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J. Hermoso de Mendoza

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1 **“Development of a molecular diagnostic test applied to experimental abattoir surveillance**  
2 **on bovine tuberculosis”.**

3  
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24 **Abstract**

25 One of the most essential systems applied to the eradication of bovine tuberculosis by  
26 *Mycobacterium bovis* is the epidemiologic surveillance of animals slaughtered in abattoir by  
27 means of inspection and sample taking of lesions compatible with tuberculosis, confirming the  
28 existence of the disease through culture and molecular detection, which takes weeks before a  
29 result can be obtained. An interesting alternative is to develop high-throughput molecular  
30 systems for the direct detection of *M. bovis* on biological samples. In this sense, our research  
31 has developed a molecular detection system by means of a real time based PCR process which  
32 is applied directly to bovine biological samples and it allows to differentiate between  
33 *Mycobacterium tuberculosis* Complex, *Mycobacterium avium* Complex and other atypical  
34 mycobacteria that are interesting from the veterinary point of view. The sensitivity was analyzed  
35 by applying a conventional extraction system based on guanidine thiocyanate and a robotized  
36 system based on the selective magnetic capture of mycobacterial DNA. The molecular detection  
37 system showed a high specificity and a detection threshold of only 2-3 genomes. The sensitivity  
38 depended on the DNA extraction system being used and on the kind of lesions on which it was  
39 used; the sensitivity ranged from 61.11% for samples with non visible lesions to 80.64% for  
40 chronic lesions, with an average sensitivity of 73.87% when using the manual extraction system  
41 and between 27.77 and 74.19% (average sensitivity 47.74%) when using the automated robotic  
42 system. In conclusion, our multiplex real time PCR assay represents a fully controlled, high-  
43 throughput diagnostic tool for the rapid detection of *Myobacterium* presence directly in animal  
44 clinical specimens, which could be a practical tool in the context of bovine tuberculosis abattoir  
45 surveillance programs and granuloma submission programs.

46

47 **Key words:** bovine tuberculosis, molecular diagnostic, epidemiologic surveillance.

48        **Introduction**

49    Bovine tuberculosis (caused by *Mycobacterium bovis*) is still an infectious disease that causes  
50    substantial damages in the agricultural sector in many developed countries; it is nowadays  
51    subjected to expensive eradication programmes in most EU countries, Spain among them  
52    (Reviriego Gordejo and Vermeersch, 2006). The development process of the eradication  
53    programmes usually shows a tendency to establish epidemiologic surveillance systems in  
54    abattoirs, where not only the sanitary inspection of all the animals slaughtered for consumption  
55    purposes is carried out but also, the systematic collection of granulomas or lesions which are  
56    compatible with tuberculosis for their subsequent culture, disease confirmation and  
57    epidemiological trace back of the outbreak, which usually ends in the total culling of the herd  
58    that have tested positive by means of the agent isolation (Evans, 1972; Kaneene et al., 2006;  
59    More and Good, 2006; Radunz, 2006). In Spain, the development of the eradication programme  
60    is allowing a progressive decreasing of the prevalence, (0.30% of prevalence in animals in the  
61    year 2005. Source: RASVE-MAPA <http://rasve.mapa.es/>) in such a way that it is advisable to  
62    start the abattoir epidemiological surveillance systems, which were initially developed in  
63    parallel with the field eradication system, by means of diagnosis and slaughtering, and they will  
64    be possibly the main tool for the eradication system in the future. Under these circumstances, it  
65    is necessary to develop diagnostic systems which are directly applied to biological samples and  
66    enable a high level of achievement and good levels of sensitivity and specificity, allowing a  
67    quick diagnosis in the laboratory by using the samples taken in the abattoir.

