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# A serological assay using *Tropheryma whipplei* antigens for the presumptive exclusion of Whipple Disease

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Running Head: Whipple’s Disease exclusion serological assay

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## SUMMARY

Whipple's disease (WD) is a rare infection in genetically susceptible persons, caused by the bacterium *Tropheryma whipplei*. An indirect-immunofluorescence serological assay (IFA), detecting patient antibodies to the bacterium, was developed using *T. whipplei* as antigen. We hypothesised that this assay could be used to rule out WD in patients in whom the diagnosis was being considered, based on a high immunoglobulin (Ig) G antibody titre to *T. whipplei*. In this study 16 confirmed WD patients and 156 age-matched controls from across Australia were compared serologically. WD patients mostly under-produce IgG antibody to *T. whipplei*, with titres of ≤1:32 being common. While at an antibody titre of <1:64 antibody titre the assay sensitivity for WD was only 69% (95% CI 41-89%), its specificity for not being WD was 91% (95% CI 85-95%). This specificity increased to 95% (95% CI 90-98%) at an antibody titre of <1:16. Patients with antibody titres of >1:64 were unlikely to have WD. At this titre, seroprevalence of *T. whipplei* IgG antibody was 92% (223/242) in Australian blood donors. Unlike other serological assays which are used to confirm a specific infection, this novel assay is designed to rule out WD infection with a specificity in Australia of 91%. Further validation of this assay, by trialing in other countries, should now be undertaken, as its usefulness is dependent on there being a high background seropositivity to *T.whipplei* in the general population at the location in which the assay is being used to rule out WD.

## INTRODUCTION

Whipple disease (WD) is a rare infection due to *Tropheryma whipplei* with an estimated prevalence of 1.1-9.8 cases per million persons and an annual incidence of 1-6 per 10 million persons[1-3]. In a small case series from Germany, Spain and north-western Italy, the maximum incidence of WD was in persons between 40-60 years of age, of whom 72-96% were middle-aged Caucasian men[2-7]. In contrast to these findings, a large retrospective population-based study carried out between 2012 and 2017 in the United States (US) found that men and women were affected at similar rates and that WD was more common in people aged greater than 65 years[3].

The clinical spectrum of WD is wide[1,5,8,9]. In classical WD, infection with *T. whipplei* probably occurs years before the first clinical manifestations, which are most often chronic diarrhea, due to gastroenteritis, or migratory arthralgia[1,9]. In localised WD *T. whipplei* can infect the joints, bones, brain, heart, skin, eyes, lymph nodes, lung and/or kidney[1,8,9]. A broad spectrum of clinical severity has been observed, ranging from asymptomatic carriage[5,8] to organ failure and death. Fatal outcome is associated with delayed diagnosis as well as misdiagnosis as an autoimmune/inflammatory disorder with consequent immunosuppressive treatment[9].

It has been observed that patients with confirmed WD frequently have reduced or undetectable *T. whipplei*-specific (Ig) G antibody levels. WD patients may not be able to mount an adequate IgG antibody response, which may be associated with a subtle immune defect. In contrast, individuals who do not go on to develop clinical WD appear to mount a strong IgG antibody response when exposed to *T. whipplei*. While healthy individuals not yet exposed to the bacterium (mostly

71 children and young adults) are necessarily IgG seronegative, they respond normally when exposed.
72 The aim of this study was to compare the sera of patients with confirmed WD to individuals
73 without WD and to evaluate the diagnostic utility of a *T. whipplei* screening IFA serology assay
74 as a tool to help distinguish patients with WD from those without WD. Seropositivity to *T. whipplei*
75 in healthy, age and sex matched blood donors in Australia was measured as the control for this
76 study of WD patients.

77
78
79 **PATIENTS, MATERIALS AND METHODS**
80
81
82 **Study Design**

83 A case-controlled comparative study of *T. whipplei* IFA was conducted from November 2019 to
84 October 2022 at the Australian Rickettsial Reference Laboratory (ARRL), Geelong, Victoria,
85 Australia, according to the *National Statement on Ethical Conduct in Human Research (2007)*
86 guideline (Barwon Health ethics reference number: 19/135). All sera tested in this study were de-
87 identified.

