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1 **Determination of decisional cut-off values for the optimal diagnosis of bovine**
2 **tuberculosis with a modified IFN γ assay (Bovigam[®]) in a low prevalence area in France**

3

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19

20 **Abstract**

21 The Bovigam[®] gamma interferon (IFN γ) assay was used to complement official skin-test
22 screening in a low bovine tuberculosis (bTB) prevalence region in France.

23 The aim of our work was to determine decisional cut-off values for protein purified
24 derivatives (PPD) and ESAT6-CFP10 antigens (R) in order to optimize the efficacy of the

25 modified Bovigam[®] test, in this low-prevalence area, for optimal classification of infected or
26 non-infected herds following positive skin tests.

27 The sensitivity of the IFN γ assay relative to *post-mortem* bTB-positive animals (Se_r) was
28 studied in 60 cattle from 20 bTB-infected herds. Its absolute specificity (Sp) was studied in
29 492 cattle from 25 bTB-free herds from a bTB-free zone. Its operational specificity (relative
30 to the positive skin test) (Sp_r) was also studied in 547 skin-test positive cattle from 172 bTB-
31 free herds from an infected zone.

32 Using normalized interpretations for individual (PPD or R) results, the cut-off values at 0.02
33 for PPD and 0.01 for R were obtained with a view to employing them in low prevalence areas
34 with no previously observed non-specific reactions to SITT.

35 Concerning its use after positive skin tests, cut-off values were set at 0.05 for PPD and at 0.03
36 for R. The choice of an interpretation method considering positive results with PPD and/or R
37 (PPDUR), justified in a high risk context, provided a test Se_r of 93% [84-98] and Sp_r of 71.8%
38 [67.9-75.6]. Analysis of positive results with PPD and R ($PPD \cap R$), ideal for low-risk
39 contexts, provided a test Sp_r of 94.3% [92.0-96.1] and Se_r of 77% [64-87].

40 Thus, adapting the criteria to the region's infection status and to the conditions for its
41 application is essential for the appropriate use of the IFN γ assay.

42

43 Keywords: Bovigam[®] IFN γ assay, ESAT6-CFP10, Diagnosis, Cut-off, Sensitivity,
44 Specificity.

45

46 **Introduction**

47 Bovine tuberculosis (bTB) is an important zoonosis found throughout the world which is
48 particularly difficult to control and eradicate. France has been officially bTB-free since 2001,
49 however, the number of infected herds in the south-west rose to 10-20 per year between 2003
50 and 2006 (Anonymous, 2009), despite strict bTB regulations.

51 BTB-infection status in a herd is officially established if *Mycobacterium bovis* is confirmed
52 by bacteriology from at least one animal, or if bTB-like lesions are detected by histology from
53 a positive single intradermal comparative cervical tuberculin test (SICTT) from the animal. In
54 France, official screening of bTB is based on the skin test. The frequency of skin test controls
55 depends on the area's bTB prevalence. Pre-movement controls have not been mandatory since
56 2005.

57 A single intradermal cervical tuberculin test (SITT) (Benet J.J., 2008) is carried out first,
58 which, if positive (*i.e.* detecting an increase in skin thickness at the bovine tuberculin injection
59 site of more than 2 mm), leads to the herd being disqualified and activity being suspended
60 until a SICTT is carried out six weeks later. In the event of a negative SICTT result (*i.e.* if the
61 bovine reaction is less than or equal to 2 mm or if the bovine reaction is greater than 2 mm
62 but the difference between bovine and avian reactions is less than 1 mm), the herd is
63 considered as bTB-free; on the contrary, if the SICTT result is positive (*i.e.* if the bovine
64 reaction is greater than 2 mm and the difference between bovine and avian reaction is greater
65 or equal to 1 mm), diagnostic culling of the affected animal is carried out. However, there are
66 certain disadvantages to the skin test such as subjectivity in result interpretation, lack of
67 specificity for the SITT and lack of sensitivity for the SICTT.

68 Because until 2005 animals testing positive to the SITT were under-declared, bTB
69 surveillance in this region was almost entirely based on *post-mortem* examination at the
70 slaughterhouse. Indeed, notification of a positive SITT reaction leads to a long suspension of

71 the herd's status at least until a further SICTT is carried out. Besides, due to the very low bTB
72 prevalence in France, the positive predictive value (PPV) of the SITT is consequently too low
73 and thus the test is considered unreliable by veterinary practitioners.

74 An alternative diagnostic test, the gamma interferon (IFN γ) assay, allows early detection of
75 bTB (Buddle *et al.*, 1995, 2009; Wood *et al.*, 2001; Gormley *et al.*, 2005; De la Rua-
76 Domenech *et al.*, 2006a, 2006b; Vordermeier *et al.*, 2006). In contrast to the skin test, the
77 IFN γ assay is a laboratory test that can be standardized, whose results can be interpreted
78 objectively, independent of veterinary practitioners. Moreover, it can be carried out without
79 any delay between the two analyses.

