MOLECULAR DIAGNOSIS

Strategy for detection and identification of bacteria based on 16S rRNA genes in suspected cases of Whipple's disease

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The 16S ribosomal RNA (rRNA) gene of the phylogenetic subdivision containing gram-positive bacteria with a high G + C content was detected specifically in clinical specimens from patients suspected of having Whipple's disease. The primary structure of 16S rDNA amplified from clinical samples was determined by cloning and sequencing. Two sorts of sequences were identified: one corresponded exactly to the rRNA sequence of *Tropheryma whippelii* (GenBank accession no. M87484) while the other was related to that of members of the genus *Corynebacterium*. No sequence related to *Mycobacterium* spp. or *Rhodococcus equi* was observed. Exhaustive examination of negative specimens with broad-range eubacterial primers detected one sequence related to Enterobacteriaceae and another related to *Enterococcus* spp. To speed identification of *T. whippelii*, a nested amplification method was devised. A first amplification specific for the gram-positive bacteria subdivision was performed, followed by a second amplification with *T. whippelii*-specific primers. The amplified *T. whippelii* product was checked by digestion with *Ava*II, *Stu*I, and *Pst*II endonucleases. These techniques were applied to DNA extracted from seven intestinal biopsy samples, two cerebrospinal fluid samples and one articular fluid from patients suspected of having Whipple's disease. *T. whippelii* 16S rDNA was found in two of the biopsy samples, one of the cerebrospinal fluid samples and in the articular fluid.

Introduction

Whipple's disease, defined initially as an 'intestinal lipodistrophy' of the small intestine, is rare, with only 617 cases reported in the world between 1907 and 1986 [1]. The most common clinical presentation is a malabsorption syndrome with diarrhoea, weight loss and abdominal pain. Migratory arthritis or severe arthralgia often occur for years before diarrhoea develops. The disease is also associated with fever, lymphadenopathy, anaemia and cardiac involvements (pericarditis, endocarditis) [2]. Because of the systemic nature of the illness, increased pigmentation of skin, polyarthritis, ascites, hepatomegaly and splenomegaly, pleuritis, ophthalmic (chronic retinitis and uveitis) or central nervous system involvements (dementia, ophthalmoplegia, paralysis of gaze, myoclonus or cerebella ataxia) are also seen [1, 3, 4].

Histological diagnosis is usually by microscopic examination of the duodenal lamina propria. Large macrophages that contain positive periodic acid-Schiff (PAS) inclusions are observed [5]. PAS-positive granulations are sickle-shaped and brilliantly purple-red (magenta) in colour. These characteristic cells were first described as sickle-particle-containing (SPC) cells [6]. The irregular aggregates responsible for PAS-positivity of macrophages are glycoproteins, probably derived from degenerate bacterial membranes [7]. These aggregates are generally considered sufficient for a diagnosis of Whipple's disease in the intestinal bowel.

However, histological diagnosis poses many problems. Histologically, AIDS cases with *Mycobacterium avium*-intracellular bowels lesions resemble Whipple's disease [8]. Differentiation is by acid-fast staining which is positive for *Mycobacterium* and negative for Whipple's disease agent [9]. *Corynebacterium* spp. and *Rhodococcus equi* infections also mimic the histological aspect of Whipple's disease by the presence of PAS-positive inclusions in macrophages [2, 10]. In extra-intestinal tissue, the typical

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lesion is more difficult to observe [2]. Peripheral lymph nodes show less intense PAS-positive reactions than SPC cells [11]. PAS-positive macrophages can also be seen in other diseases, and these changes are not considered to be of diagnostic value [12]. Synovial fluid smears show PAS-positive material in both polymorphonuclear and synovial cells similar to that observed in other inflammatory arthropathies [13]. Furthermore, the beginning of Whipple's disease may mimic sarcoidosis with polyvisceral granulomatous dissemination. Whipple's disease-specific SPC cells appear later [4, 12, 14].

