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Genotyping reveals a wide heterogeneity of *Tropheryma whipplei*

Wenjun Li,[1] Florence Fenollar,[1] Jean-Marc Rolain,[1] Pierre-Edouard Fournier,[1] Gerhard E. Feurle,[2] Christian Müller,[3] Verena Moos,[2] Thomas Marth,[4] Martin Altwegg,[5] Romana C. Calligaris-Maibach,[6] Thomas Schneider,[7] Federico Biagi,[8] Bernard La Scola[1] and Didier Raoult[1]

Correspondence
Didier Raoult
didier.raoult@gmail.com

[1]Unité des Rickettsies, IFR 48, CNRS UMR 6020, Faculté de Médecine, Université de la Méditerranée, 27 Boulevard Jean Moulin, 13385 Marseille Cedex 05, France

[2]DRK Krankenhaus Neuwied, Germany

[3]Universitätsklinik für Innere Medizin IV, Klinische Abteilung Gastroenterologie und Hepatologie, Medizinische Universität, Wien, Austria

[4]Department of Internal Medicine, St Josef Hospital, Zel, Germany

[5]Bio-Analytica AG, Maihofstrasse 95a, 6000 Luzern 6, Switzerland

[6]Department of Medical Microbiology, University of Zurich, Zurich, Switzerland

[7]Department of Infectious Diseases, Charité, Campus Benjamin Franklin, Berlin, Germany

[8]Gastroenterology Unit, Fondazione IRCCS, Policlinico San Matteo, University of Pavia, Italy

Tropheryma whipplei, the causative agent of Whipple's disease, is associated with various clinical manifestations as well as an asymptomatic carrier status, and it exhibits genetic heterogeneity. However, relationships that may exist between environmental and clinical strains are unknown. Herein, we developed an efficient genotyping system based on four highly variable genomic sequences (HVGSs) selected on the basis of genome comparison. We analysed 39 samples from 39 patients with Whipple's disease and 10 samples from 10 asymptomatic carriers. Twenty-six classic gastrointestinal Whipple's disease associated with additional manifestations, six relapses of classic Whipple's disease (three gastrointestinal and three neurological relapses), and seven isolated infections due to *T. whipplei* without digestive involvement (five endocarditis, one spondylodiscitis and one neurological infection) were included in the study. We identified 24 HVGS genotypes among 39 *T. whipplei* DNA samples from the patients and 10 *T. whipplei* DNA samples from the asymptomatic carriers. No significant correlation between HVGS genotypes and clinical manifestations of Whipple's disease, or asymptomatic carriers, was found for the 49 samples tested. Our observations revealed a high genetic diversity of *T. whipplei* strains that is apparently independent of geographical distribution and unrelated to bacterial pathogenicity. Genotyping in Whipple's disease may, however, be useful in epidemiological studies.

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Abbreviations: HVGS, highly variable genomic sequence; ITS, internal transcribed spacer; UPGMA, unweighted pair group method with arithmetic mean.

The GenBank/EMBL/DDBJ accession numbers for the HVGS marker sequences of *T. whipplei* are given in Table 1.

A table showing a summary of clinical manifestations of Whipple's disease patients and asymptomatic carriers together with *T. whipplei* genotypes, and dendrograms showing the phylogenetic organization of the 24 HVGS genotypes constructed using the neighbour-joining and parsimony methods, are available as supplementary data with the online version of this paper.

INTRODUCTION

Infection with *Tropheryma whipplei* causes a variety of clinical manifestations, such as classic Whipple's disease marked by histological lesions in the gastrointestinal tract associated with diarrhoea, lymphadenopathy, arthritis, blood culture-negative endocarditis and isolated neurological infection without these typical histological lesions (Dutly & Altwegg, 2001; Fenollar *et al.*, 2007a; Fenollar & Raoult, 2001; Marth & Raoult, 2003). In addition, *T. whipplei* DNA has been detected in the environment and in

asymptomatic carriers (Dutly *et al.*, 2000; Ehrbar *et al.*, 1999; Maiwald *et al.*, 1998; Street *et al.*, 1999). However, the prevalence of *T. whipplei* in the environment is still controversial because cultivation of *T. whipplei* directly from environmental samples is extremely difficult. The recent culture of *T. whipplei* from stools suggests a faeco-oral transmission of the disease (Raoult *et al.*, 2006).

