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1 **Interference of paratuberculosis with the diagnosis of tuberculosis in a goat flock**
2 **with a natural mixed infection**

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13 **Abstract**

14 Detection of infected animals is a key step in eradication programmes of tuberculosis.
15 Paratuberculosis infection has been demonstrated to compromise the specificity of the
16 diagnostic tests. However, its effect on their sensitivity has not been clarified. In the
17 present study, skin tests and the interferon-gamma (IFN- γ) assay were evaluated in a
18 goat flock (n=177) with a mixed tuberculosis-paratuberculosis infection in order to
19 assess the possible effect of paratuberculosis on their sensitivity. Culture of
20 mycobacteria was performed as the gold standard to determine the true infection status.
21 All techniques showed lower sensitivities than previously described; the single
22 intradermal tuberculin (SIT) test and the IFN- γ assay detected 71% (62.4-78.6, 95%
23 C.I.) of the infected animals; the single intradermal cervical comparative tuberculin
24 (SICCT) test detected only 42.7% (34.1-51.7, 95% C.I.) of infected animals. The
25 highest level of sensitivity was obtained when SIT test and IFN- γ assay were combined
26 in parallel (90.8%, 84.5-95.2, 95% C.I.). Sensitivities of the tests were also assessed by
27 comparing animals suffering tuberculosis and animals with a mixed infection; tests were
28 found to be more effective in the former group. Paratuberculosis seems to have a major
29 effect in the sensitivity of the diagnostic tests under study, and therefore must be taken
30 into account; in particular, the use of the SICCT test should be questioned when both
31 tuberculosis and paratuberculosis are present.

32 *Keywords:* tuberculosis; paratuberculosis; goat; skin test; SIT; SICCT; IFN- γ ;
33 sensitivity.

34 1. Introduction

35 Caprine tuberculosis can be caused by *Mycobacterium bovis* and *M. caprae*
36 (Gutierrez et al., 1995; Aranaz et al., 2003); both pathogens represent a serious concern
37 because of the economic impact and possible spread to bovine herds. Moreover, it
38 represents a potential source of infection to humans through unpasteurised dairy
39 products and direct contact (Gutierrez et al., 1997; Kubica et al., 2003).

40 Paratuberculosis, caused by *Mycobacterium avium* subsp. *paratuberculosis*
41 (*Map*), also causes reduced production. The involvement of *Map* in the development of
42 Crohn's disease has not been demonstrated but its zoonotic potential is still under study
43 (Grant, 2005). In Spain, several studies indicate that it is present in all domestic
44 ruminant species with variable prevalences (Reviriego et al., 2000; de Juan et al.,
45 2006a).

46 Several diagnostic tests are used to ascertain the tuberculosis status. The single
47 intradermal tuberculin (SIT) test measures the cell mediated immune (CMI) response to
48 a *M. bovis* purified protein derivative (PPD). As some of the proteins in PPD tuberculin
49 are shared among different mycobacterial species (Aagaard et al., 2003), false positive
50 reactions have been described in animals exposed to other mycobacteria (de la Rúa-
51 Domenech et al., 2006). To limit those false positive results, the single intradermal
52 comparative cervical tuberculin (SICCT) test compares the response to the bovine PPD
53 against an avian PPD (Paterson et al., 1958). Therefore specificity of the test is
54 increased, though its sensitivity might be hampered, especially in adult animals
55 (Collins, 2006).

56 The interferon-gamma (IFN- γ) assay is an *in vitro* alternative that has
57 demonstrated a higher sensitivity compared with the intradermal tuberculin tests (Wood
58 et al., 1992; Liebana et al., 1998; Gonzalez-Llamazares et al., 1999) and can detect

59 infected animals at an earlier stage (Gormley et al., 2006); therefore, it has been
60 proposed as an ancillary test in cattle (Council Directive 64/432/EEC, amended by (EC)
61 No. 1226/2002 of 8 July 2002, Anon. 2002).

62 In certain areas of the European Union co-infection with *M. bovis* or *M. caprae*
63 and *Map* may occur in a same herd. The effect of paratuberculosis in the induction of
64 false positive responses has been already studied in cattle (Walravens et al., 2002; Dunn
65 et al., 2005), but little has been investigated about the possible effect of a natural mixed
66 tuberculosis-paratuberculosis infection in their sensitivity. Previous studies
67 demonstrated a decreased sensitivity of these tests when applied to cattle previously
68 infected with *M. avium* (Amadori et al., 2002; Hope et al., 2005). In a preliminary
69 study, the sensitivity of SICCT and IFN- γ test in a cattle herd with dual tuberculosis-
70 paratuberculosis infection (Aranaz et al., 2006) was lower than in previous reports, but
71 the selection of animals may have resulted in an artificially biased higher sensitivity.