80 Due to the rise in the incidence of bTB in the region, preventive measures have been
81 strengthened and the Bovigam[®] IFN γ assay (Prionics), has been included as an additional
82 diagnostic tool to reinforce the skin test findings.

83 Since 2006, the Bovigam[®] assay has been included in surveillance programs to allow the rapid
84 confirmation of positive SITTs (serial use) and also for simultaneous use with SITTs for
85 epidemiological studies (parallel use) in this French region. ESAT6 and CFP10,
86 *M. tuberculosis* complex specific antigens (from the Statens Serum Institut, SSI), have also
87 been used to improve IFN γ assay specificity (Aagaard *et al.*, 2006), especially in population
88 groups testing positive to the SITT. In addition, several modifications to the original protocol
89 (Faye *et al.*, 2008 and Schiller *et al.*, 2009) have been introduced to optimize the test in a
90 context of low bTB prevalence.

91 The aim of our work was to determine decisional cut-off values for the protein purified
92 derivatives (PPDs) and specific antigens (ESAT6 and CFP10) in order to obtain optimal
93 results in terms of sensitivity and specificity from this modified use of the Bovigam[®] assay in
94 a low-prevalence area, in the region's infected or non-infected cattle population or after
95 positive SITT responses.

96

97 **Materials and methods**

98 The IFN γ assay sensitivity study, relative to *post-mortem* bTB positive confirmation (Se_r),
99 was conducted retrospectively using the results obtained during the 2006-2007, 2007-2008
100 and 2008-2009 bTB control programs in the affected French south-west region.

101 The IFN γ assay absolute specificity (Sp) study was performed in early 2008 in the same
102 region (Faye *et al.*, 2008).

103 Cut-off values to determine optimal Se_r and Sp values were obtained by combining the results
104 of the two studies (Se_r & Sp).

105 However, since the IFN γ assay was widely used as a routine SITT confirmatory tool, the
106 operational specificity of the IFN γ assay, *i.e.* its relative specificity to positive SITTs (Sp_r),
107 was also studied. This was carried out using the results of the 2007-2008 bTB control
108 program.

109

110 **I. Description of the IFN γ method employed**111 **I.1. Cellular stimulation and IFN γ dosage: Micro-method**

112 The guidelines for the use of the Bovigam[®] kit were those provided by the manufacturer,
113 although the initial volumes for cellular stimulation were reduced (micro-method) as similar
114 results are obtained with either of these methods (Schiller *et al.*, 2009).

115 Cellular stimulation was performed in duplicate in a 96-well plate. The bloods were processed
116 on the day of collection (6-8 hours max delay). Each aliquot of blood (250 μ l) was stimulated
117 with either (i) 25 μ l of avian tuberculin (PPD A = A) or bovine tuberculin (PPD B = B) at
118 20 μ g/ml (Prionics PPDs), (ii) 25 μ l of the recombinant peptides ESAT6 and CFP10 (R, from
119 SSI) at 5 μ g/ml of final blood concentration for each, (iii) 25 μ l of the Pokeweed Mitogen
120 (PWM, from Sigma), an immunocompetence control at 5 μ g/ml of final blood concentration,

121 (iv) 25 μ l of the Phosphate Buffer Saline (PBS = N) as a negative control for cellular
122 stimulation.

123 After 16-24 h incubation at 37°C in a humidified atmosphere, the plate was centrifuged for
124 10 minutes at 500 g to facilitate plasma collection.

125 IFN γ concentrations were determined for each supernatant plasma (tested in duplicate), using
126 the sandwich enzyme immunoassay (EIA) method (see Bovigam[®] documentation). Bovigam[®]
127 negative (NC) and positive (PC) controls were used. An additional external positive control, a
128 homemade tracer consisting of a pool of positive IFN γ cattle plasma with a constant and well
129 known optical density (OD) value, was also used in order to evaluate the test's
130 reproducibility.

131

132 **I.2. Method of interpreting results**

133 The different control values (NC and PC) used for validating EIA were those of the
134 manufacturer. The control values (PWM and PBS) used for validating the samples' quality
135 were the following: $OD_{PWM}-OD_N > 0.40$ for PWM (Faye *et al.*, 2008) and $OD_N < 0.30$ for PBS
136 (as previously described by Coad *et al.*, 2008). The level of variation between duplicate
137 samples was below 10 %.

138 A new result interpretation calculation formula was used. It takes into account the NC and PC
139 results per analysis plate, and thus absorbance variations due to analytical conditions
140 (recommended by the French standard for EIA (NF U47-019)) and used by Olsen *et al.*,
141 2005): $(OD_B-OD_A)/(OD_{PC}-OD_{NC})$ for PPD and $(OD_R-OD_N)/(OD_{PC}-OD_{NC})$ for R. (Faye *et*
142 *al.*, 2008).

