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1 **Effect of paratuberculosis on the diagnosis of bovine tuberculosis in a cattle herd**  
2 **with a mixed infection using interferon-gamma detection assay**

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17 **Abstract**

18 Interferon-gamma (IFN- $\gamma$ ) detection assay is being applied as an ancillary test to  
19 tuberculin tests in the diagnosis of bovine tuberculosis to detect the maximum number  
20 of infected animals. Among possible factors influencing the performance of  
21 tuberculosis-diagnostic tests, paratuberculosis, a widespread disease in Spain and other  
22 European countries, has been pointed out as a cause of false positive reactions. Still, its  
23 effect on the sensitivity of these tests in cattle has yet to be fully characterized. The  
24 impact of paratuberculosis in the apparent sensitivity of IFN- $\gamma$  assay was studied in a  
25 bullfighting cattle herd with a mixed tuberculosis-paratuberculosis infection, using  
26 culture of *Mycobacterium bovis* and *Mycobacterium avium paratuberculosis* as the gold  
27 standard to determine the infection status of every animal. A total of 218 animals were  
28 slaughtered and sampled for bacteriology after blood sampling. IFN- $\gamma$  assay showed a  
29 lower apparent sensitivity in animals with a mixed infection (50%) compared to all  
30 animals suffering tuberculosis (78.3%). This finding indicates that the presence of  
31 paratuberculosis in tuberculosis-infected herds could imply a serious impairment in the  
32 sensitivity of IFN- $\gamma$  detection test.

33 **Keywords:** tuberculosis; paratuberculosis; cattle; IFN- $\gamma$ ; diagnosis.

## 34 1. Introduction

35 Bovine tuberculosis is a zoonotic disease affecting cattle worldwide, causing  
36 economic losses due to eradication programs and trade limitations (Pollock and Neill,  
37 2002). In spite of great efforts the Officially Tuberculosis Free status has not yet been  
38 achieved in some countries throughout Europe. This lack of success has been attributed,  
39 among other causes, to the insufficient sensitivity of the diagnostic tests under certain  
40 field conditions (Monaghan et al., 1994; Morrison et al., 2000).

41 In Spain prevalence of bovine tuberculosis has followed a decreasing trend from  
42 11.14% (herd level) and 4.2% (individual level) in 1986 (when the national eradication  
43 program was implemented) to 1.76% and 0.42% in 2006 (Anon., 2008). At the moment,  
44 detection and removal of the maximum number of *M. bovis* infected animals is the  
45 crucial step. Therefore, the increase of the sensitivity of diagnostic tests is a key  
46 objective. For this reason the interferon- $\gamma$  (IFN- $\gamma$ ) detection assay was introduced in the  
47 EU legislation (Council Directive 64/432/EEC, amended by (EC) No. 1226/2002 of 8  
48 July 2002, Anon. 2002) as an ancillary test and adopted by the Spanish program (Anon.,  
49 2008). The conditions for the optimal use of this technique, already demonstrated in  
50 several countries (Wood et al., 1990; Gormley et al., 2006), must be determined for the  
51 different epidemiological situations in Spain.

52 To our knowledge the sensitivity of the IFN- $\gamma$  test in cattle naturally co-infected  
53 by *M. bovis* and *Mycobacterium avium paratuberculosis* (*Map*) has not been studied  
54 under field conditions. A decreased sensitivity was reported by Amadori et al. (2002)  
55 and Hope et al. (2005) in cattle previously infected with *M. avium* under experimental  
56 conditions, but this might not reflect the field situation of a naturally mixed infection  
57 with a *M. tuberculosis* complex member and *Map*. Aranaz et al. found a decreased  
58 sensitivity in tuberculosis diagnostic tests in a cattle herd with a dual infection, but the

59 number of animals with positive culture was low, and selection of slaughtered animals  
60 could have produced a biased higher sensitivity (Aranaz et al., 2006).

61 The current study was designed to determine the possible effect of natural co-  
62 infection by *M. bovis* and *Map* on the performance of IFN- $\gamma$  detection assay for the  
63 diagnosis of tuberculosis in a bullfighting cattle herd.

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## 64 2. Materials and methods

### 65 2.1. Herd and design of the study

66 The study was performed in a bullfighting cattle herd with a natural mixed  
67 tuberculosis-paratuberculosis infection. The whole herd (n=680) was subjected to  
68 analysis by single intradermal comparative cervical tuberculin (SICCT) test, IFN- $\gamma$   
69 detection assay and an ELISA for the detection of antibodies against *Map*. All animals  
70 sent to the slaughterhouse (n=218) because of positive results in any test or for  
71 zootechnical reasons were sampled to perform bacteriology.