Several questions remain about the pathogenicity of *T. whipplei*. In theory, some strains may exhibit specific pathogenic virulence leading to classic Whipple's disease, others may cause endocarditis or central nervous system disease, and other strains may be apathogenic. Finally, it is not clear whether a differential geographical distribution exists among *T. whipplei* strains.

Completion of the genome sequences for two *T. whipplei* strains, TW08/27 and Twist, may allow a rational selection of appropriate genomic sequences for typing this bacterium (Bentley *et al.*, 2003; Raoult *et al.*, 2003). In this study, we selected four highly variable genomic sequences (HVGSs) in the aligned genome sequences of strains TW08/27 and Twist. Indeed, we have previously demonstrated that the greatest discriminatory power resides in the most variable sequence fragments between two closely related bacterial genomes (two strains of a species or two closely related species) as genotyping targets. Such a strategy has been successful for *Rickettsia* species (Fournier *et al.*, 2004; Zhu *et al.*, 2005), *Bartonella* species (Foucault *et al.*, 2005; Li *et al.*, 2006) and *Yersinia pestis* (Drancourt *et al.*, 2004). The four HVGSs were used to evaluate the genetic diversity of 49 *T. whipplei* strains detected in 39 patients with Whipple's disease and 10 asymptomatic carriers.

METHODS

Patients. Forty-nine samples were analysed in this study, including 39 specimens taken from 39 patients with a diagnosis of Whipple's disease on the basis of clinical manifestations, histological analysis and PCR assays, as previously reported (Fenollar *et al.*, 2002, 2004, 2007a; Lepidi *et al.*, 2003). The presentations linked to *T. whipplei* infections were defined as gastrointestinal or classic Whipple's disease characterized by positive periodic acid-Schiff (PAS)-staining lesions on small-bowel biopsies associated with additional manifestations, and as isolated infections characterized by the lack of the histological lesions on small-bowel biopsies, such as blood culture-negative endocarditis, isolated spondylodiscitis or isolated neurological infection. Relapses of classic Whipple's disease were divided into gastrointestinal and neurological relapses, depending on the clinical manifestations and the involved organs. Ten samples from 10 asymptomatic carriers were also included, corresponding to healthy people or patients without clinical manifestations of Whipple's disease and/or an excluded diagnosis of Whipple's disease based on the analysis of small-bowel biopsies, as previously reported (Ehrbar *et al.*, 1999; Fenollar *et al.*, 2007b; Schoniger-Hekele *et al.*, 2007).

Study design. Detection of *T. whipplei* in specimens was carried out using PCR targeting the 16S–23S rRNA internal transcribed spacer (ITS), the *rpoB* gene and/or a repeated sequence, as described in previous studies (Drancourt *et al.*, 2001; Fenollar *et al.*, 2002, 2004). Each *T. whipplei* strain detected in a human specimen was given a unique code containing ordinal information, i.e. country where the sample was collected, clinical manifestation, and organ or tissue from which the specimen was taken (Fig. 1 and Supplementary Table S1). For example, FrDDb1 stands for one *T. whipplei* strain detected in the duodenal biopsy (Db) of a French patient (Fr) with classic Whipple's disease (D=digestive).

DNA preparation. Total genomic DNA was extracted from each specimen using the QIAamp Tissue kit (Qiagen), as recommended by the manufacturer.

Selection of HVGSs. Four fragments were selected by identifying the most variable sequence fragments between *T. whipplei* strains Twist and TW08/27. To this end, the BLAST2 program (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) was used to align the genomic sequences of *T. whipplei* strains Twist (Raoult *et al.*, 2003) (GenBank accession no. AE014184) and TW08/27 (Bentley *et al.*, 2003) (GenBank accession no. BX072543). Then, genomic sequences with low sequence homology, with sizes ranging from 300 to 500 bp and flanked by conserved sequences, were selected and used as genotyping markers in this study.