143 Consequently, the 0.1 cut-off recommended for the Bovigam[®] kit has been adapted with a
144 formula including median $OD_{PC}-OD_{NC}$ at 2.40: $(OD_B-OD_A)/(OD_{PC}-OD_{NC}) = 0.10/2.40 = 0.04$

145 (Faye *et al.*, 2008). The PC and NC OD values (for each analysis plate) are those obtained
146 from our sensitivity and specificity studies.

147 Thus, the individual positive results of the IFN γ assay were as follows:

148 Positive result in PPD if $(OD_B - OD_A) / (OD_{PC} - OD_{NC}) \geq$ selected cut-off

149 Positive result in R if $(OD_R - OD_N) / (OD_{PC} - OD_{NC}) \geq$ selected cut-off

150 The final result of the IFN γ assay is determined according to the combined individual results
151 of PPD and R. It is divided into 3 categories: positive (POS, positive individual results),
152 negative (NEG, negative individual results) and divergent (DIV, different individual results),
153 (Faye *et al.*, 2008).

154

155 **II. Specimen description and processing**

156 **II.1. IFN γ assay sensitivity study in relation to *post-mortem* bTB positive** 157 **confirmation (Se_r)**

158 The IFN γ assay sensitivity study in relation to *post-mortem* bTB confirmation (Se_r) was
159 carried out retrospectively in 73 cattle from 28 recognized bTB-infected herds (*i.e.* where at
160 least one animal has been found to be culture-positive for *M. bovis*) from a high-prevalence
161 zone in the studied region. All these cattle presented bTB-like lesions at the slaughterhouse
162 and were thus considered bTB-positive (as described by Gormley *et al.*, 2005). In addition,
163 40 of these 73 animals were further analyzed and confirmed as bTB-infected by different
164 direct diagnostic methods: 26 at least by culture and 14 at least by *Mycobacterium*
165 *tuberculosis* complex PCR and/or histology (Faye *et al.*, 2008).

166 Collection of these blood samples was carried out between 3 and 10 days after the SITT.

167

168 **II.2. IFN γ assay absolute specificity study (Sp)**

169 IFN γ assay absolute specificity (Sp) was studied in 492 cattle from 25 bTB-free herds (*i.e.*
170 where no cattle have been found to be culture-positive since 2001) from a bTB-free zone
171 (no positive animal in any of the screening tests (SITT and SICTT) and no recognized
172 outbreak for at least 10 years), (Faye *et al.*, 2008). These herds are still considered as bTB-
173 free to date.

174 Collection of these blood samples was carried out on the same day as the SITT.

175

176 **II.3. IFN γ assay specificity study in relation to positive SITT (Sp_r)**

177 IFN γ assay specificity relative to positive SITT (Sp_r) was studied in 578 cattle testing positive
178 to SITT from 176 bTB-free herds (*i.e.* where no cattle have been found to be culture-positive
179 since 2001). Unlike the above-mentioned absolute specificity study, these herds were located
180 in a bTB-infected zone (*i.e.* a zone where several bTB outbreaks had taken place in recent
181 years). These herds were subsequently monitored annually and to date they are still
182 considered bTB-free.

183 Collection of these blood samples was carried out 3 days after the SITT.

184

185 **III. Statistical methods**

186 Different cut-off values were evaluated in a simulation to determine their effects on the
187 sensitivity (Se_r) and specificities (Sp_r & Sp) of the test (Lauzi *et al.*, 2000). The R statistical
188 software (R version 2.9.2 (August 2009), R Foundation for Statistical Computing, Vienna,
189 Austria, <http://www.R-project.org>) was used to estimate sensitivity and specificity values
190 (with a 95% confidence interval, 95% CI).

191 To establish a set of conditions (a cut-point) to provide both the optimal test's sensitivity and
192 specificity, receiver-operator characteristic (ROC) analyses of single variables were used
193 (Ryan *et al.*, 2000).

194

195 **Results**196 **I. Individual Sensitivity relative to *post-mortem* bTB positive confirmation (Se_r) &**
197 **Absolute Specificity (Sp) and final Se_r & Sp at selected cut-off values for optimal**
198 **individual Se_r & Sp**

199 In the Se_r study, 13/73 animals yielded invalid results with PPD stimulation. The OD value
200 for A (OD_A) was high, as was that of B (OD_B) (each >2.5 OD units), with an OD_B/OD_A ratio
201 ranging from 0.850 to 1.150. Indeed, beyond 2.5 OD units, the graph representing OD as a
202 function of the $IFN\gamma$ concentration was not linear (absorbance saturation). Therefore, only the
203 remaining 60 cattle (from 20 herds) were used in our analysis.

204 Plasma from these invalid-result animals was diluted tenfold in PBS which allowed us to
205 overcome the OD saturation problem and assign them as real positive infected animals (data
206 not shown). The limited number of samples made it impossible to draw valid statistical
207 conclusion.

208

209 **I.1. Combined results of individual Se_r & Sp studies of the $IFN\gamma$ assay**210 **I.1.1. PPD Se_r & PPD Sp at different cut-off values**

211 Table 1a shows the PPD Se_r figures for different cut-off values obtained from the Se_r study.
212 PPD Sp values are shown in Table 1a using different cut-off values obtained from the Sp
213 study (Faye *et al.*, 2008).