### 72 2.2. Diagnostic methods

#### 73 2.2.1. SICCT test

74 The SICCT test was conducted by the Official Veterinary Services according to  
75 European and national legislation (EU Council Directive 64/432/CEE and RD  
76 2611/1996) after blood sampling for IFN- $\gamma$  detection using bovine and avian PPDs (CZ  
77 Veterinaria, Porriño, Spain).

#### 78 2.2.2. IFN- $\gamma$ detection test

79 Heparinized blood samples were collected from every animal and delivered to  
80 the laboratory within eight hours of collection at room temperature. Stimulation with  
81 avian and bovine PPDs was carried out as previously described (Wood et al., 1990;  
82 Liebana et al., 1998), and plasma samples were analysed in duplicate using a sandwich  
83 EIA for detection of bovine IFN- $\gamma$  (Bovigam™ Bovine Gamma Interferon Test,  
84 Prionics, Schlieren, Switzerland). Results were interpreted as previously described  
85 (Aranaz et al., 2006). Animals were considered positive if the mean optical density  
86 (OD) of a sample stimulated with bovine PPD minus the mean OD of nil antigen was  
87 greater than 0.05 and greater than the same value of the sample stimulated with avian  
88 PPD (interpretation prescribed in the Spanish eradication program).

### 89 2.2.3. Serology of paratuberculosis

90 Plasma samples were tested in duplicate using the absorbed ELISA kit  
91 Parachek™ (Prionics, Schlieren, Switzerland) according to the manufacturer's  
92 instructions. Animals were considered positive when its mean OD minus the plate  
93 negative control was greater than 0.100 as recommended by the manufacturer.

## 94 **2.3. Bacteriology**

### 95 2.3.1. Tissue collection

96 Animals were slaughtered at the abattoir, and samples were taken at the post-  
97 mortem inspection for culture of *M. bovis* (retropharyngeal, mediastinal and bronchial  
98 lymph nodes, and lung) and *Map* (mesenteric and ileocecal lymph nodes and ileocecal  
99 valve). Samples were stored at -20°C until further analysis.

### 100 2.3.2. *M. bovis* culture and characterization

101 Tuberculosis compatible lesions were selected when present; otherwise, a pool  
102 of tissues was used. Culture of samples was performed after decontamination with  
103 0.37% hexadecylpyridinium chloride (Corner and Trajstman, 1988) as described  
104 elsewhere (Liebana et al., 1998). All isolates were identified by PCR (Wilton and  
105 Cousins, 1992) and characterised using spoligotyping (Kamerbeek et al., 1997).

### 106 2.3.3. *Map* culture and characterization

107 Tissue samples of each animal were pooled and processed as described before  
108 (Greig et al., 1997) and inoculated onto four selective culture media (de Juan et al.,  
109 2006). Isolates were identified as *Map* based on their mycobactin- dependency and  
110 presence of specific elements IS900 and f57 (Coetsier et al., 2000). The type of strain  
111 was determined using the PCRs described by Collins et al. (2002) and by Castellanos et  
112 al. (2007).

## 113 **2.4. Evaluation of diagnostic test**



114           The infection status of each animal was determined using their culture results as  
115   the gold standard. Results from animals with a mixed infection (both *M. bovis* and *Map*  
116   were recovered from their tissue samples) were then compared with all animals infected  
117   by *M. bovis* to evaluate the apparent sensitivity of the IFN- $\gamma$  assay, and with all animals  
118   infected by *Map* for the assessment of the apparent sensitivity of the Parachek ELISA.

119           Statistical analysis was performed by means of the software PEPI 4.0 (Computer  
120   Program for Epidemiologist), J.H. Abramson and P.M. Gahlinger.

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### 121 3. Results

#### 122 3.1. Bacteriology results

123 A total of 218 animals were slaughtered and sampled for bacteriology during the  
124 study. Ninety-eight were culled based on their positive results in the diagnostic tests for  
125 tuberculosis: six animals were positive to the SICCT test, 81 to the IFN- $\gamma$  assay and 11  
126 to both techniques. Eighty-two other animals were slaughtered as they had positive  
127 values in the serology of paratuberculosis. The remaining 38 animals were slaughtered  
128 for zootechnical reasons (inadequate temper, poor reproductive rate or lameness). *M.*  
129 *bovis* and *Map* were isolated from 46 and 111 animals respectively (Table 1). Two of  
130 the animals negative to all tests showed lesions compatible with tuberculosis at the  
131 abattoir inspection, and both had subsequently *M. bovis* and *Map* positive-culture.

132 The 46 *M. bovis* isolates belonged to six spoligotypes, from which two were  
133 much more prevalent: SB0298 (n=29), SB0339 (n=12), SB0121 (n=2), and SB0120,  
134 SB1313 and SB1321 (n=1). Regarding the *Map* isolates, 80 (72.1%) belonged to the  
135 type II and 31 (27.9%) were type III.