The atypical intestinal histology and diagnostic problems of extra-intestinal Whipple's disease [15–18] are good reasons to look for other diagnostic methods. Electron microscopy can help in identifying macrophage inclusions. These are heterophagic vacuoles containing fragments of bacterial cell walls. The ultrastructural pattern of the rod-shaped wall corresponds to that of gram-positive bacteria with an additional trilamellar membrane external to the cell wall, specific to the Whipple's disease bacillus [7, 19, 20]. Unfortunately, this technique is not available in all laboratories.

No bacterium has been isolated reproducibly from Whipple's disease lesions by classical bacteriological methods. Occasionally, unidentified *Corynebacterium* spp. or *Streptococcus* spp. have been isolated [2]. New methods able to detect and identify bacteria without culture have been proposed [21]. PCR has been used to amplify bacterial 16S rRNA genes (rDNA) from infected tissues of patients with Whipple's disease. From the novelty of the 16S rDNA sequence, the bacillus was named *Tropheryma whippelii* and two specific PCR primers have been described [22, 23]. PCR has detected *T. whippelii* sequences in duodenal mucosa [16, 24–27], peripheral blood [24, 28], bone marrow [26], cells derived from pleural effusion [24], vitreous tissue [16] and aortic valve tissue [29].

As infections caused by the *M. avium* complex, *Corynebacterium* spp. and *R. equi* may be syndromes or have histopathology similar to Whipple's disease, this paper describes a new approach for identification of suspected Whipple's disease. This strategy initially amplifies the 16S rDNA of gram-positive bacteria with a high G + C content directly and specifically from clinical specimens. Assays were performed with typical digestive Whipple's disease specimens as well as neurological or rheumatological cases. When this initial amplification was negative, universal primers were used in repeat experiments. In both cases, the 16S rDNA amplicon was sequenced and compared with 16S rRNA sequences available in databases. Identification was achieved when the specimen sequence was identical with one from the databases. Otherwise, the unknown sequence was positioned on the phylogenetic tree. Taking into account the initial

amplification results, a nested amplification method was devised for the detection of any gram-positive bacterium and the specific identification of *T. whippelii* in order to speed identification and reduce labour associated with DNA sequencing.

Materials and methods

Bacterial strains

To evaluate the specificity of the PCR, genomic DNA extracts from the following bacteria, representing the major subdivisions of the Eubacteria [30], were used as controls: *Terrabacter tumescens* strain CIP 102515; *Rothia dentocariosa* strain CIP 8183; *Arthrobacter globiformis* strain CIP 8184; *Micrococcus luteus* strain CIP A270; *Corynebacterium xerosis* strain ATCC 373; *Propionibacterium acnes* strain ATCC 6919; *Gardnerella vaginalis*; *Mycobacterium avium* (high G + C gram-positive branch); *Staphylococcus aureus*; *Bacillus cereus* (low G + C gram-positive branch); *Afipia felis* strain ATCC 53690 (Proteobacteria α subgroup); *Alcaligenes faecalis*; *Burkholderia cepacia* (Proteobacteria β subgroup); *Escherichia coli* strain K12; *Salmonella enterica*; *Pseudomonas aeruginosa* (Proteobacteria γ subgroup) *Helicobacter pylori* (Proteobacteria ϵ subgroup) and *Bacteroides fragilis* (Flavobacterium phylum).

R. dentocariosa was grown on Columbia Agar (Diagnostics Pasteur, Marnes-la-Coquette, France) supplemented with defibrinated horse serum 20% v/v and incubated at 37°C. *A. globiformis*, *Ter. tumescens* and *M. luteus* were grown on Columbia Agar at 20°C, 25°C and 30°C, respectively. All other species were grown on Tryptocasein Soya Agar (Diagnostics Pasteur) at 30°C.

