorescence microscope.

If the Gimenez staining and the immunofluorescence staining were positive (Fig. 2), the shell vial supernatant and inoculated cells were harvested, inoculated into a 25-cm2 (internal surface) cell culture flask with 5 ml of medium, and incubated at 37°C under a 5% CO2 atmosphere to establish the isolate. When cultures were positive, small, pale-pink, poorly staining bacilli were observed with Gimenez staining and small, brightly fluorescent bacilli were observed with immunofluorescent staining. A primary isolation of T. whippelii was defined as a positive specific immunofluorescence or Gimenez staining in the supernatant of the inoculated shell vials at the 1st passage. A strain of T. whippelii was considered to be established when at least four subcultures were obtained. Histological and immunohistochemistry analysis were performed on 5-μm-thick formalin-fixed and paraffin-embedded biopsy sections, as previously described (1, 21, 32, 33).

PCR and sequencing were performed on both the fresh biopsy specimen and the isolate. The biopsy sample was ground mechanically and resuspended in 500 μl of sterile deionized water in a sterile tube. Five hundred microliters of cell culture supernatant was centrifuged at 12,000 × g for 5 min. The resulting pellet was resuspended in 90 μl of Tris-EDTA buffer for PCR studies. The DNA was extracted using Qiagen (Hilden, Germany) columns (QIAamp tissue kit) as described by the manufacturer. To perform PCR, the 16 to 23S rDNA intergenic spacer region was amplified and sequenced using primers bw3 and bw4, domain III of the 23S rRNA gene was amplified and sequenced using primers TW-23SmF and TW-InsR2, and the rpoB gene was amplified and sequenced using primers TWRPOB.F and TWRPOB.R, as previously described (6, 14, 16). PCR products were detected by 1% agarose gel electrophoresis analysis and ethidium bromide staining. Amplicons were purified using Qiagen columns (QIAquick Spin PCR purification kit).

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We compared the number of strains isolated from the valves with the number of strains isolated from the duodenal biopsy specimens by using Fisher’s exact test.
test with Epi Info (version 6.04a; Centers for Disease Control and Prevention, Atlanta, Ga.). A $P$ value of $<0.05$ was considered statistically significantly different.

**RESULTS**

Since 1999, after the first successful isolation of *T. whipplei* in our laboratory, we have never observed the growth of this bacterium when we did not expect it. Each time a *T. whipplei* strain was isolated, a diagnosis of Whipple’s disease had been established previously by histological or PCR analysis. All together, we obtained primary isolation of seven isolates and establishment of three strains (Table 1 and Fig. 3). The mean time for the primary detection was 30 days (standard deviation, ±20 days). *T. whipplei* was isolated more frequently from sterile specimens (5 of 8) than from contaminated samples (2 of 10), but this difference was not statistically significant ($P = 0.14$). However, the statistical results should be regarded with caution because the number of isolates is still small.

Our first isolate of *T. whipplei*, the Twist-Marseille strain, was obtained from a cardiac valve (31). The strain is now in its 36th passage, and its genome has been entirely sequenced (GenBank accession number AE0116852). Three new strains (Endo 2, Endo 3, and Endo 4) were isolated from three of the four other infected cardiac valves from patients with negative-blood-culture endocarditis. Of these, two strains were subcultured three times and one was subcultured only once. All the valves presented positive PCR amplification and immunohistochemistry. Three isolates were identified as genotype 2A and two as genotype 1A.

Another endocarditis strain (Endo 5) was also isolated, from a blood culture obtained from a patient (patient 15) for whom conventional blood culture was negative. This blood culture was performed at the beginning of antibiotic therapy. *T. whipplei* growth was observed 15 days after inoculation. Cardiac valve removal was performed for this patient approximately 5 weeks after the beginning of antibiotics. No bacterial growth was observed for this cardiac valve, but PCR and immunohistochemistry yielded positive results. The two other blood samples cultured were taken 5 and 7 weeks after the beginning of antibiotic therapy and were both sterile. PCR was performed only for the two last blood samples, and both were negative.