#### 136 3.2. Impact of infection status in tuberculosis diagnosis

137 Animals were classified based on their culture results according to the criteria  
138 detailed above. Proportion of positive animals and apparent sensitivity were determined  
139 and compared in each group. Compulsory skin test was performed according to the  
140 eradication program; however the results are not analyzed in detail in this study because  
141 the dangerous handling of this bullfighting herd may have impaired the carrying out of  
142 the test, therefore making difficult the interpretation of results.

143 Regarding IFN- $\gamma$  assay, a low apparent sensitivity of 78.3% (36 out of the 46  
144 animals with *M. bovis*-positive culture) was observed, though a detailed analysis of the  
145 results revealed it could be mostly attributed to the animals with a mixed infection

146 (Table 2). In fact, among these animals only eight of the 16 (50%) were positive  
147 reactors in the IFN- $\gamma$  assay (Table 2), although when their avian PPD readings were  
148 ignored the number of detected animals raised to 11 (68.8%) (Table 3). All of the false  
149 negative reactors to the IFN- $\gamma$  test in this group (n=8) were also negative in the SICCT  
150 test. Remarkably, six out of these eight negative animals were positive in the Parachek  
151 ELISA, while most IFN- $\gamma$  reactors (six out of eight) showed negative results in that  
152 ELISA (Table 3). None of these IFN- $\gamma$  negative animals presented disseminated  
153 tuberculous lesions which could indicate anergy due to advanced progression of  
154 infection.

### 155 **3.3. Impact of infectious status in paratuberculosis diagnosis.**

156 The ELISA for detection of *Map* antibodies detected a lower proportion of *Map*  
157 infected animals in the group with a mixed infection (eight out of 16, 50%) compared to  
158 all *Map* infected animals (81 out of 111, 73%) (Table 2).

159 No association was observed between the type of *Map* strain infecting an animal  
160 and the probability of being detected by any method.

161 **4. Discussion**

162 In spite of investing great efforts in the fight against tuberculosis, achievement  
163 of complete eradication has been inconsistent in certain regions or countries (Neill and  
164 Pollock, 2000). Wildlife reservoirs (Corner, 2006) and dissemination due to movement  
165 of infected animals (Johnston et al., 2005; Collins, 2006) have been pointed out as  
166 possible causes of this failure. Other major factors hampering eradication programs are  
167 limitations of routine diagnostic tests (mainly skin tests), in particular concerning their  
168 sensitivity (Monaghan et al., 1994; Morrison et al., 2000). Diagnostic accuracy is a key  
169 issue in the test-and-slaughter programs applied in Spain and many other countries,  
170 especially at the present low-prevalence stage, when detection of all infected animals is  
171 crucial (Pollock et al., 2001).

172 In the present study the proportion of *M. bovis* infected animals detected by the  
173 IFN- $\gamma$  assay seems to be greatly affected by the presence of a concomitant *Map*  
174 infection; the apparent sensitivity of the test in this herd was 78.3%, but in the group of  
175 animals with a mixed infection it dropped to 50%. The impact of paratuberculosis  
176 compromising the specificity of tuberculosis diagnostic tests has been demonstrated  
177 (Cagiola et al., 2004; Dunn et al., 2005); however, its effect on the sensitivity in cattle  
178 had not been determined in natural conditions. Nevertheless, paratuberculosis could be  
179 of great importance, as it is widespread in certain areas of the European Union (Nielsen  
180 and Toft, 2007) and other parts of the world (Kennedy and Benedictus, 2001). A  
181 statistically significant relationship (C.I. 95%) was found between the recovery of *Map*  
182 from tissue samples and response to the IFN- $\gamma$  test in *M. bovis* infected animals. While  
183 93.3% (28 out of 30) of animals with *Map* negative culture results did respond to the  
184 assay, only 50% of cattle yielding *Map* by culture was detected. Furthermore, among  
185 the ten false negative reactors found in the whole herd, eight of them (80%) were

186 suffering a concomitant *Map* infection.

187         These results corroborate the findings of a previous report in a goat flock  
188 (Alvarez et al., 2007), where paratuberculosis seemed to have a major effect on the  
189 sensitivity of tuberculosis diagnostic tests, especially on skin test, as the IFN- $\gamma$  assay  
190 was less affected. On the other hand, in the present study the ability of IFN- $\gamma$  test to  
191 detect tuberculosis-infected animals is more influenced by paratuberculosis; these  
192 differences could be due to the host species or the strains present in each herd.

193         In the group of cattle with dual infection, 50% (8/16) of the animals were false  
194 negative reactors in the IFN- $\gamma$  assay, although this apparent sensitivity increases to  
195 68.8% if interpretation is performed overlooking values obtained with avian PPD (Table  
196 3). Further studies would be needed in order to disclose the advisability of adapting  
197 interpretation criteria of the IFN- $\gamma$  assay under these circumstances.

