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1 **Title**

2 Phenotypic diversity of *Tropheryma whipplei* clinical isolates

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13

14

15 **Abstract**

16 *Tropheryma whipplei* is a bacterial pathogen responsible for a wide range of infections in
17 humans, covering asymptomatic carriage, acute infections, chronic isolated infections and
18 classic Whipple's disease. Although the bacterium is commonly found in the environment, it
19 very rarely causes disease. Genetic comparison of clinical isolates has revealed that main
20 variations were found in region encoding *T. whipplei* surface glycoproteins called WiSP.
21 However, no association has been made between the genetic diversity and the clinical
22 manifestations of the infection. In this study we evaluated the phenotypic diversity of 26
23 clinical isolates from different origins and taken from patient with different infection
24 outcomes. MRC5 and macrophages cells were infected, and bacterial uptake, survival and the
25 pro-and anti-inflammatory potential of the different clinical isolates was assessed. No
26 significant difference of phagocytosis was found between the different isolates; however, we
27 found that bacterial replication was increased for bacteria expressing high molecular weight
28 WiSP. In addition, we found that the expression of the genes coding for IL-1 β and TGF- β was
29 significantly higher when MRC5 cells were stimulated with isolates from chronic infections
30 compared to isolates from localized infections while no significant differences were observed
31 in macrophages. Overall, our study revealed that, as previously observed at the genetic level,
32 phenotypic diversity of *T. whipplei* isolates is associated with the expression of different
33 WiSP, which may result in subtle differences in host responses. Other host factors or genetic
34 predisposition may explain the range of clinical manifestations of *T. whipplei* infections.

35

36 **Keywords**

37 *Tropheryma whipplei*; clinical isolates; pathogenicity; WiSP; Whipple's disease.

38

39 **1. Introduction**

40 *Tropheryma whipplei* is the causative agent of Whipple's disease, a rare systemic disease
41 primarily characterized by weight loss, chronic diarrhea and abdominal pain, although
42 additional manifestations including endocarditis or central nervous system manifestations
43 may occur [1]. Advances in molecular biology and epidemiology have revealed that beside
44 classical Whipple's disease (CWD), *T. whipplei* is also responsible for a wide spectrum of
45 extra-digestive chronic isolated infections resulting in endocarditis, osteoarticular infections,
46 uveitis, encephalitis and adenitis [2-4]. In addition, *T. whipplei* infections can also manifest as
47 acute diseases such as bacteremia, pneumonia or gastroenteritis and asymptomatic carriers
48 exist in the general population. Several studies have suggested that contamination occurs
49 through the fecal-oral route [5]. Indeed, the bacterium is frequent in sewage samples and its
50 prevalence is higher in fecal samples from sewer workers [6, 7]. This is in sharp contrast with
51 the rarity of the condition, suggesting that beside exposition, additional host susceptibility
52 factors play a critical role in the development of the disease.

53 *T. whipplei* is a rod-shaped bacterium, measuring about 2 μm in length and 0.25-0.5 μm in
54 diameter [8, 9] and classified, based on the 16S-23S intergenic sequence among the Gram-
55 positive bacteria with high GC%, between actinomycetes and the *Cellulomonadacea* family
56 [10]. In 2003, the complete genome sequences were obtained from the strains TW08/27 and
57 Twist, isolated from the cerebrospinal fluid and a cardiac valve, respectively [11-13].
58 Genomic comparison of these two strains revealed a strong nucleotide similarity (> 99%),
59 associated with a large chromosomal inversion, which is thought to result from recombination
60 events mediated by repetitive sequences present in genes encoding the WiSP membrane
61 proteins [13].