Before the cardiac surgery or the blood sampling, all patients received antibiotic therapy. Previous antibiotic therapy did not prevent the isolation of *T. whipplei* from sterile specimens but may have prevented the establishment of strains. There was an apparent trend for isolates obtained from patients treated with antibiotics for more than 7 days not to become established in culture. Both strains obtained from pa-
tients treated for fewer than 7 days could be propagated beyond initial isolation, while none of the six strains obtained from patients treated longer ($P = 0.03$) could be continued in long-term culture. None of the patients with negative-blood-culture endocarditis had intestinal symptoms, and their duodenal biopsy specimens were negative by PAS staining, immunochemistry, and PCR.

The second strain of *T. whipplei* isolated in our laboratory was obtained from a duodenal biopsy specimen (Slow 1) of a patient with a relapsing digestive Whipple’s disease (32). Because the duodenal biopsy specimens were naturally contaminated, we used colistin, cephalotin, and amphotericin B as antimicrobial agents to control contamination. Unfortunately, the strain could not be continued after 3 passages. Another strain (Slow 2) was isolated from a duodenal biopsy specimen. This isolate, for which colistin, amphotericin B, and ciprofloxacin were used as antimicrobial agents, is currently in its 10th passage. The antibiotic solution, when used without vancomycin, did not affect the culture, as two primary isolations and one strain establishment were obtained for two duodenal biopsy specimens, results comparable to those for sterile samples treated without antibiotics (2 of 8 versus 5 of 8, respectively [$P = 1$]). Addition of vancomycin to the antibiotic solution had a profound effect on the isolation of *T. whipplei*: none of the six samples exposed to vancomycin-containing solutions grew. In contrast, 7 of 12 samples in solutions without vancomycin grew ($P = 0.03$), and 3 of these became established ($P = 0.05$).

The prescription of antibiotics for more than 7 days before
sampling (excluding samples cultured with vancomycin) prevented culture of T. whipplei from any samples. Four of four strains from patients without antibiotic therapy were primary isolates, and three of four became established, versus three of eight \((P = 0.3)\) and none of eight \((P = 0.07)\), respectively, for patients treated with antibiotics for 7 days. Four strains were isolated from the 10 genotype-1A samples, and 3 strains were isolated from the 5 genotype-2A samples. Two of the genotype-1A isolates and one genotype-2A isolate were successfully established.

\section*{DISCUSSION}

The epidemiology and pathogenesis of Whipple’s disease are still poorly understood. The isolation of infecting bacteria can serve, therefore, as a basis for the evaluation of much-needed improved diagnostic assays and as a way to enhance our understanding of the diversity and epidemiology of T. whipplei and the infections that it causes. In 1997, the first attempt at isolation of the bacterium by using intestinal biopsy samples from a patient with Whipple’s disease failed because of the inevitable bacterial overgrowth (35). The isolation of T. whipplei from tissues of two heart valves, obtained from two patients with Whipple’s disease endocarditis, was reported after inoculation in interleukin-4-deactivated macrophages (35).

However, the two strains could not be established and propagated, and the work was not reproduced. Since 1999, two isolates of T. whipplei have been recovered after inoculation of HEL cells in shell vials by our team (31, 32). In vitro, growth has been observed not only in HEL or MRC5 cells (17) but also in HeLa cells in an acidic vacuole at pH 5 (35).

The centrifugation-shell vial system is a cell culture technique for the culture of viruses and facultatively or strictly intracellular bacteria. We have used this technique routinely in our laboratory for several years, and it has led to the recovery of fastidious bacteria including Rickettsia spp. (3, 19), Coxiella burnetii (29), Bartonella spp. (20), Francisella tularensis (9), Chlamydia trachomatis (27), and mycobacteria (10) from various clinical specimens. The data reported here represent the first collection of established T. whipplei isolates obtained from clinical specimens and the first isolation of the bacterium from blood. In addition, another strain of T. whipplei was isolated from cerebrospinal fluid by an American-English team using our protocol (2).