88

89 Adult patients aged more than 18 years were included. Grounds for exclusion were pregnancy,
90 active malignancy, primary immunodeficiency, and HIV with CD4 lymphocyte count < 200.
91 Subjects were considered WD cases if they displayed consistent symptoms with supportive
92 diagnostic test results, such as positive periodic acid-Schiff (PAS) staining, positive *T. whipplei*-
93 specific immunohistochemistry staining or positive *T. whipplei* PCR or 16s rRNA PCR of a tissue
94 biopsy.[10-13]

95

96 **Sample size and collection**

97 Given the limited number of WD patients in Australia, we employed a convenience sampling
98 strategy. Patients (n=16) with confirmed WD as described above were recruited from across
99 Australia. Of the 16 WD cases, eleven had sera already in storage at ARRL. The remaining five
100 cases had serum sample collection organized through their local doctors and pathology services.
101 In addition, 242 sera were obtained from healthy individuals via the Australian Red Cross
102 Lifeblood; 156 of these sera were age and sex matched to WD patients to act as controls and all
103 sera were tested for antibodies to T. whipplei. Data such as gender, age, clinical manifestations,
104 IgM, IgG and IgA titres to T. whipplei, 16S rRNA,13 T. whipplei PCR and histology results were
105 obtained from WD patients and controls.

106 **Diagnostic techniques**

107

108 **Culture and IFA slide preparation**

109 A French strain of *T. whipplei* was cultivated in liquid axenic medium at 35◦C for several weeks.[14]
110 The *T. whipplei* cells were pelleted and resuspended in phosphate-buffered saline (PBS). To
111 optimise antigen concentrations for the final IFA, slides were prepared using doubling dilutions of
112 *T. whipplei* antigen and subjected to sandwich IFA after incubation with a strongly reacting
113 positive serum sample. Antigen aliquots were stored at –70◦C until required. When thawed, they
114 were spotted onto clean 3×10-well glass slides. After air drying, antigens were fixed with acetone
115 for 5 min. Slides could then be stored at –70◦C for later use.

116

117 **Indirect-immunofluorescence assay (IFA)**

118 All sera from the WD patients and controls were tested by in-house *T. whipplei* IFA. Known
119 reactive and non-reactive sera were tested on the same slide as the assay samples. Initial 1:8

120 dilutions of test and control sera were prepared using a 2% casein PBS buffer. Serial doubling
121 dilutions from 1:8 to 1:4096 were prepared and tested by IFA. Each serum dilution was spotted in
122 duplicate onto pre-prepared *T. whipplei* slides. Slides were incubated for 30 minutes under humid
123 conditions at 35ºC and then washed for 5 minutes in a 1:10 dilution of PBS and allowed to dry.
124 Fluorescein-conjugated goat-anti-human antibodies, [1:100 anti-human IgA (KPL, Cat # 02-10-
125 01), 1:100 anti-human IgM (KPL, Cat # 02-10-03) and 1:100 anti-human IgG (KPL, Cat # 02-10-
126 02)] were applied, and slides were incubated, washed and dried as above. Fluorescence mounting
127 fluid (Dako North America, Inc., USA) and a coverslip were applied. Microscopic evaluations of
128 the IFA were carried out under 400x magnification using an LED Leica DMLS fluorescence
129 microscope. The assay was repeated in duplicate for all sera. Reading of the slides were blinded.
130 Antibody titres for IgM, IgG and IgA were defined as the highest serum dilution giving strong
131 immunofluorescence (like the reactive control serum).
132

133 **Preparation of positive control sera for IFA**

134 IFA-positive *T.whipplei* human sera were kept in frozen storage as 5 uL aliquots until required.
135 They had been previously titrated and diluted in PBS to reach a fluorescence endpoint of 1:512
136 and used as contols on each IFA slide.
137

138 **Statistical analysis**

139 Statistical comparisons of groups, by 2-sided Fisher exact test was done on the quantitative data.
140 Sensitivity was defined as the proportion of WD patients correctly identified at a particular
141 antibody cutoff[15]. Specificity was defined as the proportion of healthy controls correctly identified
142 as not having WD[15]. Significance was set at $p<0.05$.