68    In this sense, it is worthwhile pointing out the molecular diagnostic systems, especially those  
69    ones based on the real time PCR technology (Q-PCR), due to the fact that they are faster and  
70    show more automation possibilities. So, although a lot of real time molecular detection systems,  
71    which are directly applied to biological samples, have been recently developed in order to carry  
72    out human tuberculosis diagnosis (caused by *M. tuberculosis*) (Broccolo et al., 2003; de Kantor  
73    and Portuondo, 1974; Desjardin et al., 1998; Eishi et al., 2002; Heginbothom et al., 2003;  
74    Kramme et al., 2003; Kraus et al., 2001; Lachnik et al., 2002; Miller et al., 2002; Shrestha et al.,  
75    2003). The development of procedures for *M. bovis* has been very limited and there has existed

76 just one significant research made on bovine tissue samples (Taylor et al., 2001). The main  
77 difference between the systems developed for the human and bovine species is not based on the  
78 kind of genetic marker, but on the extraction system to be used, due to the difficulty in  
79 extracting mycobacterial DNA in bovine samples, as they have few bacteria and because of the  
80 structure of the biological sample itself, which shows strong fibrosis and calcification that  
81 hamper the access to the DNA to be detected (Liebana et al., 1995). The result is a limited  
82 sensitivity, which varies between 70-75% (Bollo et al., 1998; Liebana et al., 1995; Taylor et al.,  
83 2001) and is lower than that of the culture (Taylor et al., 2001). In order to solve this problem,  
84 mycobacterial DNA selective capture systems have been developed (SC) (Brugiere et al., 1997;  
85 Mangiapan et al., 1996; Roring et al., 1998; Roring et al., 2000; Taylor et al., 2001); these  
86 systems are liable to be automated in robotic platforms. In this way, as well as developing a  
87 molecular detection system, we also evaluate that procedure (SC) by comparing it to the  
88 conventional DNA extraction systems by means of organic solvents and denaturing agents.

89

## 90 **2. Material and Methods**

### 91 *2.1 Bacterial strains*

92 The mycobacterial strains used for sensitivity and specificity testing and for optimization of the  
93 real time PCR are listed in Table 1. The strains were Institute Pasteur Collection (CIP) strains.  
94 All mycobacterial strains were cultured in liquid Dubos medium. Additionally, 7 strains  
95 belonging to 7 different species were also used in specificity tests. Some of these species are  
96 established pathogens causing lymphadenitis, and other ones are normal inhabitants of the  
97 bovine oropharynx, as the species belonged to the genera *Actinobacillus*, *Actinomyces*,  
98 *Arcanobacterium*, *Bordetella*, *Corynebacterium*, *Haemophilus* and *Streptococcus*. The  
99 identification of the reference strains was carried out by molecular means (Cousins et al., 1991;  
100 Liebana et al., 1996).

101 *2.2. Biological material*

102 Retropharyngeal and mediastinal lymph nodes were collected from 125 cattle. These cattle were  
103 either intradermal skin test positive and identified as having TB like-lesions (confirmed by  
104 Ziehl-Neelsen staining process and microscopic examination), during routine meat inspection,  
105 or without lesions (NVL). The samples were divided into similar portions, keeping one of them  
106 at -80°C, in case the tests were to be repeated. The Hexadecylpyridinium Chloride method  
107 (Corner, 1988) was used as a decontamination procedure, showing in parallel and in duplicate  
108 two Lowenstein-Jensen test tubes with pyruvate and two Lowenstein-Jensen test tubes with  
109 glycerine (Pronadisa, Spain). Residual decontaminated tissue was stored at -20°C for sequence  
110 capture. The identification of the *Mycobacterium tuberculosis complex*, isolated from culture,  
111 was carried out by molecular means (Cousins et al., 1991; Liebana et al., 1996). Each sample  
112 was identified in duplicate, including negative controls (just water) and positive ones (*M. bovis*  
113 CIP 105050).

114

115 *2.3 DNA extractions from culture*

116 A loopful, taken from a Lowenstein–Jensen pure culture, grown with pyruvate and positively  
117 identified as belonging to the *Mycobacterium tuberculosis complex*, was transferred to a  
118 microcentrifugal vial containing 100 µl 10 mM Tris–HCl/1 mM EDTA and incubated for 20  
119 min at a temperature of 99°C. After centrifugation, the supernatant was stored at -20°C until  
120 used. Serial dilutions of *M. bovis* CIP 105050 genomic DNA (1 µg/ml to 1 femtograms/ml)  
121 were used as positive controls.