214 The results presented in Table 1a show that PPD is more specific than sensitive (cut-off at
215 0.04, 83% [95% CI, 72-92%] for PPD Se_r and 99.4% [95% CI, 98.2-99.9%] for PPD Sp).

216 A ROC curve of PPD $[(OD_B-OD_A)/(OD_{PC}-OD_{NC})]$ was plotted from these PPD Se_r and Sp
217 values (Figure 1a). The optimal PPD Se_r (93% [84-98]) and PPD Sp (98.0% [96.3-99.0]) were
218 obtained when the cut-off is about 0.02 (Figure 1a and Table 1a).

219

220 I.1.2. R Se_r & R Sp at different cut-off values

221 ESAT6–CFP10 (R) Se_r values are shown in Table 1b using different cut-off values obtained
222 from the Se_r study. The R Sp figures, using different cut-off values obtained from the Sp
223 study, are shown in Table 1b.

224 The results presented in Table 1b show that R is more specific than sensitive (cut-off at 0.04,
225 80% [68-89] for R Se_r and 99.4% [98.2-99.9] for R Sp) as described in the literature.

226 A ROC curve of R [(OD_R-OD_N)/(OD_{PC}-OD_{NC})] was plotted from these R Se_r and Sp values
227 (Figure 1b). The optimal R Se_r (97% [89-100]) and R Sp (95.9% [93.8-97.5]) were obtained
228 when the cut-off is about 0.01 (Figure 1b and Table 1b).

229

230 I.2. Final Se_r & Sp of the IFN_γ assay at selected cut-off values for optimal**231 individual Se_r & Sp****232 I.2.1. Final Se_r at cut-off values 0.02 and 0.01 for PPD and R calculation methods**
233 respectively

234 The final Se_r of the IFN_γ assay, at cut-off values 0.02 and 0.01 for PPD and R calculation
235 methods respectively, are shown in Table 2. PPD∩R results correspond to positive results
236 while PPDUR results correspond to positive and divergent results.

237 The Se_r PPD∩R [% (POS)] and the Se_r PPDUR [% (POS + DIV)] obtained were estimated at
238 90% [82-98] and 100% [95-100] respectively (Table 2).

239

240 I.2.2. Final Sp at cut-off values 0.02 and 0.01 for PPD and R calculation methods
241 respectively

242 The final Sp of the IFN_γ assay, at cut-off values 0.02 and 0.01 for PPD and R calculation
243 methods respectively, are shown in Table 2.

244 The Sp PPD \cap R [1 - % (POS) = % (NEG + DIV)] and Sp PPDUR [1 - % (POS + DIV) = %
 245 (NEG)] obtained were estimated at 100% [99.2-100] and 93.9% [91.4-95.8] respectively
 246 (Table 2).

247

248 **II. Individual Sensitivity relative to *post-mortem* bTB positive confirmation (Se_r) &**
 249 **Specificity relative to positive SITT (Sp_r) and final Se_r & Sp_r at selected cut-off values**
 250 **for optimal individual Se_r & Sp_r**

251 In the Sp_r study of the IFN γ test, 31/578 animals yielded invalid results in PPD. Therefore,
 252 only the remaining 547 cattle (172 herds) were subsequently kept for our study.

253 Plasma from these invalid-result animals was diluted tenfold in PBS which allowed us to
 254 overcome the OD saturation problem and assign them as real negative infected animals (data
 255 not shown).

256

257 **II.1. Combined results of individual Se_r & Sp_r studies of the IFN γ assay**

258 **II.1.1. PPD Se_r & PPD Sp_r at different cut-off values**

259 For the different cut-off values obtained from the Se_r study, the PPD Se_r figures are given in
 260 Table 1a. For the different cut-off values obtained from the Sp_r study, the PPD Sp_r figures are
 261 also given in Table 1a.

262 The results, presented in Table 1a, show that PPD is more sensitive than specific (cut-off at
 263 0.04, 83% [72-92] for PPD Se_r and 73.3% [69.4-77.0] for PPD Sp_r) in contrast to the results
 264 obtained in the absolute specificity (Sp) study.

265 A second ROC curve of PPD [(OD $_B$ -OD $_A$)/(OD $_{PC}$ -OD $_{NC}$)] was plotted from these PPD Se_r
 266 and Sp_r values (Figure 1c). The optimal PPD Se_r (83% [72-92]) and PPD Sp_r (75.5% [71.7-
 267 79.0]) were obtained when the cut-off is about 0.05 (Figure 1c and Table 1a).

268

269 **II.1.2. R Se_r & R Sp_r at different cut-off values**

270 The R Se_r figures, for different cut-off values obtained from the Se_r study, are given in
 271 Table 1b. The R Sp_r figures, for different cut-off values obtained from the Sp_r study, are also
 272 given in Table 1b.