72 No investigation regarding the sensitivity of diagnostic tests in goat flocks with a
73 dual infection has been published, in spite of the possible impact on control programs.
74 The aim of the present study was to evaluate the sensitivity of SIT, SICCT and IFN- γ
75 tests in a goat flock with a natural tuberculosis-paratuberculosis infection in order to
76 determine the possible effect of paratuberculosis on their accuracy in detecting
77 tuberculosis infection.

78

79 2. Material and methods

80 2.1. Flock and design of the study

81 This study was performed on a flock of Spanish *Guadarrama* breed with a
82 previous diagnosis of both *M. caprae* and *Map* infection. The flock was composed of
83 177 goats older than 2 years that had never been vaccinated against paratuberculosis.
84 Seroprevalence against *Map*, estimated by ELISA (*Mycobacterium paratuberculosis*
85 Antibody Test Kit, Parachek™ Johne's Absorbed EIA), was 64.4%. All goats were
86 analysed using the SIT test, the SICCT test and the IFN- γ detection assay. Animals were
87 euthanized and necropsied for the collection of samples for bacteriology.

88 The goats included in this field trial were not experimental animals. Handling of
89 the animals, sampling and euthanasia were performed by veterinarians according to
90 Spanish Legislation.

91 2.2. Diagnostic tests

92 2.2.1. Skin tests

93 SIT and SICCT were performed after blood sampling for IFN- γ using official
94 bovine and avian PPDs (CZ Veterinaria, Porriño, Spain). Animals were inoculated 0.1
95 mL containing 0.1 mg of bovine PPD (2500 CTU) on the left side of the neck and 0.1
96 mL (2500 IU) avian PPD on the right side. The results were determined by measuring
97 the increase of the skin-fold thickness 72 h later.

98 Regarding the SIT test, animals were classified as positive if a bovine reaction
99 (increase of the skin-fold thickness after the application of the bovine PPD) of 2 or more
100 millimetres or the presence of clinical signs such as oedema, exudation, necrosis, pain
101 or inflammation at the injection site were observed (severe interpretation).

102 Animals were considered positive for the SICCT test if a positive bovine
103 reaction of 2 or more millimetres was observed which was greater than the avian

104 reaction, or if the presence of clinical signs at the injection site of the bovine PPD were
105 observed (severe interpretation).

106 2.2.2. *In-vitro* test (IFN- γ test)

107 Blood samples were stimulated with the avian and bovine PPDs used in the skin
108 tests as previously described (Liebana et al., 1998). Plasma was tested in duplicate using
109 a sandwich EIA kit for bovine IFN- γ (BovigamTM Bovine Gamma Interferon Test)
110 according to methods described by the supplier (Prionics, Schlieren, Switzerland). The
111 interpretation of the test has been described elsewhere (Aranaz et al., 2006). Two cut-off
112 points were selected to evaluate the sensitivity: the “severe interpretation” considered an
113 animal positive when the mean OD of its sample stimulated with bovine PPD minus the
114 mean OD of nil antigen was greater than 0.05, and greater than the same value of the
115 sample stimulated with avian PPD; the “standard interpretation” declared an animal
116 positive if the same data was above 0.1. Interpretation of the test was also performed
117 just using the results of the stimulation with bovine PPD to check if significant
118 differences could be observed.

119 2.3. Bacteriology

120 2.3.1. Tissue collection

121 Samples from lung and retropharyngeal, mediastinal and bronchial lymph nodes
122 were taken for culture of *M. tuberculosis* complex; samples from ileocecal valve and
123 adjacent tissue, and mesenteric lymph nodes were taken for *Map* culture. All samples
124 were stored at -20°C until analysis.

125 2.3.2. *M. tuberculosis* complex culture

126 Samples for culture included tuberculosis compatible lesions and the adjacent
127 areas, or a pool of the collected tissues if no macroscopic lesion was observed. Tissue
128 samples were homogenized with sterile distilled water and decontaminated with 0.35%

129 hexadecylpyridinium chloride (HPC) for 30 minutes (Corner and Trajstman, 1988),
130 centrifuged at 1068 g for 30 min and cultured onto Coletsos and 0.2% (w/v) pyruvate-
131 enriched Löwenstein-Jensen media (bioMérieux España and Biomedics, Madrid,
132 Spain). Isolates were identified by staining for acid-alcohol fastness and PCR
133 amplification of *Mycobacterium* genus-specific 16S rRNA fragment and MPB70
134 sequence (Wilton and Cousins, 1992). Isolates were characterised using spoligotyping
135 (Kamerbeek et al., 1997).