62 In front of the various clinical entities resulting from *T. whipplei* infections, studies have been
63 conducted to address the genetic diversity of different clinical isolates. By using comparative
64 genomic hybridization analysis, the reference *T. whipplei* Twist strain was compared to 15

65 clinical isolates isolated from samples of different origins (aortic valve, whole blood, feces,
66 duodenal biopsy, muscle, cerebrospinal fluid, synovial fluid or mesenteric lymph node) taken
67 in patients with different clinical manifestations (endocarditis, CWD, CWD with neurological
68 involvement, digestive relapse of CWD, neurologic relapse of CWD) of different
69 geographical origins (France, Canada, Portugal, Germany) [14]. The results revealed that
70 compared to Twist, the genetic content of *T. whipplei* clinical isolates was highly conserved
71 and that the main genetic variation was found in the genes coding for the WiSP protein
72 family. Overall, these results showed that biological and geographic sources of *T. whipplei* are
73 not associated with clinical manifestations and that *T. whipplei* does not acquire foreign DNA
74 [14]. Moreover, the development of a genotyping system based on four highly variable
75 genomic sequences further revealed that the genetic diversity of *T. whipplei* clinical isolates
76 was high but independent of their geographical origin or clinical manifestations and **the same**
77 **genotype** could be found in patients with localized infections, CWD and even in
78 asymptomatic carriers [15].

79 Based on these results, we aimed at defining the phenotypic diversity of 26 clinical isolates
80 coming from patients with different clinical manifestations. We therefore developed an in
81 vitro system and addressed host response to infection by measuring pro-inflammatory
82 potential, bacterial uptake and bacterial survival.

83

84 **2. Material and methods**

85 **2.1. Cell Culture and *T. whipplei* isolates**

86 The Human lung fibroblast MRC5 cell line was **grown** in MEM (Minimum Essential
87 Medium) supplemented with 10% fetal bovine serum (FBS) and 1% glutamine (Sigma, Saint
88 Quentin Fallavier, France). The cells were incubated at 37°C and they were collected at 70-

89 90% of confluence. Cells were then seeded at 5.10^5 cells/well and incubated for an additional
90 24 h, before stimulation with *T. whipplei* isolates. In some experiments, monocyte-derived
91 macrophages (MDM) were used. Briefly, peripheral blood mononuclear cells were isolated by
92 ficoll density gradient from buffy coats obtained at the French Blood Bank after informed
93 consent of the donors according to the convention n°7828 established with the “Etablissement
94 Français du Sang” (Marseille, France). Monocytes were enriched using CD14-magnetic
95 microbeads (Miltenyi Biotec) and differentiated into macrophages as previously described
96 [16]. Twenty-six anonymized clinical isolates of *T. whipplei* were obtained from the
97 collection available in our laboratory and grown in axenic culture (Table 1) [17]. Utilization
98 of clinical isolates was approved by the Local Clinical Ethics Committee of IFR 48
99 (Marseille, France; n°09-021). The clinical isolates were further classified as originating from
100 patients with systemic (i.e., CWD) or localized infections as previously described [18].
101 Briefly, systemic infection or CWD is characterized by positive periodic acid-Schiff (PAS)-
102 staining lesions on duodenal biopsies and may be associated with other additional
103 manifestations such as arthritis or neurological signs, while isolated infections are
104 characterized by the lack of histological lesions on duodenal biopsies, and extraintestinal
105 manifestations with positive *T. whipplei*-specific PCR from affected extraintestinal organs.

106 2.2. RNA extraction and Real-Time Quantitative PCR (qPCR)

107 Total RNA was extracted from cells stimulated for 6 hours with *T. whipplei* (50 bacteria per
108 cell) using the RNeasy kit (Qiagen) following manufacturer’s recommendations. After DNase
109 I (Qiagen) treatment RNA concentration was evaluated using the NanoDrop
110 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA was transcribed
111 to cDNA using the MMLV-RT kit and oligo(dT) primers (Invitrogen, Waltham, MA, USA)
112 before qPCR using the SyberGreen Fast Master Mix (Roche Diagnostics) on a CFX96 Touch

113 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with the primers listed in
114 Table 2. The expression of the genes of interest was estimated based on endogenous
115 household gene *ACTB* encoding β -actin using the following formula: fold changes (FC): FC =
116 $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = (C_{t_{target}} - C_{t_{ACTB}})_{stimulated} - (C_{t_{target}} - C_{t_{ACTB}})_{unstimulated}$.