273 The results, presented in Table 1b, show that R is more specific than sensitive (cut-off at 0.04,
 274 80% [68-89] for R Se_r and 93.1% [90.6-95.0] for R Sp_r) as described in the literature.

275 A second ROC curve of R $[(OD_R - OD_N)/(OD_{PC} - OD_{NC})]$ was plotted from these R Se_r and Sp_r
 276 values (Figure 1d). The optimal R Se_r (87% [75-94]) and R Sp_r (90.7% [87.9-93.0]) were
 277 obtained when the cut-off is about 0.03 (Figure 1d and Table 1b).

278 This cut-off corresponds to 0.07 (0.03 x 2.4) with the following calculation method: $OD_R -$
 279 OD_N .

280

281 **II.2. Final Se_r & Sp_r of the IFN_γ assay at selected cut-off values for optimal**
 282 **individual Se_r & Sp_r**

283 **II.2.1. Final Se_r at cut-off values 0.05 and 0.03 for PPD and R calculation methods**
 284 **respectively**

285 The final Se_r of the IFN_γ assay, at cut-off values 0.05 and 0.03 for PPD and R calculation
 286 methods respectively, are shown in Table 3.

287 The Se_r PPD_∩R and Se_r PPD_∪R obtained were estimated at 77% [64-87] and 93% [84-98]
 288 respectively (Table 3).

289

290 **II.2.2. Final Sp_r at cut-off values 0.05 and 0.03 for PPD and R calculation methods**
 291 **respectively**

292 The final Sp_r of the IFN_γ assay, at cut-off values 0.05 and 0.03 for PPD and R calculation
 293 methods respectively, are shown in Table 3.

294 The Sp_r $PPD \cap R$ and Sp_r $PPDUR$ obtained were estimated at 94.3% [92.0-96.1] and 71.8%
295 [67.9-75.6] respectively (Table 3).

296

297 **Discussion and Conclusion**

298 In our study, a normalized interpretation formula was used to improve the test's
299 reproducibility. Combining the individual results of the Se_r (sensitivities relative to *post-*
300 *mortem* bTB positive confirmation) of PPD and ESAT6-CFP10 (R) stimulation and the Sp
301 (absolute specificities) of PPD and R made it possible to determine the cut-off values for
302 obtaining optimal values of individual Se_r and Sp .

303 The cut-off value of our PPD calculation method is estimated at 0.02 for optimal PPD Se_r of
304 93% [84-98] and Sp of 98.0% [96.3-99.0] (Table 1a), while the cut-off value of our R
305 calculation method is estimated at 0.01 for optimal R Se_r of 97% [89-100] and Sp of 95.9%
306 [93.8-97.5] (Table 1b).

307 However, Bovigam[®]'s OD_{PC} values are generally too high (average value about 2.5) and thus
308 no longer corresponding to the dose-response zone. It is thus recommended for subsequent
309 studies to dilute the kit's positive control so as to obtain OD_{PC} values in the linear zone which
310 are constant for each batch.

311 The test's specificity and sensitivity were assessed considering positive and divergent results
312 ($PPDUR$) or just positive results for each method ($PPD \cap R$).

313 If we consider $PPD \cap R$, the values of the relative sensitivity and absolute specificity of the
314 $IFN\gamma$ assay were finally estimated respectively at 90% [82-98] and 100% [99.2-100] (Table 2)
315 at previously determined cut-off values (0.01 for R & 0.02 for PPD) in a context of low
316 prevalence with no previous non-specific reactions to SITT. For $PPDUR$, relative sensitivity

317 and absolute specificity were estimated at 100% [95-100] for Se_r and 93.9% [91.4-95.8] for
318 Sp (Table 2).

319 We can then propose to interpret the test results according to different epidemiological
320 contexts. Indeed, in the case of animals from an unfavorable context, *i.e.* belonging to a
321 previously bTB-infected herd (risk of bTB resurgence) and/or a presently bTB-infected herd
322 and/or a herd epidemiologically linked to another recognized bTB-infected herd, sensitivity
323 should be favored rather than specificity. The PPDUR interpretation should then be chosen.
324 For this type of interpretation, we obtained excellent test sensitivity (Se_r of 100%) with good
325 specificity values (Sp of 93.9%).

326 Conversely, in a favorable context, *i.e.* a herd considered free of bTB for several years and
327 presenting no other epidemiological link with other infected herds, or a herd from a bTB-free
328 zone, specificity should be favored rather than sensitivity. The PPD \cap R interpretation would
329 thus be more suitable. Here, we obtain excellent test specificity (Sp of 100%) with acceptable
330 test sensitivity (Se_r of 90%).

331 However, since the IFN γ assay was mostly used after a positive SITT, we also studied the
332 operational specificity of the test (Sp_r). The results obtained show that R is more specific than
333 PPD at a selected cut-off value of 0.04 (93.1% [90.6-95.0] for R Sp_r (Table 1b) & 73.3%
334 [69.4-77.0] for PPD Sp_r (Table 1a)). The use of R clearly allows the specificity of the IFN γ
335 assay to be improved (Van Pinxteren *et al.*, 2000; Buddle *et al.*, 2001, 2003, 2009;
336 Vordermeier *et al.*, 2001, 2006; Wood *et al.*, 2001 and Aagaard *et al.*, 2006; De la Rua-
337 Domenech *et al.*, 2006a; Coad *et al.*, 2008).