Clinical specimens

Whipple's disease was suspected on the basis of clinical or histopathological features, or both. Specimens consisting of non-fixed frozen biopsies of fundus, ileum or jejunum, mesenteric lymph node, cerebrospinal or articular fluid were received from 10 different patients with suspected Whipple's disease (Table 1). Clinical presentations were variable. Six patients (cases 1–6) had gastritis, duodenal lesions or jejuno-colitis, associated with weight loss, abdominal pain and fever. Case 5 and case 6 [31] had arthritis, in one instance on prosthetic material, complicating a diagnosis of Whipple's disease. Cases 7, 8, 9 [32] and 10 had suspected neurological Whipple's disease without gastrointestinal symptoms. PAS-positive macrophages in the endoscopic biopsies had been seen only in cases 1, 5 and 7 (Table 1). Ten other specimens from patients with no evidence of Whipple's disease were included as negative controls. These specimens consisted of two diarrhoeal stools, two hepatic biopsies (with granulomatous lesions), five gastric biopsies (with gastritis) and one sample of articular fluid.

Table 1. Clinical specimens from suspected cases of Whipple's disease and results obtained following examination by different PCR methods

Case no.	Specimen	PAS-stained granulations in intestinal macrophages	Identification following amplification and sequencing		Identification following nested PCR	
			High G+C gram-positive primers	Universal primers	Gram-positive primers	<i>T. whippelii</i> -specific primers
1	Intestinal biopsy	+	<i>T. whippelii</i>	ND	+	<i>T. whippelii</i>
2	Intestinal biopsy	–	<i>Corynebacterium</i> spp.	ND	+	–
3	Intestinal biopsy	–	negative	<i>Enterococcus</i> spp.	+	–
4	Intestinal biopsy	–	ND	ND	–	–
5	Intestinal biopsy	+	ND	ND	+	<i>T. whippelii</i>
6	Articular fluid	–	ND	ND	+	<i>T. whippelii</i>
7	Intestinal biopsy	+	ND	ND	–	–
8	Intestinal biopsy	–	Negative	Enterobacteriaceae	–	–
9	Cerebrospinal fluid	–	ND	ND	+	<i>T. whippelii</i>
10	Cerebrospinal fluid	–	ND	ND	–	–

ND, not done.

DNA extraction

Genomic DNA was extracted from bacteria or clinical samples with a procedure used previously for studying cat scratch disease [33]. A negative control (all the reagents without sample) was included in each batch of experiments to ensure that none of the extraction buffers or reagents was contaminated with target DNA.

16S rDNA amplification before sequencing

Reaction mixtures were prepared under semi-sterile conditions following published guidelines to avoid contamination [34]. PCR amplification (see below) was performed with a DNA Thermal Cycler Model 480 (Perkin Elmer). A blank control without template DNA was included in each batch of experiments and yielded no PCR product, demonstrating that contamination was not a problem. DNA extracted from *C. xerosis* was used as a positive control.

The following oligonucleotide primers were selected on the basis of 16S rDNA sequence conservation among 83 sequences (obtained from GenBank) of gram-positive bacteria with a high G + C content: primer G+, 5'-ACGGGTAGCCGGCCTGAGAGGG-3'; and primer rG+, 5'-ACCCCGTCAATTYCTTTGAGTTT-3' (Y = C or T). The target of primer G+ was located at position 290 and the target of primer rG+ was located at position 920 (*E. coli* numbering system [35]). For 16S rDNA amplification before sequencing, Bio-Taq Polymerase (Bioprobe, Montreuil-sous-bois, France) was used, with a pre-denaturation step of 5 min at 94°C, followed by 35 cycles of 94°C for 1 min, 70°C for 1 min and 72°C for 1 min. For elongation of PCR product, a final step at 72°C for 5 min was included.

To examine whether gram-positive bacteria with a high G + C content were the only bacteria associated with Whipple's disease, broad-range eubacterial oligonucleotide primers were used [36]. The target of the 5'-end primer was located at position 10 of the 16S

rDNA gene, and the 3'-end primer at position 1490 for the same gene. Amplification involved pre-denaturation for 5 min at 94°C, followed by 35 cycles of 94°C for 1 min, 49°C for 1 min and 72°C for 1 min, with a final elongation step at 72°C for 5 min. These primers were also used to demonstrate the absence of inhibitors in clinical specimens (stools and gastric biopsies) used as negative controls.