136 2.3.3. *Map* culture

137 Samples of each animal were pooled, decontaminated using 0.75% HPC for 18
138 hours (Greig et al., 1999) and inoculated onto selective media (de Juan et al., 2006b).
139 Isolate identification was confirmed by mycobactin-dependency, and specific PCR for
140 the detection of IS900 and f57 sequence (Coetsier et al., 2000). Isolates were further
141 characterized using the PCR test described by Collins et al. (2002).

142 2.3.4. Classification of the animals according to the culture results

143 To evaluate the performance of the diagnostic test for tuberculosis depending on
144 their infection status, animals were classified using the culture result as the gold
145 standard. Animals that showed lesions compatible with tuberculosis but had negative
146 culture results (n=14) were excluded from the study to avoid considering them as
147 negative.

148 Secondly, in order to determine if paratuberculosis infection could produce
149 significant differences in the reliability of the tests, tuberculosis-infected animals were
150 classified as 1) animals positive to tuberculosis: all animals from which *M. caprae* was
151 cultured regardless of *Map* culture results, and 2) animals positive to tuberculosis and
152 paratuberculosis (tbc+/ptb+): only animals from which both *M. caprae* and *Map* were
153 isolated from their tissues.

154 Statistical analysis of the data was carried out using the software PEPI 4.0
155 (Computer Program for Epidemiologist), J.H. Abramson and P.M. Gahlinger.

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156 3. Results

157 3.1. Culture results

158 After completing the *in-vivo* tests, all animals (n=177) were tested by
159 bacteriology. Fourteen animals showing tuberculosis-compatible lesions but negative to
160 *M. caprae* culture were excluded from the study to overcome a possible lack of
161 sensitivity of the culture procedure. From the remaining 163 goats, *M. caprae* was
162 isolated from 131 animals (74%), and 30 (18.4%) were positive for the culture of *Map*
163 (Table 1).

164 All the *M. caprae* isolates belonged to the spoligotype SB1084, and all *Map*
165 isolates were classified as cattle type (type II).

166 3.2. Assessment of the diagnostic tests

167 Sensitivity for each test was calculated using the culture results as the gold
168 standard (Table 2). The SICCT test was the least sensitive, detecting only 56 out of 131
169 (42.7%) infected animals, even though a severe interpretation was applied. The tests
170 that detected the maximum number of infected animals were both SIT test and IFN- γ
171 assay when the cut-off point was fixed in 0.05 (“severe interpretation”). If the results of
172 the stimulation with avian PPD were ignored in the interpretation of the IFN- γ assay,
173 the sensitivity did not change significantly, regardless of the cut-off point (Table 2).

174 Animals with a positive culture of *M. caprae* are represented on the basis of their
175 skin tests results in Fig. 1. SIT test detected 37 (28.2%) more infected animals than
176 SICCT, but 38 animals remained undetected by both skin tests. Twenty-six of those
177 animals were detected by the IFN- γ assay, and thus 12 remained undetected. *Map*
178 infection was confirmed by culture in 5 out of those 12 animals.

179 When the different tests are combined in parallel, the best sensitivity was
180 obtained when SIT and “severe” IFN- γ assay are used together (90.8%) (Table 3).

181 Results of SIT test and IFN- γ assay in the tuberculosis-infected animals (n=131)
182 are compared in Table 4. The agreement found between the 2 tests was very low
183 (Kappa=0.036, Confidence Interval 95%, -0.135-0.207). The same result was obtained
184 when the IFN- γ assay results were compared with the SICCT readings (Kappa=0.036,
185 C.I. 95%, -0.109-0.181). The regression lines for positive and negative animals in the
186 IFN- γ test were also determined and compared (Fig. 2). Both lines showed a similar
187 equation ($y=0.69x+2.2$ and $y=0.64x+1.9$).

188 Following the criteria detailed above, results obtained from all tuberculosis-
189 infected animals (n=131) were compared with those from the sub-group of animals with
190 mixed infection (tbc+/ptb+) (n=24) (Table 5). SIT test showed a lower sensitivity in the
191 animals with the dual infection (54.2%) than that achieved in the overall group (71%);
192 differences were found to be statistically significant (C.I. 95%). SICCT test also
193 performed worse in the tbc+/ptb+ group, but the differences were not found to be
194 significant (C.I. 95%). IFN- γ assay showed a similar sensitivity in both groups
195 regardless the threshold applied. The small differences found between the 2 groups were
196 not statistically significant (C.I. 95%).