338 Thus, as has already been described by others (Rothel *et al.* 1990; Wood *et al.*, 1991, 2001;
339 Ryan *et al.*, 2000 and Gormley *et al.*, 2005), the Bovigam[®] assay appears to be a useful
340 additional diagnostic tool for the detection of bTB in low-prevalence areas when used with
341 specific peptides and an adapted protocol.

342 The combination of results from the individual Se_r and the individual Sp_r studies
343 (specificities, relative to positive SITTs, of PPD and R) made it possible to determine cut-off
344 values for obtaining optimal individual values.

345 The cut-off value of our PPD calculation method was estimated at 0.05 for optimal PPD Se_r
346 of 83% [72-92] and Sp_r of 75.5% [71.7-79.0] (Table 1a); the cut-off value of our R calculation
347 method was estimated at 0.03 for optimal R Se_r of 87% [75-94] and Sp_r of 90.7% [87.9-93.0]
348 (Table 1b).

349 If we consider $PPD \cap R$, the values of the relative sensitivity and specificity of the $IFN\gamma$ assay
350 were finally estimated respectively at 77% [64-87] and 94.3% [92.0-96.1] (Table 3) at
351 previously determined cut-offs (0.03 for R & 0.05 for PPD), in a low prevalence context and
352 in a positive SITT population, while they were estimated at 93% [84-98] for Se_r and 71.8%
353 [67.9-75.6] for Sp_r (Table 3), with regard to the PPDUR method. Then, in an unfavorable
354 context, the PPDUR interpretation would provide good test sensitivity (Se_r about 93%) with
355 suitable test specificity (Sp_r about 71.8%). Conversely, in a favorable context, the $PPD \cap R$
356 interpretation would provide good test specificity (Sp_r about 94.3%) with suitable test
357 sensitivity (Se_r about 77%). In these population groups, the loss in specificity led to a
358 modification of the employed cut-off values and thus, a decrease in the sensitivity. This effect
359 is more noticeable for PPD than for R.

360 In retrospective studies, *i.e.* Se_r and Sp_r studies, the totality of the assays could not be carried
361 out with same delay after SITT. We did not take into account this variability in performing the
362 test (3-10 days after SITT), even though the literature shows it may sometimes affect the
363 results (Schiller et al., 2010).

364 On the whole, the values for individual sensitivity and specificity depend on the specific field
365 conditions when applying the test (the total population or just the positive SITT population).
366 As a result, their corresponding decisional cut-offs also vary. Subsequent to the choice of

367 decisional cut-off values, the result interpretation method (PPD \cap R or PPDUR method) is
368 selected according to a favorable or unfavorable epidemiological context.

369

370 To conclude, the present work made it possible to determine cut-off values of 0.02 and 0.01
371 for PPD and R respectively for the use of the modified IFN γ assay in parallel to SITT, in a
372 particular region in Dordogne, *i.e.* in a population without previous SITT's non-specific
373 reactions and with a low bTB prevalence context. Concerning the use of this IFN γ assay after
374 positive SITTs and in a low prevalence area, we obtained cut-off values of 0.05 and 0.03 for
375 PPD and R respectively. These cut-off values could also be suitable for other areas where
376 cross reactions with environmental mycobacteria might occur.

377 The different data obtained show that, according to the region's infection status and the
378 conditions of the test's application, it is necessary to study individual (PPD and R)
379 sensitivities and specificities so as to define optimal decisional cut-off values for PPD and R.
380 Moreover, the PPDUR interpretation method should be used when a bTB high-risk estimate is
381 established (based on an unfavorable epidemiological context) whereas a PPD \cap R
382 interpretation method should be used for a low-risk assessment (favorable epidemiological
383 context).

384 Finally, adapting the analysis criteria (cut-offs & interpretation method) to the region's
385 infection status (for example low or high bTB prevalence, frequency of non-specific reaction,
386 etc.) and to the test application conditions (for example confirmation of previous SITT results,
387 total or partial depopulation, etc.), is essential for the appropriate use of our modified IFN γ
388 assay.

389

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399 **Conflict of Interest Statement**

400 The authors have not declared any conflict of interest.

401

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479 **Tables**

480 **Table 1. Results (% - 95% CI) of individual Se_r , Sp and Sp_r of the IFN γ assay for different cut-off values,**
 481 **obtained in Se_r (n=60), Sp (n=492) and Sp_r (n=547) studies:** a. Results of PPD Se_r , PPD Sp and PPD Sp_r
 482 (result calculation: $(OD_B - OD_A) / (OD_{PC} - OD_{NC})$); b. Results of R Se_r , R Sp and R Sp_r (result calculation: $(OD_R -$
 483 $OD_N) / (OD_{PC} - OD_{NC})$).