Design of primers, PCR amplification and sequencing. Amplifications of the ITS, the *rpoB* gene and/or repeated sequences were performed using the previously described conditions and the tws3f/tws4r (Fenollar *et al.*, 2002), TWRPOB.F/TWRPOB.R (Drancourt *et al.*, 2001) and 5303F/5303R primer pairs (Fenollar *et al.*, 2004), respectively. Primers for amplifying and sequencing the four HVGSs were selected using the Primer 3.0 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). All primers were obtained from Eurogentec and are shown in Table 1. PCR reactions were carried out in a PTC-200 automated thermal cycler (MJ Research). Five microlitres of 3 ng μl^{-1} of each DNA solution were amplified in a 25 µl reaction mixture containing 50 pM each primer; 200 µM (each) dATP, dCTP, dGTP and dTTP (Invitrogen); 1.5 U Hotstar Taq DNA polymerase (Qiagen); 2.5 µl 10× PCR buffer; and 1 µl 25 mM $MgCl_2$. The following conditions were used for amplification: initial heat activation at 95 °C for 15 min was followed by 40 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C, and extension for 1 min at 72 °C. Amplification was completed by holding the reaction mixture for 5 min at 72 °C to allow complete extension of the PCR products. PCR products were purified by using the MultiScreen PCR filter plate (Millipore), as recommended by the manufacturer. Amplicons were sequenced in both directions using BigDye 1.1 chemistry (Applied Biosystems) on an ABI 3130*xl* automated sequencer (Applied Biosystems), as recommended by the manufacturer. Sterile water was used as a negative control in each PCR assay. All sequences were checked twice in both directions to ensure the reliability of the typing method.

Sequence analysis and phylogenetic analysis. Nucleotide sequences were edited using the Autoassembler package (Perkin-Elmer). For each HVGS, a sequence type was defined as a sequence exhibiting unique mutation(s). HVGS genotypes were defined as unique combinations of the four HVGS types.

The genotypic distribution of *T. whipplei* strains associated with four clinical manifestations was analysed to evaluate the correlation between HVGS genotypes and distinct clinical manifestations of Whipple's disease.

Multiple alignment of sequences was carried out using the CLUSTAL W software (Thompson *et al.*, 1994). For phylogenetic analysis, sequences of the four HVGSs were concatenated. Phylogenetic relationships among *T. whipplei* genotypes were inferred using the unweighted pair group method with arithmetic mean (UPGMA), neighbour-joining and maximum-parsimony methods within the MEGA 3.1 software (Kumar *et al.*, 2001). All different HVGS sequence types were deposited in GenBank (Table 1).