117 **2.3. Uptake and survival of *Tropheryma whipplei***

118 Cells were stimulated with *T. whipplei* (50 bacteria per cell) for 4 h, washed to eliminate free
119 bacteria, and incubated for 12 days in MEM containing 10% FBS and 1% glutamine.
120 Bacterial quantity was assessed by qPCR, as previously described [19]. Briefly, DNA was
121 extracted using the QIAamp DNA Mini Kit (Qiagen) and PCR was performed using
122 SyberGreen Fast Master Mix using primers specific for *T. whipplei* (Table 2).

123 **2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and** 124 **periodic acid Schiff (PAS) staining**

125 Bacteria were resuspended in PBS and then lysed by sonication (3 times at 50%) in TS buffer
126 (7M urea, 2M thiourea, 1% CHAPS). Debris were removed by centrifugation at 10,000 g for
127 20 minutes at 4°C. The protein content was determined by the Bradford method [20]. The
128 samples were resolved on 10% SDS-PAGE gel and glycoproteins were detected by PAS
129 staining as previously described [21]. Briefly, the gel was fixed in 1.5 % trichloroacetic acid
130 and acetic acid, incubated with 1% periodic acid for 50 minutes, rinsed with distilled water
131 and stained with Schiff's reagent for 50 minutes in the dark. Gel analysis and clustering was
132 performed with the GelJ 2.0 software.

133

134 **2.5. Statistical analysis**

135 Statistical analysis was performed with the GraphPad Prism Software, using one-way
136 ANOVA, Mann-Whitney U test and Chi-square test. Differences were considered significant
137 when $p < 0.05$.

138

139 **3. Results and discussion**

140 **3.1. Uptake and survival of *T. whipplei* clinical isolates**

141 Firstly, we aimed to determine if the internalization and survival of the different strains of *T.*
142 *whipplei* can discriminate one from another. MRC5 cells were infected with the different
143 strains at 50 bacteria per cell for 4 hours, washed to remove free bacteria and incubated for 12
144 days. Bacterial uptake and survival of *T. whipplei* were evaluated by qPCR after 4 h and 12
145 days respectively. Bacteria were efficiently phagocytosed by MRC5 cells and no significant
146 difference was found between the different strains (Fig. 1A). However, significant differences
147 were observed when considering bacterial replication after 12 days ($p = 0.0004$ by one-way
148 ANOVA, Fig. 1B). Indeed, some isolates efficiently replicate while other were barely
149 detected. It was previously shown **that** reduction of *T. whipplei* surface protein glycosylation
150 was associated with decreased bacterial replication [22]. We thus assessed the glycosylation
151 status of whole bacterial lysate of individual clinical isolates by staining acrylamide gel with
152 PAS. As seen in Fig. 2A, a band at nearly 100 kDa and which probably corresponds to
153 GpTw110 was present in all the isolates; however, as previously reported, the glycosylation
154 profile was markedly different for each clinical isolate [22]. Further analysis using the GelJ
155 software revealed that the clinical isolates could be clustered based on the presence of high
156 molecular weight (HMW) and low molecular weight (LMW) glycoproteins, respectively (Fig.
157 2B). Interestingly, clinical isolates expressing HMW glycoproteins were clustered together
158 and correspond to strains isolated from systemic infections (9/11) while those isolated from