Culture of T. whipplei is currently achievable, but it is fastidious work. A difference exists between primary isolation and establishment of a strain. Successful isolation has not always led to propagation of the isolate. Only three of seven T. whipplei isolates were successfully established for more than 4 passages. This is in line with previous studies that have shown the
potential of the shell vial technique for isolation of fastidious organisms but not necessarily for further propagation (19, 20). In these studies, we could establish only 15 of 34 (44%) *Rickettsia* strains and 65 of 81 (80%) *Bartonella* strains isolated by the shell vial method. The inability of these isolates to grow may be explained by specimens not being inoculated immediately after isolation, small numbers of organisms, and/or antibiotic therapy given prior to biopsy. Our experience with *C. burnetii* (29), *Bartonella* spp. (20), and *Rickettsia conorii* (19) demonstrated that samples must be collected prior to the initiation of an antibiotic regimen if the bacterium is to be successfully isolated. However, in our present study, most of our patients had antibiotic therapy before the sampling. Five of our seven isolates were from patients with previous antibiotic therapy. Our data show that the critical factor is the length of the antibiotic therapy before the sampling. Antibiotic therapy for more than 7 days before sampling significantly affects the establishment of the bacterium in culture, as evidenced by the fact that none of the bacteria isolated from patients on therapy for more than 7 days could be grown.

In addition, due to the presence of intestinal flora in duodenal biopsies, antibiotics must always be used in the culture medium to decontaminate the samples. It is therefore necessary to design specific antibiotic protocols. Our first antibiotic protocols were based on preliminary results derived from studying the in vitro antibiotic susceptibility of our first isolate (unpublished data). Ciprofloxacin, cephalothin, and colistin were used at concentrations which appeared not to inhibit the growth of *T. whipplei*. When these protocols were used, *T. whipplei* could be isolated and established. However, the principal limitation of these antibiotic mixes, directed against the intestinal flora, is that they could not always inhibit the growth of *Staphylococcus*. Problems with staphylococcal contamination led us to use vancomycin, with the result that cultures of duodenal biopsy specimens exposed to this drug were negative. This finding is in keeping with 16S rDNA sequence results that suggest that *T. whipplei* might be classified among gram-positive organisms (34), and it also suggests that vancomycin might be active in vivo against Whipple’s disease. Since vancomycin must be avoided in the culture medium, we have no antibiotic regimen to propose to control staphylococcal overgrowth. Thus, more information about *T. whipplei*’s antibiotic susceptibility profile is necessary to improve the culture of duodenal biopsy specimens.

In our study, *T. whipplei* strains of the two most common genotypes, 1A and 2A, were found and were successfully cultivated. In addition, there is no difference in primary isolation and establishment between these two different genotypes of *T. whipplei*.

Several factors have allowed the successful culture of *T. whipplei*. The first is patience, as the growth of *T. whipplei* is observed after an average of 30 days of incubation and sometimes more. The second factor is the treatment of patients, as our data clearly demonstrate that samples must be collected before the completion of 1 week of antibiotic treatment in order to isolate and establish the bacterium. Third, for contaminated samples, antimicrobial protocols consisting of colistin (10 μg/ml), amphotericin B (1 μg/ml), and ciprofloxacin (1 μg/ml) or cephalothin (2 μg/ml) can be used but vancomycin should absolutely be avoided. Currently, culture cannot be considered a practical tool for the diagnosis of Whipple’s disease, but obtaining several more strains may be useful for better understanding of the pathology of Whipple’s disease. The one great disadvantage of this method of culturing *T. whipplei* is that culture is currently performed only in specialized laboratories with the capacity for cell culture (26), making it necessary to improve the culturing technique in order to more efficiently isolate and propagate this organism. Finally, we conclude that the isolation and establishment of *T. whipplei* in culture was not an epiphenomenon and that we have defined a strategy for accomplishing this on a routine basis. Our success will no doubt improve as we gain more experience in isolating this very fastidious organism.

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REFERENCES