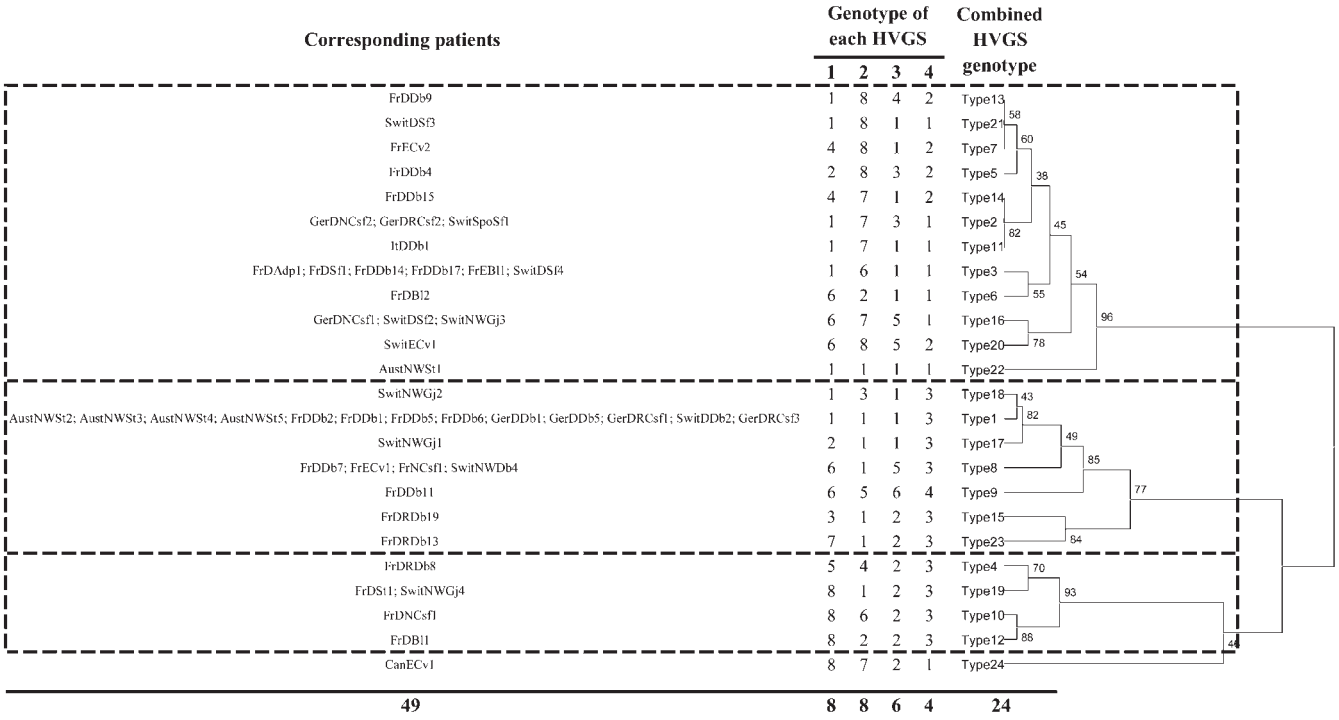

Fig. 1. Dendrogram showing the phylogenetic organization of the 24 HVGS genotypes, constructed using the UPGMA method. Sequences from the four HVGSs were concatenated. Bootstrap values are indicated at the nodes. Abbreviations: Fr, France; It, Italy; Ger, Germany; Can, Canada; Swit, Switzerland; Aust, Austria. D, digestive Whipple's disease; E, endocarditis due to *T. whipplei*; N, neurological Whipple's disease; Spo, spondylodiscitis; NW, without clinical manifestation of Whipple's disease; R, relapse. Db, duodenal biopsy; Cv,cardiac valve; Csf, cerebrospinal fluid; Bl, blood; Adp, adenopathy; Sf, synovial fluid; Gj, gastric juice; St, stool.

RESULTS

Patients

The presentations linked to Whipple's disease were: 26 classic gastrointestinal Whipple's disease associated with additional manifestations; six relapses of classic Whipple's disease, including three gastrointestinal and three neurological relapses; and seven isolated infections due to *T. whipplei*, including five endocarditis, one spondylodiscitis and one neurological infection (Fig. 1, Supplementary Table S1). From the 10 patients without Whipple's disease, there were five stool samples taken from five Austrians, and four gastric juice samples and one duodenal biopsy taken from five Swiss (Fig. 1, Supplementary Table S1).

Genotyping *T. whipplei* based on four HVGSs

The four selected regions exhibited 92.51, 97.48, 93.42 and 89.22 % identity between *T. whipplei* strains Twist and TW08/27, contained 14, 8, 10 and 11 variable positions, and classified the 49 strains in 8, 8, 6 and 4 sequence types, respectively (Tables 1 and 2).