159 localized infections (10/12) expressed LMW glycoproteins ($p = 0.0018$ by Chi-square test,
160 Fig. 2B). Finally, clustering analysis revealed a third group strongly expressing glycoproteins
161 of intermediary range. We then grouped the clinical isolates based on whether they were
162 isolated from patients with systemic or localized infection. Bacterial uptake by MRC5 cells
163 was not significantly different between strains isolated from systemic or localized infections
164 (Fig. 1A). Interestingly, and although not significant, we found that strains isolated from
165 systemic infections seemed to be less efficiently phagocytosed by MDM (Supplementary Fig.
166 1), suggesting that HMW glycoproteins may interfere with bacterial uptake in professional
167 phagocytes. Similarly, we observed a tendency in which systemic strains seem to replicate
168 more efficiently in MRC5 cells than strains isolated from localized infection (Fig. 1B).
169 Overall, these data suggest that the uptake and replication pattern of *T. whipplei* are associated
170 with the presence of HMW glycoproteins and are in accordance with previous observation
171 that show that prolonged passages of *T. whipplei* were associated with reduced glycosylation
172 and reduced intracellular replication in macrophages. However, we cannot rule out that strains
173 that replicate efficiently express genes that are absent in those that replicate poorly, or
174 inversely. Nevertheless, these strains have a high rate of genomic conservation and clinical
175 isolates from various geographical and biological sources have a high conservation rate [14].
176 Question remains regarding the diversity of these glycoproteins. It has been suggested that
177 these WiSP glycoproteins exhibit amino acid hypervariation that could be responsible for
178 various pathological features and immune system evasion [14]. However, further studies are
179 needed to determine whether they all originate from recombination events or if they are
180 differently glycosylated.

181 3.2. Host responses

182 It is fairly admitted that the initial interaction between bacterial determinants and cell
183 receptors condition subsequent host responses. As seen above, the glycoprofile varies greatly
184 with the clinical isolates, suggesting that all these strains express different sets of
185 glycoproteins. We then wondered whether strains from systemic infection elicit a distinct
186 response from strains isolated in patients with localized infections. MRC5 cells were infected
187 with the different strains of *T. whipplei* for 6 hours and the expression of the inflammatory
188 (TNF and IL-1 β) or immunomodulatory (TGF- β and HLA-G) mediators was assessed by
189 qPCR. We found that expression of TNF was mainly downregulated by *T. whipplei* and
190 varied greatly between the different clinical isolates; However, they were no significant
191 difference when considering strains isolated from systemic and localized infection,
192 respectively (Fig. 3A). We recently found that *T. whipplei* Twist induced HLA-G expression
193 and that HLA-G expression was inversely correlated with that of TNF [23]. In line with this
194 finding, we found that most of the clinical isolates also induced HLA-G expression (Fig. 3B).
195 Similarly, we did not find significant difference when considering strains isolated from
196 systemic and localized infection, respectively (Fig. 3C), suggesting that HLA-G expression
197 and TNF repression is a general feature of *T. whipplei* infection. We next measured IL-1 β ,
198 another cytokine whose expression has previously been associated with *T. whipplei* infection
199 [19]. Remarkably, we found that induction of IL-1 β expression was restricted to a subset of
200 *T. whipplei* isolates which were mainly isolated from systemic infections ($p = 0.0191$ by
201 Mann-Whitney U test, Fig. 3D). Finally, we assessed the expression of TGF- β , the
202 expression of which is associated with immunoregulation. Again, we found that induction of
203 TGF- β expression by *T. whipplei*-infected cells varied greatly depending on the clinical
204 isolates, However, when considering strains isolated from systemic or localized infection, we
205 found that strains from systemic infections induced a stronger expression of TGF- β by MRC5
206 cells than strains from localized infection ($p = 0.0164$ by Mann-Whitney U test, Fig. 3D).