198         Interestingly, 75% of these dually infected animals with no response in the IFN-  
199  $\gamma$  assay were positive in the serology of *Map*, in opposition to what happened to most  
200 animals positive in the IFN- $\gamma$  test in the same group (that were seronegative for *Map*)  
201 (Table 3). The negative association between results in IFN- $\gamma$  assay and serology of *Map*  
202 in animals with a mixed infection was also observed in the previous report in goats  
203 (Alvarez et al., 2007) as from eight false negative reactors to the IFN- $\gamma$  assay found in  
204 that study, seven (87.5%) had high titers of antibodies against *Map* (unpublished data).

205         Regarding the ELISA for detection of antibodies against *Map*, it is remarkable  
206 that a lower apparent sensitivity was observed in the group of animals with a mixed  
207 infection compared to the whole group of animals infected with *Map*. However, this fact  
208 would have no consequences under field conditions because animals would be culled  
209 due to positive response to the tuberculosis diagnostic tests.

210         In conclusion, our results would suggest that both *M. bovis* and *Map* influence

211 the overall immune response developed in the dually-infected animals, and may  
212 compromise the reliability of diagnostic tests that are based in the measurement of  
213 specific response generated against each pathogen. Differences in the proportion of *M.*  
214 *bovis*-infected animals reacting to the IFN- $\gamma$  assay were observed depending on their  
215 paratuberculosis status: animals with dual infection were more likely to produce false  
216 negative reactions to this test. This fact highlights the importance of ascertaining the  
217 paratuberculosis status of a herd when implementing a strategic use for the IFN- $\gamma$  assay,  
218 not only because its effect on the specificity but also for its impact on the sensitivity.  
219 More studies should be performed with larger numbers of animals representing the  
220 actual bovine population in order to confirm this finding.

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326

Table 1.

*Mycobacterium bovis* and *Mycobacterium avium* subsp. *paratuberculosis* culture results of slaughtered animals (n=218)

Culture results	<i>Map</i> +	<i>Map</i> -	Total
<i>M. bovis</i> +	16	30	46
<i>M. bovis</i> -	95	77	172
Total	111	107	218

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Table 2.

Apparent sensitivities with confidence intervals (95%) of the IFN- $\gamma$  detection test and the Parachek<sup>TM</sup> ELISA in the groups formed based on culture results

<b>Diagnostic test</b>	<b>Tbc+ animals (n=46)<sup>a</sup></b>	<b>Ptb+ animals (n=111)<sup>b</sup></b>	<b>Tbc+/Ptb+ (n=16)<sup>c</sup></b>
IFN- $\gamma$ detection test	78.3 (63.6-89)	-	50 (24.6-75.3)
Parachek ELISA	-	73 (63.7-81)	50 (24.6-75.3)

328 <sup>a</sup> Animals with *M. bovis* positive isolation (regardless their *Map* culture results).

329 <sup>b</sup> Animals with *Map* infection (regardless their *M. bovis* culture results).

330 <sup>c</sup> Animals with positive cultures of both *M. bovis* and *Map*.

331

Table 3.

Results of IFN- $\gamma$  assay and Parachek ELISA in animals suffering a tuberculosis-paratuberculosis dual infection (n=16)

Animal	Bovine IFN- $\gamma$ <sup>a</sup>	Avian IFN- $\gamma$ <sup>b</sup>	Parachek ELISA <sup>c</sup>
1	<b>0.635</b>	0.132	-0.012
2	<b>0.529</b>	0.080	0.008
3	<b>0.314</b>	0.263	-0.025
4	<b>0.212</b>	0.069	-0.025
5	<b>0.161</b>	0.044	<b>0.384</b>
6	<b>0.157</b>	0.036	-0.020
7	<b>0.115</b>	0.036	-0.026
8	<b>0.051</b>	0.016	<b>0.126</b>
9	0.035	0.077	<b>1.048</b>
10	0.136	0.139	<b>0.883</b>
11	0.239	0.888	<b>0.400</b>
12	0.070	0.141	<b>0.242</b>
13	0.007	0.005	<b>0.213</b>
14	0.007	0.017	<b>0.210</b>
15	0.018	0.024	0.013
16	0.017	0.002	-0.007

332 Optical densities above the cut-off point (positive animals) are presented in bold letters.

333 <sup>a</sup> Results after the in-vitro stimulation with bovine PPD minus the value obtained after  
334 stimulation with PBS.

335 <sup>b</sup> Results after the in-vitro stimulation with avian PPD minus the value obtained after  
336 stimulation with PBS.

337 <sup>c</sup> Results in the ELISA for detection of *Map* antibodies.