Amplification products were detected by electrophoresis on agarose 0.8% w/v gels in Tris-acetate buffer (0.04 M Tris-acetate, 0.002 M EDTA, pH 8.1), with a 1-kb DNA Ladder (Gibco BRL) as the DNA size standards.

16S rDNA cloning and sequencing

To facilitate cloning, a 12-base dUMP-containing sequence—CUACUACUACUA—was added to the 5'-end of PCR primers [37]. The resulting PCR product was cloned in plasmid pAMP1 with the CLONAMP™ System (Gibco BRL). Twelve clones/specimen were chosen for amplification in *E. coli* strain MC1061. The presence of the insert was verified by restriction digestion with *Bam*HI and *Eco*RI [38]. Two clones/specimen were sequenced by 'cycle sequencing' (Amersham International) with universal primers complementary to plasmid or conserved regions of 16S rDNA labelled with [γ P³²]-ATP (Isotopchim, Ganagobie-Peyruis, France) [39].

Phylogenetic interpretation

Partial sequences obtained were compared with rRNA sequences from the GenBank (Bethesda, MD, USA) and European Molecular Biology Laboratory (Cambridge) databases provided with the Lasergene software (DNASTar, London). If no sequence was identical, the nearest sequences were aligned manually. Identification of the partial sequence was performed with phylogenetic analysis by the Maximum Parsimony Method with the PAUP program (heuristic option) [40] and by

the Distance Method with the Kimura parameters [41] included in the programs DNADIST and NEIGHBOR [42] contained in the PHYLIP Inference Package [43].

Nested PCR for diagnosis

16S rDNA from the gram-positive phylum was amplified with Hi-Taq Polymerase (Bioprobe). Primer A_L—5'-AGRGTTYGATYCTGGCTCAGGAYG-3' (R = A or G)—was selected on the basis of sequence conservation in the gram-positive phylum of Eubacteria (both low and high G + C content organisms in the gram-positive bacteria branch), and had a target at the 5'-end, position 10, of the 16S rRNA gene. The second primer was rG+ (see above), but was used in this experiment at low stringency (65°C). Amplification involved predenaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 1 min, with a final elongation step at 72°C for 5 min.

DNA extracts from the strains listed above were used as positive or negative controls. One sample known to contain *T. whippelii* 16S rDNA sequences (case 1) was included as a control. When no amplification was detected, or when a 'blur' was seen on the agarose gel, the amplification was interpreted as being inhibited by compounds found in the sample. The PCR was then repeated with a sample diluted 10- and 100-fold.

Depending on the amount of gram-positive 16S rDNA product amplified, the nested PCR was done with 1 µl of either a 1 in 10 or 1 in 100 dilution of the initial PCR product. Two internal oligonucleotide primers were used to identify *T. whippelii*. These were primer W185—5'-CGACCCATGAGGGCATCCTC-3' (position 185)—and primer rW830—5'-GCGGTGGAAC-CACCCCCACG-3' (position 830). Nested PCR was performed with predenaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. The amplified products were detected by electrophoresis as described above.

The resulting amplification products were checked by digestion with *Ava*II, *Stu*I or *Pst*I. These enzymes cleave *T. whippelii* 16S rDNA at position 625 with *Ava*II, 489 with *Pst*I and 415 with *Stu*I, yielding products of specific sizes. 16S rDNA of phylogenetically related species (*Ter. tumescens*, *Dermatophilus congolensis*, *Clavibacter xyli*, *R. dentocariosa*, *A. globiformis*, *M. luteus*) have different cleavage sites on the corresponding rDNA fragment, resulting in restriction fragments of different sizes. Therefore, 10 µl of each PCR amplification product were digested, according to the conditions recommended by the manufacturer of the enzymes (New England Biolabs), and analysed by electrophoresis on an agarose (Bioprobe) 1% w/v gel containing NuSieve Agarose (FMC Bio Products, Rockland, ME, USA).