207 Overall, these data suggest that in MRC5 cells, *T. whipplei* induces HLA-G which is
208 associated with the downregulation of TNF, and that the expression of TGF- β and IL-1 β is
209 mostly restricted to systemic strains which express HMW glycoproteins. Finally, given that *T.*
210 *whipplei* mainly infects macrophages in vivo and that macrophage responses are probably
211 central in the pathophysiology of *T. whipplei* infections, we wondered whether strains from
212 systemic and localized infections induced different macrophage polarization profiles. MDM
213 were infected with the different strains of *T. whipplei* for 6 hours and the expression of M1
214 (TNF, IL-6 and IL-1 β) and M2 (IL-10, TGF- β and IL1-ra) genes was assessed by qPCR.
215 Expression of most of these genes varied greatly between the different clinical isolates,
216 however, when considering isolates from localized or systemic infection, we were not able to
217 find significant differences (Fig. 4). Hence, although the clinical isolates differed at least by
218 the expression of specific sets of WiSP glycoproteins, there are no major phenotypic
219 differences between the different clinical isolates. These results are in agreement with the
220 comparative analysis of sixteen clinical isolates by CGH array designed with *T. whipplei*
221 Twist genome which revealed a high degree of conservation between these isolates and that
222 the diversity was mainly associated with WiSP proteins [14]. Consequently, our data imply
223 that the diversity of clinical manifestations of *T. whipplei* infections are not related to
224 expression of particular and specific virulence traits but rather depend on host factors or
225 genetic disposition of the host. Similarly, in a comparative study between *M. tuberculosis*
226 strains, it was shown that bacterial proteins were expressed in a similar way. However,
227 several distinct differences in antigen expression levels were detected. Indeed, variations in
228 antigen recognition were observed even between patients infected with the same genotype. It
229 is therefore possible that some differences in antigen expression between strains may become
230 evident only when the bacteria grow in the host environment [24]. Future research is needed

231 to address whether *T. whipplei* protein expression in situ is similar between patients with
232 different clinical manifestations and if so, which host factors are involved in such regulation.

233

234 **Author statement**

235 AFB drafted the manuscript. AFB, JA and EBA performed the experiments. BD supervised
236 the experiments and wrote the manuscript. All authors participated in data analysis and
237 revision of the manuscript and approved the final version.

238

239 **Declaration of competing interest**

240 The authors declare no competing financial interests.

241

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322

323 **Figure legends**

324 **Figure 1. Uptake and survival of *T. whipplei*.** MRC5 were infected with the different
325 clinical isolates for 4 hours, washed to remove free bacteria and cultured for 12 days.
326 Bacterial uptake (**A**) and survival (**B**) of individual clinical isolates (left) or categorized
327 according to clinical manifestations (right, localized or systemic infections) were assessed by
328 determining the DNA copy number by qPCR. Bars denote mean \pm SEM, $n = 3$, $p < 0.05$ by
329 Mann and Whitney U test.

330 **Figure 2. Diversity of *T. whipplei* glycoproteins.** (**A**) Whole cell lysates from *T. whipplei*
331 isolates were resolved by SDS-PAGE and gel were stained with PAS. Lane a shows Dig7;
332 lane b, Slow2; lane c, DigNeuro40; lane d, Neuro14; lane e, DigADP25; lane f, Dig54; lane g,
333 Neuro47; lane h, Art57; lane i, BCU26; lane j, Endo19; lane k, Art50; lane l, Dig49; lane m,
334 Neuro21; lane n, Endo52; lane o, Art37; lane p, Neuro1; lane q, Neuro18; lane r, Art45; lane
335 s, Art55; lane t, Art42; lane u, Twist; lane v, Endo7; lane w, Dig10; lane x, Endo27; lane y,
336 Neuro53 and lane z, Neuro20. Lane * was excluded from the analysis. (**B**) The different
337 profiles were clustered using the GelJ software, the type of infection (systemic or localized)
338 from which the isolate originates is noted as S or L, respectively.