	Cut-off	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08
a. Results of PPD Se_r, PPD Sp and PPD Sp_r % - 95% CI (number)	PPD Se_r (i)	93% 84-98 (56)	93% 84-98 (56)	87% 75-94 (52)	83% 72-92 (50)	83% 72-92 (50)	75% 62-85 (45)	75% 62-85 (45)	75% 62-85 (45)
	PPD Sp (ii)	97.6% 95.8-98.7 (480)	98.0% 96.3-99.0 (482)	99.2% 97.9-99.8 (488)	99.4% 98.2-99.9 (489)	99.4% 98.2-99.9 (489)	99.4% 98.2-99.9 (489)	99.4% 98.2-99.9 (489)	99.4% 98.2-99.9 (489)
	PPD Sp_r (iii)	61.1% 56.8-65.2 (334)	66.5% 62.4-70.5 (364)	70.4% 66.4-74.2 (385)	73.3% 69.4-77.0 (401)	75.5% 71.7-79.0 (413)	77.1% 73.4-80.6 (422)	79.5% 75.9-82.8 (435)	80.8% 77.2-84.0 (442)
b. Results of R Se_r, R Sp and R Sp_r % - 95% CI (number)	R Se_r (iv)	97% 89-100 (58)	88% 76-95 (53)	87% 75-94 (52)	80% 68-89 (48)	72% 59-83 (43)	68% 55-80 (41)	67% 53-78 (40)	62% 48-74 (37)
	R Sp (v)	95.9% 93.8-97.5 (472)	98.6% 97.1-99.4 (485)	99.4% 98.2-99.9 (489)	99.4% 98.2-99.9 (489)	99.6% 98.5-99.9 (490)	99.6% 98.5-99.9 (490)	99.8% 98.9-100 (491)	99.8% 98.9-100 (491)
	R Sp_r (vi)	60.9% 56.6-65.0 (333)	84.8% 81.5-87.7 (464)	90.7% 87.9-93.0 (496)	93.1% 90.6-95.0 (509)	94.5% 92.3-96.3 (517)	95.4% 93.3-97.0 (522)	96.0% 94.0-97.5 (525)	96.5% 94.6-97.9 (528)

484 i. Number of PPD positives in Se_r study; ii. Number of PPD negatives in Sp study; iii. Number of PPD negatives
 485 in Sp_r study; iv. Number of R positives in Se_r study; v. Number of R negatives in Sp study; vi. Number of R
 486 negatives in Sp_r study.

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Table 2. Final results (number of POS, DIV and NEG), at cut-off values 0.02 and 0.01 for the PPD and R calculation method respectively, in the Se_r study (n=60) and the Sp study (n=492) of the IFN γ assay and then the Se_r (% - 95% CI) and the Sp (% - 95% CI) of the IFN γ assay from the PPD \cap R or PPDUR interpretation method.

	Cut-offs: 0.02 PPD & 0.01 R	
Number of	Final results in Sp study	Final results in Se_r study
POS	0	54
DIV	30	6
NEG	462	0
Interpretation method	Final Sp % - 95% CI (number of negative animals)	Final Se_r % - 95% CI (number of positive animals)
PPD \cap R	100% [99.2-100] (492)	90% [82-98] (54)
PPDUR	93.9% [91.4-95.8] (462)	100% [95-100] (60)

493 Sp PPD \cap R: $1 - \% (POS) = \% (NEG + DIV)$; Sp PPDUR: $1 - \% (POS + DIV) = \% (NEG)$;
 494 Se_r PPD \cap R: $\% (POS)$; Se_r PPDUR: $\% (POS + DIV)$.

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Table 3. Final results (number of POS, DIV and NEG), at cut-off values 0.05 and 0.03 for the PPD and R calculation method respectively, in the Se_r study (n=60) and the Sp_r study (n=547) of the IFN γ assay and then the Se_r (% - 95% CI) and the Sp_r (% - 95% CI) of the IFN γ assay from the PPD \cap R or PPDUR interpretation method.

Cut-offs: 0.05 PPD & 0.03 R		
Number of	Final results in Sp_r study	Final results in Se_r study
POS	31	46
DIV	123	10
NEG	393	4
Interpretation method	Final Sp_r % - 95% CI (number of negative animals)	Final Se_r % - 95% CI (number of positive animals)
PPD \cap R	94.3% [92.0-96.1] (516)	77% [64-87] (46)
PPDUR	71.8% [67.9-75.6] (393)	93% [84-98] (56)