122

123 *2.4. DNA extractions from bovine tissue*

124 *i. Sequence capture:* DNA sequence capture PCR, as previously described (Taylor et al., 2001),  
125 was applied to homogenates of 125 decontaminated bovine tissues with the following additional  
126 modifications. In brief, suspensions of decontaminated tissue homogenate (500 µl) were  
127 transferred to a screw-cap microcentrifuge tube containing 500 µl of 0.1-mm-diameter glass  
128 beads (Cell Disruption Media, 0.1 mm. Scientific Industries Inc.). The pellet and beads were

129 suspended in 500  $\mu$ l of lysis buffer (0.01 M Tris-HCl (pH 7.8), 0.01 M EDTA and 0.5% SDS).  
130 Samples were agitated in a FastPrep 101 bead shaker at 6m/s for 1 min. Proteinase K (Takara  
131 Bio Inc. Shiga, Japan) was added to a final concentration of 20 mg/ml and the mixture was  
132 incubated at 55°C over-night in a ThermoMix Confort (Eppendorf, Hamburg, Germany) under  
133 constant agitation at 550 r.p.m. The samples were shaken in the FastPrep as before. Following a  
134 brief centrifugation, the resulting supernatant was subjected to an automated protocol in an Evo  
135 Freedom 150 (Tecan, Maennedorf, Switzerland) liquid handling robot.  
136 The EVO Freedom 150 was equipped with 8 disposable tip adapter, the Te-MagS™ instrument  
137 (Tecan, Maennedorf, Switzerland) (48 tubes of 1.5 ml format), carrier for primary 2 ml sample  
138 tubes, for reagents, for disposable tips and PCR microplates positioned on the work table. A  
139 robotic manipulator arm (RoMa) transports the microplates from the pipeting positions to the  
140 Te-MagS™ instrument. The magnetic separation module is controlled from the Tecan Gemini  
141 Software (Tecan, Maennedorf, Switzerland) by serial interface.  
142 500  $\mu$ l were taken from the supernatant of each sample and they were subjected to a 99° C  
143 denaturing process for 15 minutes using the Te-MgS™ instrument. Afterwards, the capture  
144 biotinized oligonucleotides were added at room temperature (Ec16S.1390 y Mb23S.44n) (2.5  
145 pm of each one) in 5 M of NaCl. The homogenate was shaken in the Te-MgS™, allowing in  
146 this way the hybridization of the capture probes for 2 hours at 65°C under constant agitation.  
147 After the hybridization process 10  $\mu$ l of Streptavidin Magnetic Particles (Roche Diagnostics,  
148 Penzber, Germany), dissolved in binding buffer (Binding Buffer: 10 mM Tris-ClH, 1mM EDTA  
149 in 100 mM NaCl pH 7.5) and they were incubated for 3 hours, allowing in this way the  
150 instrument Te-MagS to carry out the movement of the particles inside the screw-cap tube. The  
151 mangnetic beads joined to the binding made up of the capture probe and the bacterial DNA were  
152 washed twice using the mangnetic separator of the Te-MagS in 750  $\mu$ l of wash-buffer (Wash-  
153 buffer TEN1000: 10mM Tris-ClH, (pH 7.5) 1mM EDTA, 1M NaCl,) and then in sterile  
154 deionized H<sub>2</sub>O.  
155 Magnetic beads were resuspended in 25 ml of sterile deionized H<sub>2</sub>O and stored at -20°C prior to  
156 PCR amplification. Immediately prior to amplification, captured mycobacterial DNA was

157 released from magnetic beads by heat treatment at 99°C for 5 min. Following a brief  
158 centrifugation, the resulting supernatant was subjected to the appropriate amplification in the  
159 Real Time PCR system.

160

161 *ii. Guanidinium thiocyanate DNA purification.* For the extraction of DNA directly from bovine  
162 tissue biological samples, the reagent Tri-Reagent was used (Molecular Research Centre,  
163 Cincinnati, USA), based on the Guanidinium thiocyanate action, according to the instructions of  
164 the manufacturer and in the way described in other studies (Brisson-Noel et al., 1991;  
165 Chakravorty and Tyagi, 2001). This extraction was carried out in parallel, both on the same  
166 samples described before and on 500 µl of decontaminated tissue homogenate. On both kinds of  
167 extraction negative (just water) and positive extraction controls were included; these controls  
168 consisted of DNA alicutas (10ng/ml) of the *M. bovis* CIP 105050 strain.