339

340 **Figure 3. Host responses to *T. whipplei* infection.** MRC5 cells were stimulated for 6 h with
341 *T. whipplei* clinical isolates and expression of *TNF* (**A**), *HLAG* (**B**), *IL1B* (**C**), and *TGFB* (**D**)
342 was evaluated by qRT-PCR after normalization to the *ACTB* endogenous control ($n=3$). Data
343 are shown for each individual clinical isolate (left) or categorized according to clinical
344 manifestations (right, localized or systemic infections). $p < 0.05$ by Mann and Whitney U test.

345

346 **Figure 4. Macrophage responses to *T. whipplei* infection.** MDM were stimulated for 6 h
 347 with *T. whipplei* clinical isolates and expression of M1 (*TNF*, *IL6*, and *IL1B*) and M2 (*IL10*,
 348 *TGFB* and *IL1RN*) was evaluated by qRT-PCR after normalization to the *ACTB* endogenous
 349 control (n=3). Isolates were categorized according to clinical manifestations (localized or
 350 systemic infections). $p < 0.05$ by Mann and Whitney U test.

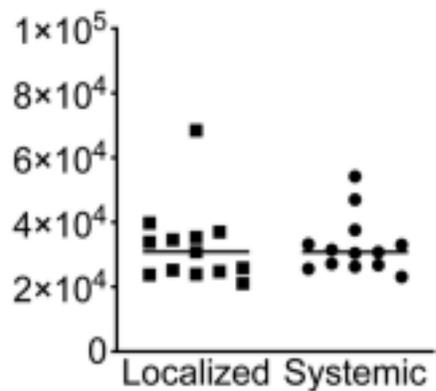
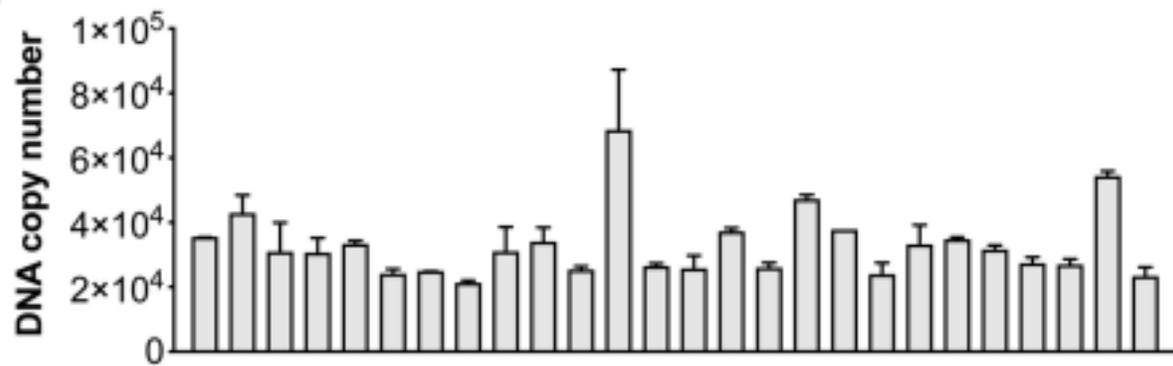
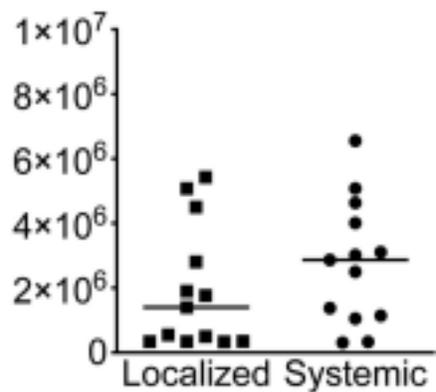
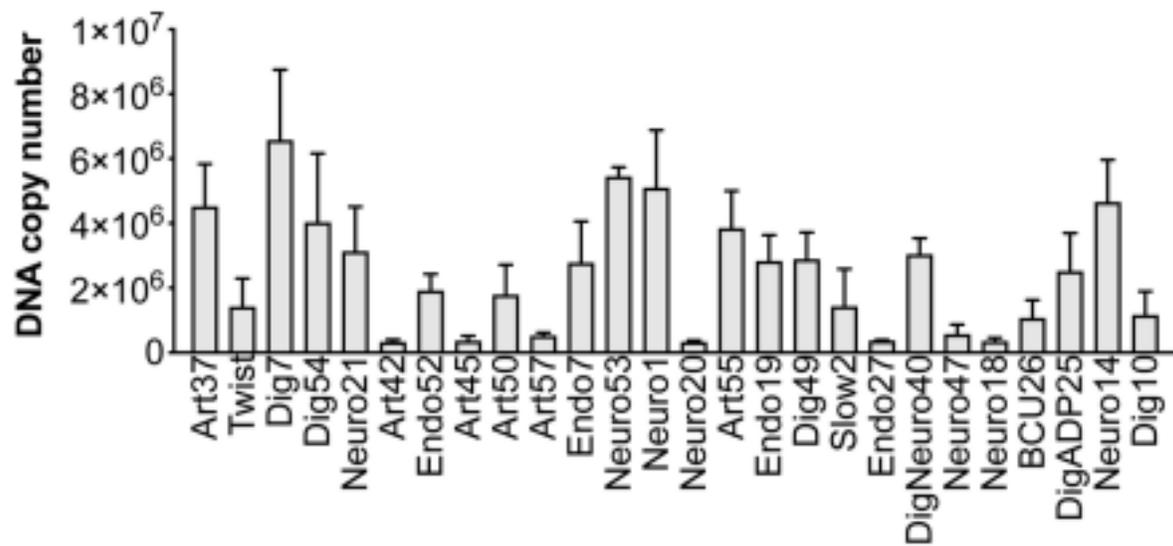
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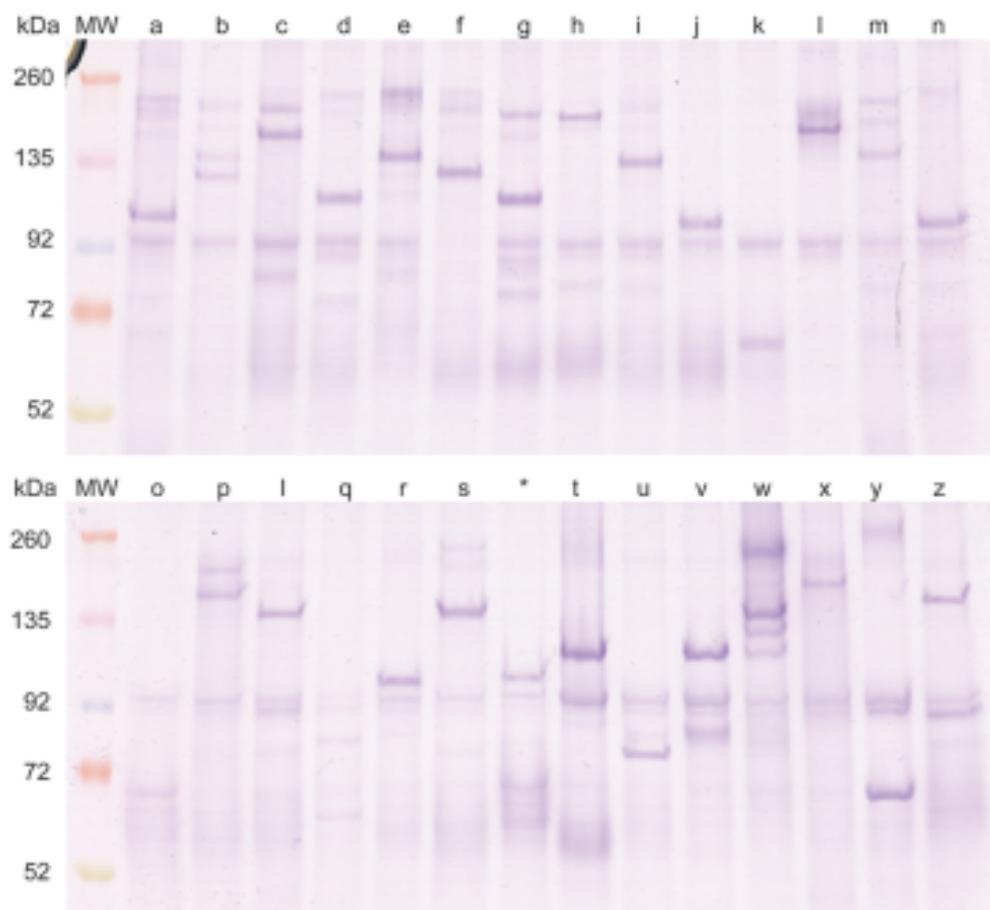
352 Tables