By combining sequence types of the four sequences, the 49 tested *T. whipplei* strains were classified into 24 HVGS genotypes (Table 1). Of these, genotype 1, which was identified in 13 *T. whipplei* strains (26.5 % of the 49 tested *T. whipplei* DNA samples) was the most common in this study (Fig. 1). Genotypes 3, 8, 2, 16 and 19 included six, four, three, three and two *T. whipplei* DNA samples, respectively, which together accounted for 36.7 % of the 49 tested *T. whipplei* strains (18/49) (Fig. 1). Each of the remaining 18 genotypes contained only one strain each (Fig. 1).

When stratifying genotypes according to clinical manifestations, 18 genotypes (1–6, 8–16, 19, 21 and 23) were identified among the 32 *T. whipplei* DNA samples detected in patients with classic Whipple's disease; five genotypes (3, 7, 8, 20 and 24) were identified among the five strains detected in patients with endocarditis; the single strain detected in a patient with spondylodiscitis was of genotype 2, which was also shared by another two strains from patients with classic Whipple's disease; the 10 strains detected in patients without Whipple's disease were classified into seven genotypes (1, 8, 16–19 and 22) (Fig. 1).

Phylogenetic analysis of 24 HVGS genotypes

Phylogenetic trees obtained from concatenation of the four HVGS sequences using the neighbour-joining and

Table 1. Genomic information for the four HVGSs, primers used for amplification and sequencing of these HVGSs, and the reference numbers of the nucleotide sequences deposited in GenBank

Nucleotide sequences from the HVGS 1 marker were deposited in GenBank under reference numbers EF363910, EF363911, EF363912, EF363913, EF363914, EF363915, EF363916 and EF363917 for genotypes 1, 2, 3, 4, 5, 6, 7 and 8, respectively. Nucleotide sequences from the HVGS 2 marker were deposited in GenBank under reference numbers EF363917, EF363918, EF363919, EF363920, EF363921, EF363922, EF363923, EF363924 and EF363925 for genotypes 1, 2, 3, 4, 5, 6, 7 and 8, respectively. Nucleotide sequences from the HVGS 3 marker were deposited in GenBank under reference numbers EF363926, EF363927, EF363928, EF363929, EF363930 and EF363931 for genotypes 1, 2, 3, 4, 5 and 6, respectively. Nucleotide sequences from the HVGS 4 marker were deposited in GenBank under reference numbers EF363932, EF363933, EF363934 and EF363935 for genotypes 1, 2, 3 and 4, respectively.

HVGS	Content of HVGS	Genotype	Size (bp)	Position on Twist genome	Position on TW08/27 genome	Identity between TW08/27 and Twist	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)
HVGS 1	TWT133 and intergenic spacer (TWT133–TWT134)	8	227	154 935–155 161	154 333–154 553	92.51 %	GCTGCGCGAAGTAATTTG	AGATACATGCGGAGATACT
HVGS 2	proS/prolyl-tRNA synthetase (TW183)	8	318	233 956–234 273	661 022–661 339	97.48 %	GCCTTGACTATGACATAATCAA	TCGGACTAAAAGTGCGACAC
HVGS 3	secA-hp (TWT131) intergenic spacer	6	150	152 732–152 881	152 147–152 297	93.42 %	TTTGTCATAGGCATTTCTGTAG	AGACCTCACTGTTATACGGAT
HVGS 4	TW183 and intergenic spacer (TWT183–TWT184)	4	162	234 449–234 610	660 680–660 846	89.22 %	CGGATCTTCACGAAATGTCC	ATAACAAGAAGCTGGATATGC

maximum-parsimony methods showed similar phylogenetic classifications to the UPGMA method. The 24 HVGS genotypes were grouped into three clusters and one single branch linked with cluster 3 (Fig. 1, Supplementary Table S1). Cluster 1 contained 12 HVGS genotypes from 21 *T. whipplei* strains, cluster 2 contained seven HVGS genotypes

Table 2. Polymorphism characteristics of the four HVGSs

The numbers in the HVGS polymorphism column show the position of each variable nucleotide with reference to the Twist strain. The allele before the number is that within the Twist strain and the alleles after the number are possible variable nucleotides within other *T. whipplei* strains. Inser, insertion; dele, deletion.