Results

16S rRNA gene sequence analysis

PCR amplification with primers G+ and rG+ and either *C. xerosis* DNA or DNA extracts from cases 1 and 2 yielded a 630-bp fragment. No PCR product was observed with a control containing no DNA template or DNA extracts from cases 3 and 8 (data not shown). The 630-bp fragments amplified from the two clinical cases were cloned and sequenced. For case 1, the sequence obtained was identical to that of *T. whippelii* 16S rDNA (accession no. 87484 [23]) (Table 1). For case 2, the sequence obtained corresponded to a *Corynebacterium* spp. 16S rDNA, (Fig. 1). On this part of the 16S rRNA, the sequence was identical to those of *C. amycolatum* and *C. asperum*.

PCR amplification with eubacterial primers applied to DNA extracts from both of the negative samples yielded a 1480-bp product. These fragments were cloned and partially sequenced. One sequence belonged to a member of the Enterobacteriaceae (case 8), while the other was related to sequences belonging to members of the genus *Enterococcus* (case 3).

Fig. 1. Phylogenetic tree derived from a comparison of the 16S rDNA sequences of *Corynebacterium* spp. by the PAUP method. GenBank accession numbers are in parentheses. The position of the sequence derived from case 2 is indicated.

Nested PCR and restriction fragment length polymorphisms (RFLPs)

The nested amplification method was tested with the 10 clinical samples (including case 1, shown above to contain 16S rDNA corresponding to *T. whippelii*, and case 2, shown to contain the 16S rDNA sequence of a *Corynebacterium* spp.), all the bacteria listed in *Materials and methods*, and 10 negative control clinical specimens. A 910-bp fragment was obtained after the first amplification with primers A_L and rG+ (specific for gram-positive bacteria) with *S. aureus*, *B. cereus*, *C. xerosis* (Fig. 2, lane 6), *P. acnes* (lane 7), *A. globiformis* (lane 8), *Ter. tumescens* (lane 9), *R. dentocariosa*, *M. avium*, *G. vaginalis*, *Mic. luteus*, the stools and gastric biopsies used as control specimens, and five clinical samples (cases 1, 2, 3, 5 and 9). No amplification product was observed for *Af. felis*, *Alc. faecalis*, *E. coli* (lane 10), *Sal. enterica*, *Ps. aeruginosa*, *Bur. cepacia*, *H. pylori*, *Bact. fragilis* (lane 11), the negative control (all the reagents without target DNA), the hepatic biopsies and articular fluid used as control specimens, and the other clinical samples (cases 4, 6, 7, 8 and 10) (lanes 4 and 5). Inhibition, evidenced by a 'blur' on the agarose gel, was detected with three samples. Sample dilution allowed subsequent amplification of gram-positive 16S rDNA from case 6 (lane 3).

The second amplification (with *T. whippelii*-specific primers W185 and rW830) performed on the 910-bp amplicon generated a 650-bp fragment for four

clinical samples: cases 1 (Fig. 3, lane 12), 5, 6 (containing inhibitors) and 9. No amplification was obtained with all the other strains or samples tested: *S. aureus*, *G. vaginalis*, *B. cereus*, *M. avium* (Fig. 3, lane 1), *C. xerosis* (lane 2), *P. acnes* (lane 3), *A. globiformis* (lane 4), *Ter. tumescens* (lane 5), *R. dentocariosa* (lane 6), *E. coli* (lane 7), *Bact. fragilis* (lane 8), the negative control (lane 15), and the five clinical samples corresponding to cases 3, 4, 7, 8 and 10 (lanes 9, 10, 11 and 13). For case 2, containing *Corynebacterium* spp. 16S rDNA (lane 14), a 910-bp fragment resulting from the first amplification persisted on the agarose gel, but no 650-bp fragment was seen.