197 **4. Discussion**

198 Bovine tuberculosis is subject of control and eradication programs in many
199 countries worldwide; these programs are based on the early detection and removal of
200 infected animals, therefore they rely on the sensitivity of available diagnostic tests. In
201 this sense, the presence of non-tuberculous mycobacteria has been pointed out as a
202 possible cause of misdiagnosis (Lauzi et al., 2000; Dunn et al., 2005). Caprine
203 tuberculosis is also important, as it can jeopardise the success of the programs for
204 bovine tuberculosis and represents a public health threat.

205 In the present study we have applied an adaptation of the diagnostic tests used in
206 cattle to a goat flock with a mixed infection of tuberculosis and paratuberculosis. The
207 sensitivity observed in all tests were lower than results published by other authors for
208 cattle (Francis et al., 1978; Wood, et al., 1992; Whipple et al., 1995) and goats
209 (Gutierrez et al., 1998, Liebana et al., 1998). As there are no established criteria for the
210 interpretation of the skin tests in goats, in this study we have adopted the criteria of the
211 European legislation for cattle but considering positive the inconclusive reactors
212 (animals with a bovine reaction of more than 2 mm and less than 4 mm with no clinical
213 signs at the injection site in the SIT test; and animals with a bovine reaction of more
214 than 2 mm but equal to or less than an avian reaction and the absence of clinical signs in
215 the SICCT test). SIT test, under a severe interpretation, showed the highest sensitivity,
216 as it detected 71% of the infected animals, which lies within previously described
217 sensitivity rates though on the lower side.

218 To avoid a possible lack of specificity of the SIT test, the application of the
219 SICCT test has been recommended in areas where a high number of avian reactors are
220 expected (Monaghan et al., 1994). However, in this study the SICCT test was an
221 unreliable test, as it could only detect 42.7% of the infected animals even when the

222 severe interpretation was applied. These data show that SICCT test is not adequate for
223 diagnosis of tuberculosis in goats if tuberculosis and paratuberculosis coexist in the
224 same flock.

225 The low sensitivities observed in the present study are most likely caused by a
226 particular cause such as the paratuberculosis co-infection. Another possible explanation
227 for the decreased sensitivity could be the presence of high rates of anergic animals in
228 the flock that would not produce a detectable immune response. However, this
229 possibility was ruled out because a high proportion of false negative reactors showed a
230 response to the stimulation with the avian PPD in the SICCT test. Therefore, a plausible
231 explanation was the masking effect of the reaction against avian PPD in animals with a
232 mixed infection.

233 To confirm if paratuberculosis infection was affecting the sensitivity of the
234 tuberculosis diagnostic tests, we have compared the results obtained from all
235 tuberculosis-infected animals with those from goats with a mixed infection. This
236 comparison revealed that SIT test performed significantly better in the former group
237 (Table 5), as the differences between sensitivities in each group were statistically
238 significant (C.I. 95%). This observation points out the possible effect of the
239 paratuberculosis co-infection in the performance of the SIT test. The same remark can
240 be done regarding the SICCT test, though in this case the differences found were not
241 statistically significant (C.I. 95%). However, this could be attributed to the small size of
242 the tbc+/ptb+ group.

243 The detection of IFN- γ has been accepted as a useful ancillary tool in the
244 diagnosis of tuberculosis in cattle. In our study, IFN- γ assay with the cut-off point fixed
245 at 0.05 showed the same sensitivity as the SIT test in the whole flock. It must be
246 remarked that ignoring the results of stimulation with avian PPD allows the detection of

247 a few more infected animals. Thus, in situations of goat populations with a mixed
248 tuberculosis-paratuberculosis infection, the IFN- γ assay might show the same sensitivity
249 but better specificity than skin tests. In addition, it might be more cost-effective to
250 perform IFN- γ assay stimulating the lymphocytes only with bovine PPD. The cut-off
251 value of the ELISA for the IFN- γ must be adjusted to obtain a higher sensitivity (in our
252 study, PPD minus the mean OD of nil antigen greater than 0.05 instead of 0.1).
253 Interestingly, the sensitivity rates of the IFN- γ assay obtained in the two groups
254 (tuberculosis-infected and mixed infection animals) showed no statistically significant
255 differences (confidence level 95%), which could imply that this test is not as influenced
256 by a concurrent paratuberculosis in a flock than the skin tests. Adverse effects of the
257 paratuberculosis on the sensitivity of the tuberculosis diagnostic tests used in this study
258 (SIT, SICCT and IFN- γ detection tests) were also reported in cattle with a dual
259 mycobacterial infection (Aranaz et al. 2006).