169

170 *2.4 Primers, biotinylated capture oligonucleotides and probe design.*

171 The objective was to develop three independent tests for a multiple and simultaneous detection  
172 of: 1<sup>st</sup>, the organisms which belong to the *Mycobacterium tuberculosis* (CMTB) Complex; 2<sup>nd</sup>,  
173 the organisms which belong to the *Mycobacterium avium* (CMA) Complex (including *M. avium*  
174 *subsp. paratuberculosis*) and 3<sup>rd</sup>, the most number of species belonging to the *Mycobacterium*  
175 genus, except those ones belonging to CMTB and CMA which will be named NTM (non  
176 tuberculosis Mycobacteria).

177 Primers and TaqMan probes were selected based on alignments of the ITS region (16S-23S  
178 rDNA internal transcribed spacer (Roth et al., 1998)) with sequences from the National Centre  
179 for Biotechnology Information (NCBI) database. DNA sequences alignments were performed  
180 using the ClustalX program (<http://www.ebi.ac.uk/clustalw/>, European Bioinformatic Institute)  
181 and were made to investigate interspecies and intraspecies variations. The alignments include  
182 multiple stains of all species of the *Mycobacterium tuberculosis* Complex, the *Mycobacterium*  
183 *avium* Complex and several species of atypical mycobacteria such as *Mycobacterium*  
184 *massiliense*, *Mycobacterium abscessus*, *Mycobacterium vaccae*, *Mycobacterium fortuitum*,

185 *Mycobacterium gordonae*, *Mycobacterium smegmatis*, *Mycobacterium kansasii* and  
186 *Mycobacterium chelonae*. An NCBI BLAST search was performed to check the specificity of  
187 the DNA sequences of primers and probes. Primers and probes were designed with the help of  
188 the Primer Express software (Applied Biosystems, Foster City, USA) and were checked with  
189 Oligo-Analyzer 3.0 (<http://biotools.idtdna.com/analyzer>), an online service of IDT Biotools  
190 (Coralville, Iowa), to ensure minimal selfcomplementarity and to prevent the presence of  
191 secondary structures. The sequence and properties of the oligonucleotides used are shown in  
192 Table 2. The complex-specific identification and primer and probe sets (detectors) were devised  
193 to simplify the assay. Thus, the three detectors share the UNIMYC-319R reverse primer. The  
194 primers and probes were selected after alignment of the micobacterial ITS sequence  
195 encompassing the 3' end of the 16s rDNA and the 5' end of the 23s rDNA. Specific  
196 amplification was obtained by using the primers and probe combinations showed in Table 2.  
197 Primers and probes were synthesized at Applied Biosystems (Cheshire, UK).  
198 The biotinylated capture oligonucleotides Ec16S.1390 (3' Biotin-  
199 aaaaaTTGTACACACCGCCCGTCA 5') and Mb23S.44n (3' Biotin-  
200 aaaaaTCTCGATGCCAAGGCATCCACC 5') (Roth et al., 1998) located in positions 156.664  
201 to 156.682 (5' end of the 16s gene) and 157.137 to 157.116 (3' start of the 23s gene) on the  
202 genome sequence of *M. bovis* AF2122/97, respectively, were used to capture de ITS region of  
203 the *Mycobacterium* genus and were synthesized at Eurogentec (Seraing, Belgium).