The RFLP pattern of the products, generating two fragments of 300 bp and 350 bp with *Pst*I (Fig. 4, lane 1), two fragments of 235 bp and 415 bp with *Stu*I (Fig. 4, lane 5), and two fragments of 220 bp and 430 bp with *Ava*II (data not shown) were used to confirm *T. whippelii* 16S rDNA sequence identity.

Of the 10 samples from suspected cases of Whipple's disease, six contained 16S rDNA of gram-positive bacteria. Only four 16S rDNA sequences corresponded to *T. whippelii* (Table 1). For the six patients with gastrointestinal symptoms, only three samples (from three patients) contained detectable *T. whippelii* 16S rDNA. Of the three samples containing bowel PAS-positive macrophages, two contained detectable *T. whippelii* 16S rDNA. Surprisingly, of four suspected cases of neurological Whipple's disease, *T. whippelii*

Fig. 2. PCR products derived from specific amplification of 16S rDNA gene fragments of the gram-positive phylum. DNA samples were: lane 1, DNA size markers; 2, Whipple's disease specimen (case 1) with rDNA sequence corresponding to *T. whippelii*; 3, Whipple's disease suspected specimen (case 7); 4, Whipple's disease suspected specimen (case 5); 5, Whipple's disease suspected specimen (case 4); 6, *C. xerosis*; 7, *P. acnes*; 8, *A. globiformis*; 9, *Ter. tumescens*; 10, *E. coli*; 11, *Bact. fragilis*; 12, DNA size markers.

Fig. 3. PCR products derived from specific amplification of 16S rDNA gene fragments of *T. whippelii*. DNA samples were: lane M, DNA size markers; 1, *M. avium*; 2, *C. xerosis*; 3, *P. acnes*; 4, *A. globiformis*; 5, *Ter. tumescens*; 6, *R. dentocariosa*; 7, *E. coli*; 8, *Bact. fragilis*; 9, sample from case 5; 10, sample from case 8; 11, sample from case 4; 12, Whipple's disease specimen (case 1) with rDNA sequence corresponding to *T. whippelii*; 13, sample from case 3; 14, Whipple's disease suspected specimen (case 2) with rDNA sequence corresponding to *C. xerosis*; 15, negative control.

Fig. 4. Restriction digest profiles following specific amplification of 16S rDNA gene fragments from *T. whippelii*. Lane 1, DNA size markers; 2, *Pst*I digest; 3, no sample; 4, DNA size markers; 5, *Stu*I digest.

16S rDNA was detected in the cerebrospinal fluid of only one case. In addition, *T. whippelii* 16S rDNA was amplified from the articular fluid of one patient with arthritis complicating a diagnosis of Whipple's disease.

Discussion

The identification strategy used in this paper was based on the current hypothesis that Whipple's disease is caused by a gram-positive bacterium. A first approach detected high G + C content gram-positive bacterial

16S rDNA by amplification and sequenced the amplicon to reach identification. A second approach amplified 16S rDNA from any gram-positive bacterium in the clinical specimens and then used the amplicon as a target for a further *T. whippelii*-specific amplification. This nested PCR should be easier to perform than sequencing in clinical laboratories. The small set of cases investigated provided information on the diagnostic ability of these assays.

When clinical signs included diarrhoea, weight loss, abdominal pain, fever and adenopathy, the diagnosis of Whipple's disease made by the physician in charge was confirmed by the detection of *T. whippelii* 16S rDNA (cases 1, 5 and 6). However, with clinically atypical disease (case 7), PAS granulations in intestinal macrophages could not be explained by the presence of gram-positive bacteria. In case 2, the partial 16S rDNA sequence was identical to the corresponding *C. amycolatum* and *C. asperum* sequences. These two organisms constitute a single genomic species [44] and seem to be the *Corynebacterium* species isolated most frequently from clinical infections [45]. Their 16S rDNA sequence could be easily differentiated (10 differences) from the 16S rDNA sequence of *C. xerosis*. Thus, the origin of the sequence detected was not a result of laboratory contamination by the *C. xerosis* sequence used as a PCR control. Unidentified *Corynebacterium* spp. and *C. bovis* have been reported from suspected cases of Whipple's disease [2, 46]. However, because the histology of case 2 was not convincing, the sequence obtained may still originate from cutaneous bacteria. Broad-range eubacterial primers occasionally amplified enterobacterial or *Enterococcus* sequences from negative samples, but these bacteria were not linked to Whipple's disease and probably originated from the intestinal flora. Diagnoses of ischaemic colitis and