## RESULTS

### Case demographics and clinical presentations

Fifteen male and one female WD patients were recruited to the study. They ranged in age from 37–82 years, with a mean age of 57 years [95% confidence interval (CI) 50–65 years] (Table 1). The clinical presentations of these 16 WD cases varied: seven had infective endocarditis; three displayed central nervous system manifestations; four had classic WD with intestinal malabsorption; and two had joint manifestations.

### Demographics of the controls

The 156 age-matched controls ranged from 31-69 years old (Table 1). The mean age of age-matched controls was 50 years (95% CI 48-52). There was no obvious difference between the WD and age-matched controls in terms of gender and age.

### IFA serology: IgG

WD patients' antibody titres are shown in Table 2 and compared with controls in Figure 1. WD patients were less able to produce IgG antibodies to *T. whipplei* than healthy age-matched controls. This difference was statistically significant when comparing antibody titres at 1:16, 1: 32 and 1:64 dilutions (Table 3).

With an IFA IgG titre cutoff set at <1:64, sensitivity (for diagnosing WD) and specificity (for diagnosing not having WD) were 69% (95% CI 41-89%) and 91% (95% CI 85-95%) respectively (Table 3).

169

170 **IgM and IgA**

171 No difference in *T. whipplei* IgM or IgA IFA antibody titres was observed between WD cases and

172 age-matched controls ($p > 0.05$). Titres of this antibody isotype did not discriminate between these

173 two groups (data not shown for controls).

174

175 **DISCUSSION**

176

177 This study appears to confirm the hypothesis that patients with WD under-produce IgG antibody

178 compared with age and sex-matched controls exposed to *T. whipplei*. This finding is consistent

179 with previous reports [16-20]. We were able to establish an antibody titre cutoff (<1:64) and arrive at

180 a potentially useful serological assay to rule out WD (specificity 91%). In patients who had been

181 naturally exposed to *T. whipplei*, and who had IgG antibody titres ≥1:64, there was a reasonable

182 likelihood that they did not have WD. Those without WD rarely have such low titers (<1:64). In a

183 previous French study, when the cutoff titre was set at 1:100, IgG did not discriminate between

184 subjects with and without WD[21]. The discrepancy between these results might be explained by the

185 different antibody cutoff titres used as well as possible technical differences in the assays. In an

186 Australian context, this assay has the potential to be useful to screen for the absence of WD in

187 patients with WD-compatible signs and symptoms.

188

189 In contrast to a previous study, we did not find serum IgA to *T. whipplei* to be useful in

190 discriminating between patients with and without WD.19 Secretory IgA in intestinal mucus

191 might correlate with recent exposure to *T. whipplei,* but it would be challenging to measure

192 routinely. The usual early serological response to a primary bacterial or viral infection would