204

### 205 *2.5 Real-time PCR analysis*

206 DNA detection was performed by amplification using the real-time 7.500 Sequence Detection  
207 System (Applied Biosystems, Foster City, USA). Reactions were carried out in duplicate in 96-  
208 well optical plates. The 25 µl reaction mixtures contained 12.5 µl of TaqMan Master Mix  
209 (which includes the heat-activated AmpliTaq Gold enzyme, Applied Biosystems, Foster City,  
210 USA), 300 nM of each specific primer and 300 nM of each MGB probe, and 3 µl of DNA.  
211 All the reactions included an internal positive control (IPC) as the inhibition detection system  
212 commercialized by Applied Biosystems (*TaqMan*® *Exogenous Internal Positive Control*

213 *Reagent, PN 4304437*). In the same way, 2 non-amplification negative controls (No  
214 amplification Control, NAC, with no DNA problems and with DNA exogenous of the cutoff  
215 IPC), 2 negative controls with no DNA problems and two positive controls (including 1ng of  
216 the *M. Bovis* CIP 105050 strain) were used.

217 The reaction conditions were as follows: 10 min at 95°C for enzyme activation and 40 cycles of  
218 15s at 95°C and 1 min at 55°C. Readings were automatically taken by software every cycle, and  
219 the logarithmic of the increment in fluorescence was plotted versus the cycle number. The  
220 threshold level was fixed at the same midexponential position for all runs.

221

### 222 2.6 Data analysis.

223 Data were analyzed using the Sequence Detection Software 2.0 (Applied Biosystems, Foster  
224 City, USA). The fluorescence emission baseline was calculated during the first 3-15 cycles and  
225 the default threshold (Ct) set at three times the standard deviation of the baseline measurement.

226 The mean value and standard deviation from three independent experiments, each performed  
227 with duplicate reactions, were used to build the standard curves for serial dilution DNA  
228 samples. The calibration curves were automatically constructed by the SDS 7.500 software.

229

## 230 3. Results

### 231 3.1 Specificity

232 The specificity was assessed with DNA from 11 mycobacterial species (Table 1) and 7 other  
233 bovine pathogens. Several pathogens that may cause lymphadenitis, such as *Streptococcus*  
234 *bovis*, *Arcanobacterium haemolyticum* and *Actinomyces bovis*, were also included in this  
235 specificity testing. No nonspecific results were obtained in the genus *Mycobacterium* specific  
236 Q-PCR, but only *Mycobacterium gordonae*, *kansasii*, *szulgai*, *gastri*, *ulcerans*, could be  
237 detected. The *M. tuberculosis*-specific Q-PCR, detected only *M. bovis*. The *M. avium*-specific  
238 Q-PCR recognized six isolates belonging to all three subspecies of *M. avium* and was negative  
239 for *M. scrofulaceum* and other species tested (Table 1).

240

## 241 3.2 Analytical sensitivity

242 In order to establish the minimum detection threshold (or assay experimental sensitivity),  
243 reference curves for each studied Complex (MTC, MAC and NTM) were carried out. For each  
244 Complex, 10 fold dilutions serial dilutions were created in triplicate; they contained from 1 µg  
245 to 1 femtograms. For the three assays, the SDS 7.500 system (Applied Biosystems) was able to  
246 detect and differentiate up to 8 logarithmic magnitude orders, with a minimum detection  
247 threshold of 10 femtograms (2-3 genomic equivalents) for the MTC and MAC detection assays  
248 and 100 femtograms for the Genus *Mycobacterium* detection assay. Regarding the MTC, a  
249 calibration straight line was obtained with a 2.77 slope, a Ct= 34,36 interception in the  
250 minimum threshold (10 fg/ml) and one  $R^2=0.957$ . For all systems generated, a strong linear  
251 relationship between the log of the starting amount of DNA and the Ct values obtained.  
252 Moreover, the sensitivity and dynamic range of the three assays were not affected by the  
253 presence of concentrations of up to 10 µg of bovine genomic DNA (data not shown).