sarcoidosis were made, respectively, for cases 3 and 8. None of the specimens studied contained *R. equi* or mycobacterial sequences.

When arthritis was the dominant clinical feature, diagnosis was by PCR assay of the gastrointestinal biopsy (case 5) or of the articular fluid (case 6) [31]. Both cases were arthritis relapses which appeared during treatment. In optical microscopy, synovial fluid smears indicated non-specific arthropathy. Detection of Whipple's bacillus by PCR assay can be an alternative to examination of the synovial membrane by electron microscopy [13]. Neurological Whipple's disease was suspected if there was an association of ophthalmoplegia, dementia and cerebella ataxia. Meninges are rarely involved in Whipple's disease, but the presence of typical PAS-positive macrophages has been noted occasionally [3, 47]. *T. whippelii* 16S rDNA was detected in the cerebrospinal fluid of case 9 only. Reversal of neurological signs and magnetic resonance images by trimethoprim-sulphamethoxazole treatment was in agreement with this diagnosis [32, 47].

In both cases 6 and 9, the duodenum showed no histological evidence of infection and *T. whippelii* 16S rDNA was found at other locations. The intestinal tract could either be free from Whipple's bacillus at the start of the disease or the bacteria could be present in insufficient numbers to be observed microscopically or not yet degraded by macrophages [7]. For arthritis without typical intestinal lesions [15], or neurological symptoms without gastrointestinal involvement [48, 49], we recommend that the PCR test be performed with articular or cerebrospinal fluid.

Detection and identification of fastidious pathogenic bacteria has traditionally been a challenge to the clinical microbiologist. When bacteria are non-cultivable, or only cultivable at very low efficiency, amplification and sequencing of rDNA seems to be the best approach. Universal primers can be used to amplify 16S rDNA from unknown bacteria causing infectious diseases and then sequenced [23, 50, 51]. However, when several species occur in the specimen, separation of the various DNA sequences by cloning is necessary, which makes the procedure laborious. A major interpretation problem arises when a sequence corresponding to a ubiquitous cutaneous, intestinal, or environmental species is found.

Specific primers for PCR amplification of *T. whippelii* rDNA have been described previously [23], but were not used in this study for the following reasons. Although the implication of *T. whippelii* in Whipple's disease is quite convincing, the occasional implication of other gram-positive bacteria has not been ruled out. Furthermore, *M. avium-intracellulare*, *Corynebacterium* spp. and *R. equi* have been found to generate PAS-positive macrophages in intestinal lesions. Amplification of sequences from high G + C gram-

positive bacteria or, for practical reasons, gram-positive sequences in general, would be less likely to miss taxonomically related bacteria possibly associated with the patient's disease. Lack of initial amplification could be followed by the use of universal primers (subject to the caveats discussed above).

Identification of the PCR product was by sequencing or by *T. whippelii*-specific amplification. For bacteria that have been identified only on the basis of their 16S rRNA, the sequencing procedure is necessary to validate the specificity of the primers. This kind of strategy limits contamination risks and reduces tedious clone screening procedures. When *T. whippelii*-specific amplification was performed to identify the gram-positive 16S rDNA PCR product, the target for the amplification was chosen to allow a confirmative restriction digestion of the second amplicon. This nested PCR had a better sensitivity and specificity than a simple PCR. When *T. whippelii*-specific amplification fails, the first amplicon should be sequenced and the sequence compared with databases. We propose that such a strategy should be used for the detection of bacteria associated with Whipple's disease.

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