193 normally involve a conversion from IgM to IgG isotypes modulated by T-cell cytokines.
194 Seroconversion or a four-fold rise in antibody titre would be expected after about a 10–14-
195 day interval.[22] Our serological findings in WD patients were contrary to this. This may be due
196 to a specific genetic predisposition to WD associated with human leukocyte antigen alleles
197 DRB1*13 and DQB1*06, which may disrupt optimum antigen presentation.[23] Interleukin
198 (IL)-16 gene polymorphisms and other polymorphisms polarise cytokine production towards
199 T-helper (Th) 2 cell activity.[24,25] This specific genetic predisposition probably explains the
200 inability to switch from IgM to IgG antibody isotypes. In addition, there appears to be a
201 decrease in Th1 and Th17 cell activity, poor synthesis of transforming growth factor-beta 1
202 and excess synthesis of IL-10 by WD patients,[17,26–28] a biological mechanism that changes B-
203 cell production of antibody from one class to another.[16,19,20]
204
205 This is the first study to test for the seroprevalence of *T. whipplei* IgG antibody in Australia.
206 The finding that 223/ 242 (92%) Australian blood donors had *T. whipplei* IgG antibody
207 detected by IFA at ≥1:64 serum dilution suggests that the microbe is widespread in the
208 Australian environment, with most adult Australians having been exposed to it. *Tropheryma*
209 *whipplei* has been detected in 37–66% of waste from sewage plants[4,28–32] and in stool samples
210 of 48% children in Laos,[33] and 12–26% of sewage plant workers[30] and 1–11% of healthy
211 individuals in France.[29] The viable bacterium can also be detected in human faeces and
212 saliva,[12] supporting the notion of human colonisation, probably through faecal-oral
213 transmission. Further work with larger sample populations will be required to fully
214 characterise rates of exposure to *T. whipplei* in relevant communities and different settings.
215

216 There are several limitations to this study. Only a single strain of *T. whipplei* was used as
217 antigens for the IFA. Thus, if there were strain-specific differences in antibody affinities, it
218 might have affected our results. Antigenic shift and loss of antigen specificity after a few
219 subcultures is also a possibility. Ideally, a few robust antigenic targets could be identified
220 through a proteomic approach for the development of more specific assays. Testing patients
221 with diseases other than WD should now be undertaken. Finally, the absent/low antibody
222 responses we observed in some healthy controls complicate the reliance on an absence of *T.*
223 *whipplei* IgG responses as a marker for active WD. We assume that the absence of antibodies
224 in healthy individuals reflects a lack of exposure to the bacterium. Additional studies to
225 differentiate asymptomatic carriers could be considered by undertaking PCR testing on saliva
226 and/or stool. The control sera were obtained from Australian Red Cross Lifeblood, which
227 does not collect blood from persons older than 70, meaning this is an inherent bias in the
228 study. However, the study findings remain the same even if the three WD patients over 70
229 years old are removed from the analysis. This study demonstrates that the detection of *T.*
230 *whipplei* IgG antibody on IFA serology is a promising screening assay to rule out WD (Fig.
231 2). The usefulness of this assay (or the confidence a doctor may have in ruling out WD in a
232 patient) will only be shown by the passage of time, as the assay is used more widely. Given
233 the rarity of WD and the invasive nature of existing diagnostic tests, an inexpensive,
234 noninvasive, screening serological assay with sufficient accuracy to reliably exclude the
235 diagnosis may be a worthwhile addition to the diagnostic armamentarium of the physician.

236

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249

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251 Conduct in Human Research (2007) of the Australian National Health and Medical Research
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253