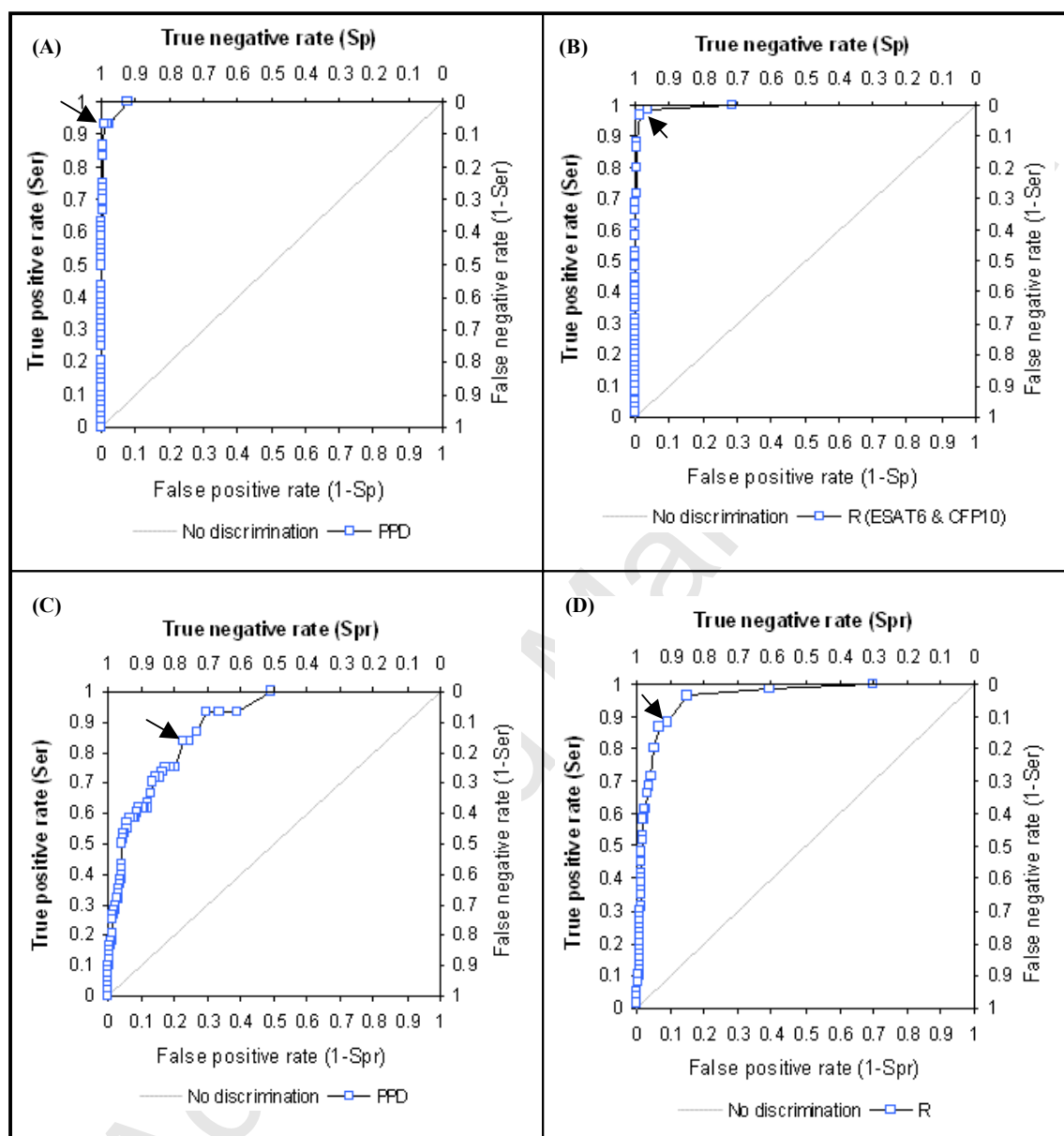
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Sp_r PPD \cap R: $1 - \% (POS) = \% (NEG + DIV)$; Sp_r PPDUR: $1 - \% (POS + DIV) = \% (NEG)$;
 Se_r PPD \cap R: $\% (POS)$; Se_r PPDUR: $\% (POS + DIV)$.

506 Figure

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 510 **Figure 1. (A):** ROC curve of PPD $[(OD_B - OD_A) / (OD_{PC} - OD_{NC})]$ from PPD Se_r & PPD Sp_r . The optimal cut-
 511 point of this calculation method at 0.02, yielding a Se_r of 0.93 and a Sp_r of 0.98, is shown (arrow); **(B):** ROC
 512 curve of R $[(OD_R - OD_N) / (OD_{PC} - OD_{NC})]$ from R Se_r & R Sp_r . The optimal cut-point of this calculation method
 513 at 0.01, yielding a Se_r of 0.97 and a Sp_r of 0.96, is shown; **(C):** ROC curve of PPD $[(OD_B - OD_A) / (OD_{PC} - OD_{NC})]$
 514 from PPD Se_r & PPD Sp_r . The optimal cut-point of this calculation method at 0.05, yielding a Se_r of 0.83 and a
 515 Sp_r of 0.76, is shown; **(D):** ROC curve of R $[(OD_R - OD_N) / (OD_{PC} - OD_{NC})]$ from R Se_r & R Sp_r . The optimal cut-
 516 point of this calculation method at 0.03, yielding a Se_r of 0.87 and a Sp_r of 0.91, is shown.

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