260 Comparison between IFN- γ assay and SIT test in the infected animals revealed a
261 low agreement, meaning they behaved differently, detecting different populations of
262 infected animals. This is also observed in Figs. 1 and 2, as the IFN- γ test results are
263 distributed independently of the quantitative skin test results throughout the whole
264 graphic. This is in line with the general assumption that some infected animals might
265 respond to only one test, so the population which is positive to each test is not exactly
266 the same (Neill et al., 1994; Pollock et al., 2005; Aranaz et al., 2006), and highlights the
267 importance of the use of the IFN- γ assay as an ancillary test.

268 Twelve out of 131 infected animals (9.16%) did not show any CMI response.
269 They are represented in the bottom left corner of Fig. 1. *Map* was isolated from five of
270 them. However, *M. caprae* was cultured from the intestinal samples of other four
271 animals, and therefore could have overgrown a possible culture of *Map*. No obvious

272 cause for the lack of response, apart from a concomitant *Map* infection, was found.
273 These animals represent a great risk, as they were not detected by any diagnostic
274 technique and therefore would have maintained the tuberculosis infection in the farm.
275 On the other side, nine of these animals showed macroscopic lesions compatible with
276 tuberculosis, and therefore could have been detected in the slaughterhouse.

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277 **Conclusion**

278 - The sensitivities of the diagnostic tests were below the values described in previous
279 reports, and paratuberculosis co-infection was considered the most likely cause.

280 - The SICCT test was the least sensitive test, probably because the paratuberculosis
281 infection induced an important reaction to the avian PPD. Therefore, the use of SICCT
282 test in goats in areas where tuberculosis and paratuberculosis are present should be
283 discouraged.

284 - Skin tests and IFN- γ detection assay detected different populations of infected animals.
285 This highlights the usefulness of IFN- γ assay as an ancillary test. In our study, IFN- γ
286 detection test seemed to be more consistent regardless the concomitant paratuberculosis
287 infection as compared to skin tests.

288 - Some infected animals (12/131, 9.16%) were undetectable for the tuberculosis
289 diagnostic tests used in this study. Nine of these “undetectable” animals (75%) could
290 have been detected in the slaughterhouse because of their visible lesions; this points out
291 *post-mortem* inspection as a still essential tool in eradication plans for tuberculosis.

292 Paratuberculosis represents a serious impairment on the reliability of routine
293 tuberculosis diagnosis tests in goats, and should be taken into account in eradication
294 programs in areas with high paratuberculosis prevalences. Moreover, the impact of
295 paratuberculosis in the sensitivity of these diagnostic tools in cattle should also be
296 addressed in further studies, because of the economic and social consequences of a
297 failure of the diagnostic tests. Above all, a great deal of common sense added to the use
298 of the best diagnosis tools and proper epidemiological studies are necessary elements of
299 the eradication programmes, as the overall assessment of the situation of each
300 epidemiological unit cannot rely exclusively on the results of the diagnostic tests.

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316 **References**

- 317 Aagaard, C., Govaerts, M., Meng, O. L., Andersen, P., Pollock, J. M., 2003. Genomic
318 approach to identification of *Mycobacterium bovis* diagnostic antigens in cattle. J.
319 Clin. Microbiol. 41, 3719-3728.
- 320 Amadori, M., Tagliabue, S., Lauzi, S., Finazzi, G., Lombardi, G., Telo, P., Pacciarini,
321 L., Bonizzi, L., 2002. Diagnosis of *Mycobacterium bovis* infection in calves
322 sensitized by mycobacteria of the *avium/intracellulare* group. J. Vet. Med. B
323 Infect. Dis. Vet. Public Health 49, 89-96.
- 324 Anon., July 9, 2002. Off. J. Eur. Union L179, 13–18.
- 325 Aranaz, A., Cousins, D., Mateos, A., Dominguez, L., 2003. Elevation of
326 *Mycobacterium tuberculosis* subsp. *caprae* Aranaz et al. 1999 to species rank as
327 *Mycobacterium caprae* comb. nov., sp. nov. Int. J. Syst. Evol. Microbiol. 53,
328 1785-1789.
- 329 Aranaz, A., de Juan, L., Bezos, J., Alvarez, J., Romero, B., Lozano, F., Paramio, J. L.,
330 Lopez-Sanchez, J., Mateos, A., Dominguez, L., 2006. Assessment of diagnostic
331 tools for eradication of bovine tuberculosis in cattle co-infected with
332 *Mycobacterium bovis* and *M. avium* subsp. *paratuberculosis*. Vet. Res. 37, 593-
333 606.
- 334 Coetsier, C., Vannuffel, P., Blondeel, N., Denef, J. F., Cocito, C., Gala, J. L., 2000.
335 Duplex PCR for differential identification of *Mycobacterium bovis*, *M. avium*, and
336 *M. avium* subsp. *paratuberculosis* in formalin- fixed paraffin-embedded tissues
337 from cattle. J. Clin. Microbiol. 38, 3048-3054.
- 338 Collins, D. M., De Zoete, M., & Cavaignac, S. M. (2002). *Mycobacterium avium* subsp.
339 *paratuberculosis* strains from cattle and sheep can be distinguished by a PCR test
340 based on a novel DNA sequence difference. J. Clin. Microbiol. 40, 4760-4762.