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257

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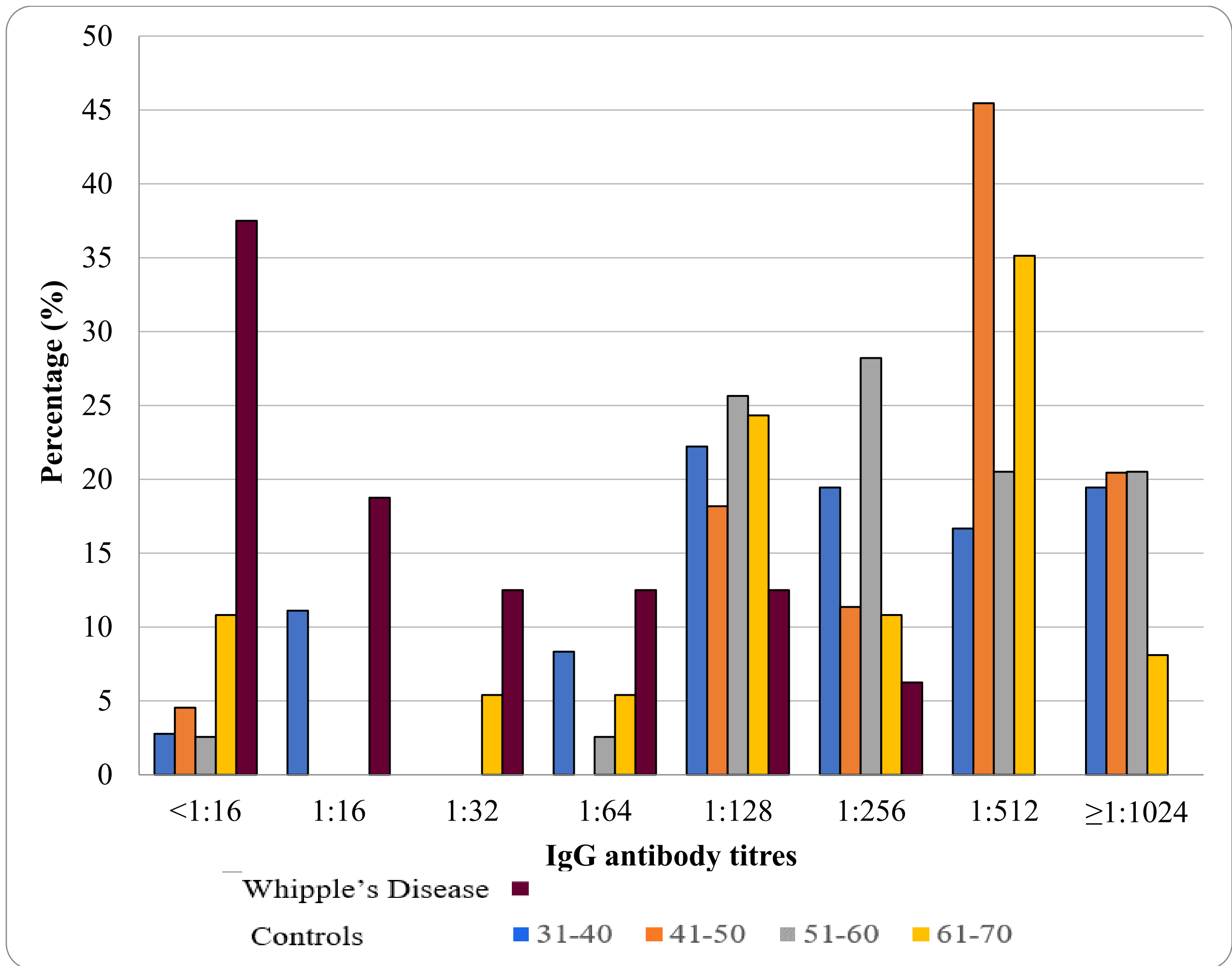
392 **FIG 1:** Distribution of immunoglobulin G (IgG) titres to *Tropheryma whipplei* in Whipple
393 disease patients and age-matched controls.

394




395

396

397 **TABLE 1:** Gender and age comparison of Whipple Disease patients (n=16) and age-matched
398 controls (n=156)

399

| Gender | Whipple's Disease | Age-matched controls |
|---|---|---|
| Female (%) | 1 (6) | 2 (1) |
| Male (%) | 15 (94) | 154 (99) |
| **Age Group** | | |
| 31-40 (%) | 4 (25) | 36 (23) |
| 41-50 (%) | 1 (6) | 44 (28) |
| 51-60 (%) | 5 (31) | 39 (25) |
| 61-70 (%) | 3 (19) | 35 (24) |
| 71-80 (%) | 2 (13) | 0 (0) |
| 81-90 (%) | 1 (6) | 0 (0) |
| Mean age | 57 | 50 |

400

401 **TABLE 2:** Indirect immunofluorescence assay antibody titres in 16 patients with Whipple
402 disease (WD)