HVGS	Number of nucleotide variations	Number of genotypes	HVGS polymorphism, with reference to the Twist strain							
HVGS 1	14	8	GGGGAT11-16dele	G24T	48inserTA	CT54-55TC	G61A	A68G	G89C	C122T
			C124T	G156A	G160A	A166G	G177T	A223G		
HVGS 2	8	8	C29T	T56C	C140T	A227G	A257G	A263G	G299A	G305A
HVGS 3	10	6	G19dele	A33G	T74AorG	A75T	G80A	G90A	97inserCTorCC	C99T
			G103A	A119G						
HVGS 4	11	4	T20C	GT23-24AG	TTT31-33GCC	43inserGGGTT	C50T	G60A	C69G	G83T
			C96T	G146A	G153T					
Total (four HVGSs)	43	24								

from 23 *T. whipplei* strains, and cluster 3 contained four HVGS genotypes from five *T. whipplei* strains (Fig. 1). Genotype 24, identified in strain Twist and isolated from the cardiac valve of a Canadian patient with endocarditis, grouped with cluster 3 but with a low bootstrap value (Fig. 1). The phylogenetic classification of *T. whipplei* strains associated with distinct clinical manifestations was also analysed to evaluate the correlation between HVGS genotypes and distinct clinical forms (Fig. 1, Supplementary Table S1). Cluster 1 contained 14, three, one, one and two *T. whipplei* strains detected in patients with classic Whipple's disease, endocarditis, spondylodiscitis, neurological relapse, and patients without Whipple's disease, respectively (Fig. 1). Nine, one, one, two, two and seven strains detected in patients with classic Whipple's disease, neurological disorder, endocarditis, gastrointestinal relapse, neurological relapse, and individuals without Whipple's disease, respectively, were grouped into cluster 2 (Fig. 1). Cluster 3 contained three, one and one strains from patients with classic Whipple's disease, a patient with gastrointestinal relapse and a patient without Whipple's disease, respectively (Fig. 1). Thus, in this study, no significant correlation between HVGS genotypes and distinct manifestations of Whipple's disease was found on the basis of genotypic and phylogenetic analysis.

DISCUSSION

In this study, we found the combination of four HVGSs, selected following comparison of the complete genomes of two *T. whipplei* strains, useful for genotyping 49 clinical strains of this bacterium. Prior to our study, because of the fastidious nature of *T. whipplei*, several targets, such as 16S rDNA, 23S rDNA, *hsp65*, *atpD*, *tuf*, *groEL*, *rpoB* and *rnpD* and 16S–23S rDNA ITS, had been studied to assess the phylogenetic organization of *T. whipplei* (Drancourt *et al.*, 2001; Hinrikson *et al.*, 1999b, 2000b; Maiwald *et al.*, 1996, 2000, 2003; Morgenegg *et al.*, 2000; Relman *et al.*, 1992; Wilson *et al.*, 1991). Most of these DNA sequences, however, are more conserved and reveal a limited genetic diversity among *T. whipplei* strains, although they contribute to some extent to genus and species classification. From sequences of domain III of 23S rDNA, two sequence types were identified among 50 *T. whipplei* strains detected in human specimens by Hinrikson *et al.* (2000a, b). Using a 620 bp fragment of the *hsp65* gene, four genetic variants were identified among eight *T. whipplei* strains, but these strains had been selected from representative strains of three ITS genotypes (Morgenegg *et al.*, 2000). ITS, currently the first-used molecular marker for typing *T. whipplei* infection, has so far allowed the differentiation of seven genotypes (Geissdorfer *et al.*, 2001a; Hinrikson *et al.*, 1999a, b; Maiwald *et al.*, 2000). In a first study, ITS was found to be identical in nine Swiss strains (Hinrikson *et al.*, 1999a). In a second study, three ITS sequence types were identified among 38 strains from 28 Swiss patients with Whipple's disease (Hinrikson *et al.*, 1999b). Subsequently, five ITS sequence types were identified in 43 patients with Whipple's disease from the USA (11 patients), Germany (28 patients), Switzerland (three patients) and Austria (one patient) (Maiwald *et al.*, 2000). More recently, a new ITS sequence type, type 7, was described in a German patient with endocarditis (Geissdorfer *et al.*, 2001a). Combination of the four HVGSs allowed the identification of 24 HVGS genotypes among the 49 studied *T. whipplei* strains. The degree of sequence identity of HVGSs between strains Twist and TW08/27 (89.2–97.5 %) was lower than that of ITS (99.32 %) (Geissdorfer *et al.*, 2001b; Hinrikson *et al.*, 1999b). Thus, compared with previously described DNA sequence-based typing methods, our HVGS-based typing method was more discriminatory.