- 341 Collins, J. D. (2006). Tuberculosis in cattle: Strategic planning for the future. *Vet.*
342 *Microbiol.* 112, 369-381.
- 343 Corner, L. A., Trajstman, A. C., 1988. An evaluation of 1-hexadecylpyridinium chloride
344 as a decontaminant in the primary isolation of *Mycobacterium bovis* from bovine
345 lesions. *Vet. Microbiol.* 18, 127-134.
- 346 de Juan, L., Alvarez, J., Aranaz, A., Rodriguez, A., Romero, B., Bezos, J., Mateos, A.,
347 Dominguez, L., 2006a. Molecular epidemiology of Types I/III strains of
348 *Mycobacterium avium* subspecies *paratuberculosis* isolated from goats and cattle.
349 *Vet. Microbiol.* 115, 102-110.
- 350 de Juan, L., Alvarez, J., Romero, B., Bezos, J., Castellanos, E., Aranaz, A., Mateos, A.,
351 Dominguez, L., 2006b. Comparison of four different culture media for isolation
352 and growth of type II and type I/III *Mycobacterium avium* subsp. *paratuberculosis*
353 strains isolated from cattle and goats. *Appl. Environ. Microbiol.* 72, 5927-5932.
- 354 de la Rúa-Domenech, Goodchild, A. T., Vordermeier, H. M., Hewinson, R. G.,
355 Christiansen, K. H., Clifton-Hadley, R. S., 2006. Ante mortem diagnosis of
356 tuberculosis in cattle: a review of the tuberculin tests, gamma-interferon assay and
357 other ancillary diagnostic techniques. *Res. Vet. Sci.* 81, 190-210.
- 358 Dunn, J. R., Kaneene, J. B., Grooms, D. L., Bolin, S. R., Bolin, C. A., Bruning-Fann, C.
359 S., 2005. Effects of positive results for *Mycobacterium avium* subsp
360 *paratuberculosis* as determined by microbial culture of feces or antibody ELISA
361 on results of caudal fold tuberculin test and interferon-gamma assay for
362 tuberculosis in cattle. *J. Am. Vet. Med. Assoc.* 226, 429-435.
- 363 Francis, J., Seiler, R. J., Wilkie, I. W., O'Boyle, D., Lumsden, M. J., Frost, A. J., 1978.
364 The sensitivity and specificity of various tuberculin tests using bovine PPD and
365 other tuberculins. *Vet. Rec.* 103, 420-425.

- 366 Gonzalez Llamazares, O. R., Gutierrez Martin, C. B., Alvarez, N. D., de la Puente
367 Redondo VA, Dominguez, R. L., Rodriguez Ferri, E. F., 1999. Field evaluation of
368 the single intradermal cervical tuberculin test and the interferon-gamma assay for
369 detection and eradication of bovine tuberculosis in Spain. *Vet. Microbiol.* 70, 55-
370 66.
- 371 Gormley, E., Doyle, M.B., Fitzsimons, T., McGill, K., Collins, J.D., 2006. Diagnosis of
372 *Mycobacterium bovis* infection in cattle by use of the gamma-interferon
373 (Bovigam®) assay. *Vet. Microbiol.* 112, 171-179.
- 374 Grant, I. R., 2005. Zoonotic potential of *Mycobacterium avium* ssp. *paratuberculosis*:
375 the current position. *J. Appl. Microbiol.* 98, 1282-1293.
- 376 Greig, A., Stevenson, K., Henderson, D., Perez, V., Hughes, V., Pavlik, I., Hines, M. E.,
377 McKendrick, I., Sharp, J. M., 1999. Epidemiological study of paratuberculosis in
378 wild rabbits in Scotland. *J. Clin. Microbiol.* 37, 1746-1751.
- 379 Gutierrez, M., Samper, S., Gavigan, J. A., Garcia Marin, J. F., Martin, C., 1995.
380 Differentiation by molecular typing of *Mycobacterium bovis* strains causing
381 tuberculosis in cattle and goats. *J. Clin. Microbiol.* 33, 2953-2956.
- 382 Gutierrez, M., Samper, S., Jimenez, M. S., Van Embden, J. D., Marin, J. F., Martin, C.,
383 1997. Identification by spoligotyping of a caprine genotype in *Mycobacterium*
384 *bovis* strains causing human tuberculosis. *J. Clin. Microbiol.* 35, 3328-3330.
- 385 Gutierrez, M., Tellechea, J., Garcia Marin, J. F., 1998. Evaluation of cellular and
386 serological diagnostic tests for the detection of *Mycobacterium bovis*-infected
387 goats. *Vet. Microbiol.* 62, 281-290.
- 388 Hope, J. C., Thom, M. L., Villarreal-Ramos, B., Vordermeier, H. M., Hewinson, R. G.,
389 Howard, C. J., 2005. Exposure to *Mycobacterium avium* induces low-level
390 protection from *Mycobacterium bovis* infection but compromises diagnosis of