403

| Study patients | Gender | Age Range | Clinical Manifestation & Diagnostic method | IgA | IgM | IgG |
|---|---|---|---|---|---|---|
| Patient 1 | M | 41-50 | Endocarditis<br>Dx Cardiac valve<br>16s rRNA + | <32 | 256 | ≤8 |
| Patient 2 | M | 51-60 | Endocarditis<br>Dx Cardiac valve<br>16s rRNA + | <32 | ≤32 | ≤8 |
| Patient 3 | M | 51-60 | Classic WD + Joint<br>Dx Duodenal and<br>knee tissues PCR + | <32 | 128 | ≤8 |
| Patient 4 | M | 51-60 | CNS WD + Joint<br>Dx Small bowel<br>biopsy PAS +; PCR +<br>CSF PCR + | <32 | 256 | ≤8 |
| Patient 5 | M | 61-70 | CNS WD<br>Dx CSF PCR + | <32 | ≤32 | ≤8 |
| Patient 6 | M | 71-80 | CNS WD<br>Dx: Vitreous PAS +<br>Gastric PCR + | <32 | ≤32 | ≤8 |
| Patient 7 | M | 31-40 | Classic WD | <32 | ≤32 | ≤8 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | Dx: Duodenum and<br>Lymph node PAS+;<br>PCR + | | | |
| Patient 8 | M | 51-60 | Endocarditis<br>Dx Cardiac valve<br>16s rRNA + | 32 | 1024 | 16 |
| Patient 9 | F | 61-70 | Classic WD + Joint<br>Lymph node<br>PAS +; PCR + | <32 | 512 | 16 |
| Patient 10 | M | 31-40 | Endocarditis<br>Dx Cardiac valve<br>16s rRNA + | <32 | 128 | 32 |
| Patient 11 | M | 31-40 | Classic WD<br>Dx Duodenum tissue<br>PAS + | <32 | 256 | 32 |
| Patient 12 | M | 61-70 | Classic WD<br>Dx Gastric tissue PAS+;<br>PCR + | 64 | ≤32 | 64 |
| Patient 13 | M | 81-90 | Classic WD<br>Dx Small bowel tissue<br>PCR + | <32 | ≤32 | 64 |
| Patient 14 | M | 51-60 | Endocarditis<br>Dx Cardiac valve<br>16s rRNA + | <32 | 512 | 128 |

| Patient 15 | M | 71-80 | Classic WD → Endocarditis (15 years later) Dx Cardiac valve 16s rRNA + | <32 | 256 | 128 |
|---|---|---|---|---|---|---|
| Patient 16 | M | 31-40 | Endocarditis Dx Cardiac valve 16s rRNA + | <32 | 256 | 256 |

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405 CNS, central nervous system; CSF, cerebrospinal fluid; Dx, diagnosis; PAS, periodic acid–Schiff
406 stain; PCR+, *Tropheryma whipplei* DNA was detected by polymerase chain reaction (PCR)
407 amplification; 16s rRNA+, at least 99% homology to *T. whipplei* TW08/27 in National Center
408 for Biotechnology Information taxonomy database; WD, Whipple disease.

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**TABLE 3:** Role of IgG titre in excluding Whipple disease (WD) in patients: serological comparison of IgG antibody titres in WD patients (n=16) and age-matched controls (n=156)

| Antibody Titres | WD | Age-matched controls | Sensitivity & Specificity | p-value[@] |
|---|---|---|---|---|
| **IgG <64 (Test +)** | 11 | 14 | Sn: 69% (95% CI 41% to 89%) | <0.001 |
| **IgG ≥ 64 (Test -)** | 5 | 142 | Sp: 91% (95% CI 85% to 95%) | |
| **IgG < 32 (Test +)** | 9 | 12 | Sn: 56 % (95% CI 30% to 80%) | <0.001 |
| **IgG ≥ 32 (Test -)** | 7 | 144 | Sp: 92% (95% CI 87% to 96%) | |
| **IgG < 16 (Test +)** | 6 | 8 | Sn: 38% (95% CI 15% to 65%) | <0.005 |
| **IgG ≥ 16 (Test -)** | 10 | 148 | Sp: 95% (95% CI 90% to 98%) | |

[@] p-values are determined by Fisher's exact probability test; Sn: Sensitivity; Sp: Specificity

417 **FIG 2:** Algorithm for ruling out Whipple's Disease using a *Tropheryma whipplei* Indirect
418 Immunofluorescence Assay (IFA).