353 **Table 1.** Primers used in this study

Target	Forward	Reverse	354
<i>ACTB</i> (human)	ggaaatcgtgcgtgacatta	aggaaggaaggctggaagag	355
<i>IL1B</i> (human)	cagcacctctcaagcagaaaac	gtgggcattggtgtagacaac	356
<i>TNF</i> (human)	gaggagagaagcaactacagacc	aggagaagaggctgaggaacaag	357
<i>HLA-G</i> (human)	ctgaccctgaccgagacct	ctcgcctcggttgtagtagcc	358
<i>TGFB1</i> (human)	tetatgacaagttcaagcaga	gacatcaaaagataaccactc	359
<i>IL6</i> (human)	ggaagggtcaggtgttttctg	ccaggagaagattccaaagatg	360
<i>IL10</i> (human)	gggggttgaggtatcagaggtaa	gctccaagagaaaggcatctaca	361
<i>IL1RN</i> (human)	cctaactcctcctcctcttcc	tctcatcaccagacttgacaca	
ITS (<i>T. whipplei</i>)	ccgaggcttatcgagattg	ggtgacttaaccttttggag	

Clinical isolate	Sample origin	Clinical manifestation	Infection: Systemic (S) / Localized (L)	Geographical origin
Art37	Articular fluid	Arthritis	L	France
Art42	Articular fluid	Arthritis	L	France
Art45	Articular fluid	Arthritis	L	France
Art50	Articular fluid	Arthritis	L	France
Art55	Articular fluid	Arthritis	L	France
Art57	Articular fluid	Arthritis	L	France
Endo7	Aortic valve	Endocarditis	L	France
Endo19	Aortic valve	Endocarditis	L	Germany
Endo27	Aortic valve	Endocarditis	L	France
Endo52	Aortic valve	Endocarditis	L	France
Twist	Aortic valve	Endocarditis	L	Canada
Neuro47	Cerebrospinal fluid	Neuro manifestations	L	France
Neuro53	Cerebrospinal fluid	Neuro manifestations	L	France
Neuro1	Cerebrospinal fluid	Neuro relapse of CWD	S	Germany
Neuro20	Cerebrospinal fluid	Neuro relapse of CWD	S	Germany
BCU26	Duodenal biopsy	CWD	S	France
Dig7	Blood	CWD	S	France
Dig49	Duodenal biopsy	CWD	S	France
Dig54	Duodenal biopsy	CWD	S	France
DigADP25	Mesenteric lymph node	CWD	S	France
DigNeuro40	Cerebrospinal fluid	CWD	S	France
Slow2	Duodenal biopsy	CWD	S	France
Neuro14	Cerebrospinal fluid	CWD with neuro involvement	S	Germany
Neuro18	Cerebrospinal fluid	CWD with neuro involvement	S	France
Dig10	Cerebrospinal fluid	CWD with neuro involvement	S	Germany
Neuro21	Cerebrospinal fluid	CWD with neuro involvement	S	Germany

A**B**

A**B**