- 391 disease in cattle. Clin. Exp. Immunol. 141, 432-439.
- 392 Kamerbeek, J., Schouls, L., Kolk, A., van, A. M., van, S. D., Kuijper, S., Bunschoten,
393 A., Molhuizen, H., Shaw, R., Goyal, M., van, E. J., 1997. Simultaneous detection
394 and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and
395 epidemiology. J. Clin. Microbiol. 35, 907-914.
- 396 Kubica, T., Rusch-Gerdes, S., Niemann, S., 2003. *Mycobacterium bovis* subsp. *caprae*
397 caused one-third of human *M. bovis*-associated tuberculosis cases reported in
398 Germany between 1999 and 2001. J. Clin. Microbiol. 41, 3070-3077.
- 399 Lauzi, S., Pasotto, D., Amadori, M., Archetti, I. L., Poli, G., Bonizzi, L., 2000.
400 Evaluation of the specificity of the gamma-interferon test in Italian bovine
401 tuberculosis-free herds. Vet. J. 160, 17-24.
- 402 Liebana, E., Aranaz, A., Urquia, J. J., Mateos, A., Dominguez, L., 1998. Evaluation of
403 the gamma-interferon assay for eradication of tuberculosis in a goat herd. Aust.
404 Vet. J. 76, 50-53.
- 405 Monaghan, M. L., Doherty, M. L., Collins, J. D., Kazda, J. F., Quinn, P. J., 1994. The
406 tuberculin test. Vet. Microbiol. 40, 111-124.
- 407 Neill, S. D., Cassidy, J., Hanna, J., Mackie, D. P., Pollock, J. M., Clements, A., Walton,
408 E., Bryson, D. G., 1994. Detection of *Mycobacterium bovis* infection in skin test-
409 negative cattle with an assay for bovine interferon-gamma. Vet. Rec. 135, 134-
410 135.
- 411 Paterson, A. B., Stuart, P., Lesslie, I. W., 1958. The use of tests on slaughterhouse cattle
412 for estimating relative potencies of tuberculins and for the calculation of
413 discrimination tests. J. Hyg. (Lond) 56, 1-18.
- 414 Pollock, J. M., Welsh, M. D., McNair, J., 2005. Immune responses in bovine
415 tuberculosis: towards new strategies for the diagnosis and control of disease. Vet.

- 416 Immunol. Immunopathol. 108, 37-43.
- 417 Reviriego, F. J., Moreno, M. A., Dominguez, L., 2000. Soil type as a putative risk factor
418 of ovine and caprine paratuberculosis seropositivity in Spain. *Prev. Vet. Med.* 43,
419 43-51.
- 420 Walravens, K., Marche, S., Rosseels, V., Wellemans, V., Boelaert, F., Huygen, K.,
421 Godfroid, J., 2002. IFN-gamma diagnostic tests in the context of bovine
422 mycobacterial infections in Belgium. *Vet. Immunol. Immunopathol.* 87, 401-406.
- 423 Whipple, D. L., Bolin, C. A., Davis, A. J., Jarnagin, J. L., Johnson, D. C., Nabors, R. S.,
424 Payeur, J. B., Saari, D. A., Wilson, A. J., Wolf, M. M., 1995. Comparison of the
425 sensitivity of the caudal fold skin test and a commercial gamma-interferon assay
426 for diagnosis of bovine tuberculosis. *Am. J. Vet. Res.* 56, 415-419.
- 427 Wilton, S., Cousins, D., 1992. Detection and identification of multiple mycobacterial
428 pathogens by DNA amplification in a single tube. *PCR Methods Appl.* 1, 269-
429 273.
- 430 Wood, P. R., Corner, L. A., Rothel, J. S., Ripper, J. L., Fifis, T., McCormick, B. S.,
431 Francis, B., Melville, L., Small, K., de Witte, K., 1992. A field evaluation of
432 serological and cellular diagnostic tests for bovine tuberculosis. *Vet. Microbiol.*
433 31, 71-79.