Recently, another typing strategy that combined three variable number of tandem repeats (VNTRs) and ITS discriminated 11 *T. whipplei* DNAs detected in patients with Whipple's disease. Each of the four markers (three VNTRs and ITS) identified two, three, six and two types among the 11 *T. whipplei* DNAs, respectively (Maiwald *et al.*, 2000). However, DNA size-based typing methods are not as discriminatory as sequence-based typing methods, because size variations may result from nucleotide insertions or deletions other than at VNTR sites. Phylogenetic analysis combining the two methods is difficult. In addition, the Maiwald *et al.* (2000) study was based on a limited number of strains.

Although the study of additional strains may be necessary, our results suggest that asymptomatic carriage and distinct clinical manifestations of Whipple's disease are not related to the genetic diversity of *T. whipplei* but rather to as yet unknown patient characteristics such as a deficient immune function, as proposed previously (Marth *et al.*, 1994, 1997).

Although Whipple's disease is a rare systemic infectious disease, and mainly endemic in Europe and America, the relationships between genetic diversity and geographical distribution of the causative agent remain unclear. From ITS sequences, it appears that two common sequence types (types 1 and 2) are observed, with similar frequencies in patients from both the USA and Europe (Hinrikson *et al.*, 1999b; Maiwald *et al.*, 2000). Only one *T. whipplei* strain from outside Europe, Twist, was included in our study. Strain TW08/27, isolated from cerebrospinal fluid of a German patient with a neurological relapse of classic Whipple's disease, had the most frequent HVGS genotype, type 1, and grouped into cluster 2 with 22 European *T. whipplei* strains (Fig. 1). Strain Twist, isolated from a cardiac valve of a Canadian patient with endocarditis due to *T. whipplei*, exhibited a specific HVGS genotype, type 24, and grouped with cluster 3 with an extremely low bootstrap value (Fig. 1). We could not draw any conclusion about the geographical distribution of *T. whipplei*. Ten HVGS genotypes were identified among 11 Swiss *T. whipplei* strains, in contrast to 15, two and three HVGS genotypes identified among 25 French, five Austrian and

seven German *T. whipplei* strains, respectively. The genetic diversity of Swiss *T. whipplei* strains seems to be higher than that observed in French, Austrian and German strains. However, more *T. whipplei* strains originating from different continents should be tested using this typing method to study the geographical distribution of *T. whipplei*. The high variability provides further evidence that positive PCR results in asymptomatic carriers are not, as was previously suggested (Dutly & Altwegg, 2001; Fenollar *et al.*, 2007a, b), due to carry-over contamination.

In conclusion, we identified four HVGSs based upon genomic comparison of *T. whipplei* strains TW08/27 and Twist. The four HVGSs classified 49 *T. whipplei* strains detected in patients with or without manifestations of Whipple's disease into 24 genotypes. However, no significant correlation between HVGS types and clinical manifestations of Whipple's disease or geographical distribution of *T. whipplei* was found. Our observations revealed a high genetic diversity of *T. whipplei* strains that apparently did not determine microbial pathogenicity and geographical distribution. Genotyping of *T. whipplei* may, however, be useful for epidemiological investigations such as the discrimination of recurrent Whipple's disease from reinfection with another strain.

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