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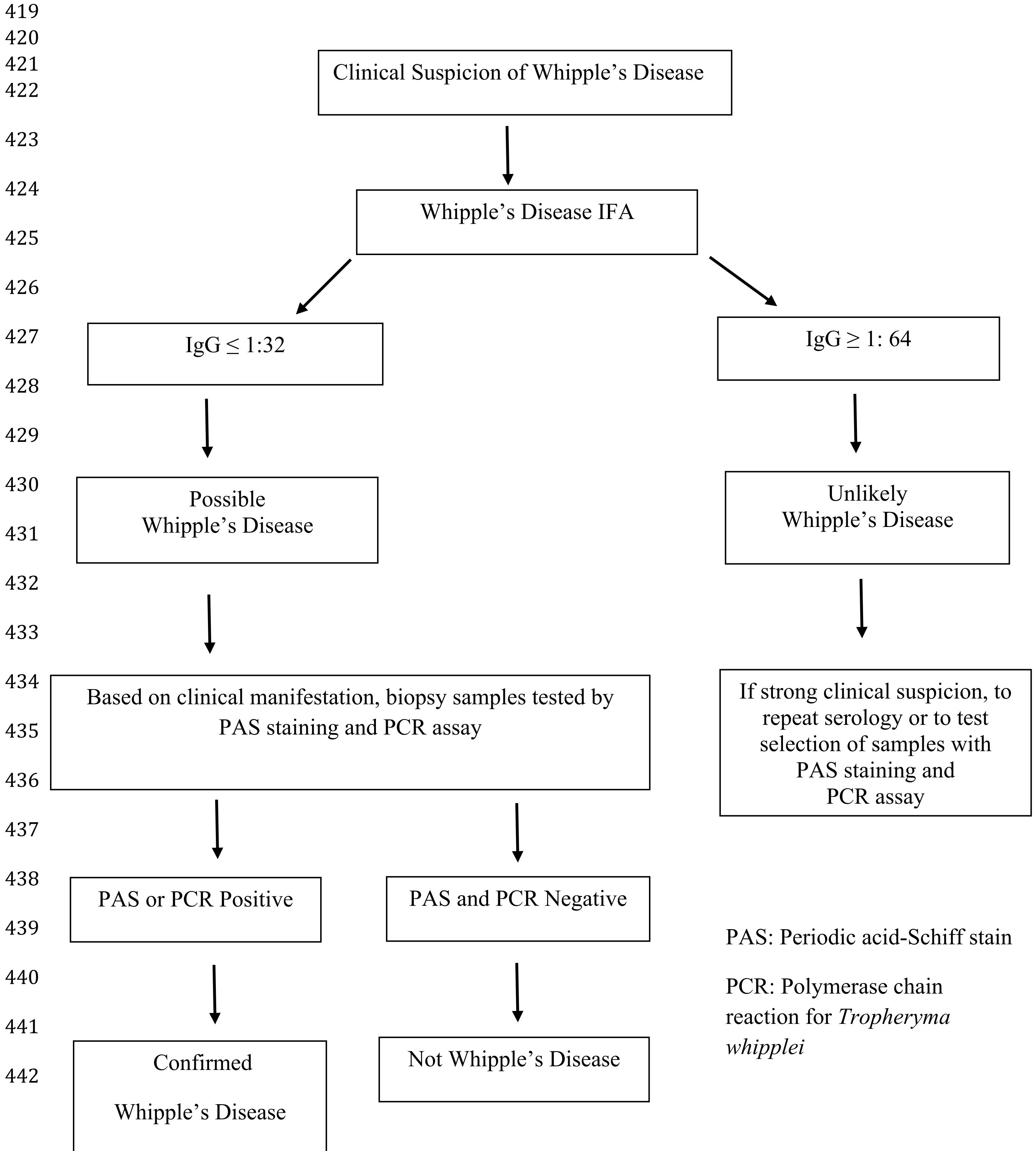


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