Table 1. Culture results of *Mycobacterium caprae* and *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) of the animals included in this study (n=163)*

Culture results	<i>Map</i> +	<i>Map</i> -	Total
<i>M. caprae</i> +	24	107	131
<i>M. caprae</i> -	6	26	32
Total	30	133	163

* After the exclusion of 14 animals with lesions compatible with tuberculosis but negative culture of *M. caprae*.

435

Table 2. Sensitivity of the diagnostic tests performed in the flock (n=163)

Diagnostic test	Positive reactors	True positives^a	S (95% CI)
SIT test (severe interpretation)	118	93	71 (62.4-78.6)
SICCT test (severe interpretation)	69	56	42.7 (34.1-51.7)
Standard IFN- γ detection test (cut-off point=0.1)	86	76	58 (49.1-66.6)
Standard IFN- γ detection test (no avian PPD) ^b	90	79	60.3 (51.4-68.7)
Severe IFN- γ detection test (cut-off point=0.05)	109	93	71 (62.4-78.6)
Severe IFN- γ detection test (no avian PPD) ^b	114	96	73.3 (64.9-80.6)

436 ^a True Positive: positive reactor animals with *M. caprae* infection confirmed by culture.

437 ^b Results obtained when the results of the stimulation with avian PPD are ignored.

438

Table 3. Sensitivity obtained combining the diagnostic tests performed in the flock

(n=163)

Diagnostic tests in parallel	Positive reactors	True positives^a	S (95% CI)
Severe SIT + Standard IFN- γ	142	115	87.8 (80.9-92.9)
Severe SIT + Severe IFN- γ	149	119	90.8 (84.5-95.2)
Severe SICCT + Standard IFN- γ	118	99	75.6 (67.3-82.7)
Severe SICCT + Severe IFN- γ	131	108	82.4 (76.5-89.8)

439 ^a True Positive: animals with a *M. caprae* infection confirmed by culture.

440

Table 4. Agreement analysis between SIT test and IFN- γ assay in the tuberculosis-culture positive animals (n=131)

	IFN-γ positive	IFN-γ negative
SIT positive	67	26
SIT negative	26	12

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Table 5. Sensitivities with confidence intervals (95%) of the tests in the groups formed based on culture results

Diagnostic test	Tbc+ ^a (n=131)	Tbc+/Ptbc+ ^b (n=24)
Severe SIT	71 (62.4-78.6)	54.2 (33-74)
Severe SICCT	42.7 (34.1-51.7)	29.2 (13-51)
Standard IFN- γ detection test (0.1)	58 (49.1-66.6)	58.3 (37-78)
Severe IFN- γ detection test (0.05)	71 (62.4-78.6)	66.7 (45-84)
Severe SIT + Standard IFN- γ	87.8 (80.9-92.9)	75 (53-90)
Severe SIT + Severe IFN- γ	90.8 (84.5-95.2)	79.2 (58-93)
Severe SICCT + Standard IFN- γ	75.6 (67.3-82.7)	70.8 (49-87)
Severe SICCT + Severe IFN- γ	82.4 (76.5-89.8)	75 (53-90)

442 ^a Animals with *M. caprae* positive cultures, regardless *Map* culture results.

443 ^b Animals with positive cultures of both *M. caprae* and *Map*.

444 Fig. 1. Culture positive animals for tuberculosis (n=131) represented based on their
445 reaction to the skin tests and IFN- γ results.

446 The continuous line represents the threshold of the SICCT test (animals over this line
447 are those detected by SICCT test). The dashed line represents the threshold of the SIT
448 test (animals over this line are those detected by SIT test).

X: Difference in mm. after the inoculation of avian PPD

449 Y: Difference in mm. after the inoculation of bovine PPD

450 ○ Animals with positive values in the IFN- γ assay

451 ▲ Animals with negative values in the IFN- γ assay

452 Fig. 2. Culture positive animals with for tuberculosis (n=131) represented based on their
453 reaction to the skin tests, and regression lines of positive (dot-dashed line; $y=0.69x+2.2$)
454 and negative (continuous line; $y=0.64x+1.9$) animals in the IFN- γ assay.

455 The dashed line represents the threshold of the SIT test (2 mm.).

456 X: Difference in mm. after the inoculation of avian PPD

457 Y: Difference in mm. after the inoculation of bovine PPD

458 ○ Animals with positive values in the IFN- γ assay

459 ▲ Animals with negative values in the IFN- γ assay