254

## 255 3.3. Clinical sensitivity

256 A total amount of 125 bovine samples, obtained from intradermal skin test positive animals,  
257 were tested by macroscopic inspection, culture, PCR identification of culture isolates and  
258 multiplex real-time TaqMan-based assay. Of the 125 samples, 75 showed lesions consistent  
259 with TB, confirmed through bacterioscopy and 50 were considered to have no visible lesions  
260 (NVL) with negative bacterioscopy. The samples with lesions were later classified according to  
261 their pathological status in *incipient lesions* (n=44) confirmed by means of bacterioscopy  
262 (Ziehl-Nielsen), showing only unilateral damage of the retropharyngeal lymph node and  
263 *generalised lesions* (n=31) with different stages of evolution (from caseous lesions scarcely  
264 spread to granulomatous lesions which showed chronic calcification. Once the samples were  
265 cultured, 36 positive cultures were obtained from the samples classified as NVL (36/50), 33 in  
266 incipient lesions y 28 in widespread lesions. There was no positive culture in 14 samples, in  
267 spite of the fact that they showed compatible lesions and positive bacterioscopies. If we  
268 consider the combination of confirmed anatomic pathology with bacterioscopy and culture as

269 the gold standard, we would have 97 animals positive to TB and 28 non-conclusive. This  
270 determines a culture sensibility of 77.6% (97/125). No mycobacteria other than *M. bovis* were  
271 detected by culture.

272 By applying the molecular detection system and using the conventional extraction system (Tri-  
273 Reagent), we detected 22 positive samples (22/36, 61.11%) to the MTC detector in the case of  
274 the NVL culture positive samples, 35 (31/44, 79.44%) in the samples with initial lesions and 25  
275 (25/31, 80.64%) in the group of the widespread lesions, which determines a total sensitivity of  
276 65.6% (82/125). Taking into account the field diagnosis as well as the bacterioscopy, we could  
277 consider 111 animals true positives (36 NVL positive culture +75 animals with compatible  
278 lesions and positive bacterioscopy), which determines a sensitivity of 73.87% (82/111). No Q-  
279 PCR positive samples were detected in the NVL group with no positive culture.

280 By applying the automated extraction system based on SC, we detected a decreasing sensitivity  
281 of up to 47.74% (1.47 times lower), with only 10 (10/36, 27.27%) positive samples in positive  
282 NVL/culture, 20 (20/44, 45.45%) in incipient lesions and 23 (23/31, 74.19%) in widespread  
283 lesions (Table 3). Organisms belonging to MAC or NTM were not detected, neither by means of  
284 a culture nor a Q-PCR process.

285 The sensitivity was positively correlated to the mean Ct for each kind of samples (Table 3 and  
286 Figure 1), with higher Ct in the samples with widespread lesions and lower in the NVL samples,  
287 as a clear indicator of the bacterial charge in each group of lesions. Along with this, the standard  
288 deviation in each group of lesions became higher, regardless of the kind of extraction system,  
289 indicating thus, great irregularity in the bacterial charges of each group.

290

#### 291 **4. Discussion**

292 The purpose of this study was to develop, by using a multiplex real time PCR approach, a high-  
293 quality post-mortem diagnostic system applicable directly to bovine clinical specimens, in the  
294 context of an experimental abattoir epidemiological surveillance program, allowing a rapid and  
295 accurate identification of the infectious agent and/or confirmation of the field diagnosis. In this  
296 regard three independent quantitative PCR methods were designed and optimized.

297 This objective was fulfilled in the case of the MTC and MAC organisms whereas the detection  
298 capacity of the genus was limited to 5 atypical mycobacteria species: *Mycobacterium gordonae*,  
299 *kansasii*, *szulgai*, *gastri*, *ulcerans*.

300 The magnitude of the detected detection thresholds (around 2-3 genomes) is similar to the ones  
301 detected in previous studies (Rodriguez et al., 1995; Taylor et al., 2001; Wards et al., 1995),  
302 while the detected experimental sensitivity (73.87%) is slightly higher than in previous studies,  
303 where it was around 70% (Liebana et al., 1995; Taylor et al., 2001; Vitale et al., 1998; Zanini et  
304 al., 2001); however, the detected sensitivity depending on the kind of lesion detected was not  
305 specified in any of the previous studies.

306 The sensitivity detected in relation to the culture can be considered similar to the one detected in  
307 previous studies carried out on the same population and kind of samples (Gonzalez Llamazares  
308 et al., 1999). The worst sensitivity detected using the robotic system (just the 48.18%) in  
309 comparison with previous studies (Roring et al., 2000; Taylor et al., 2001) is a disadvantage in  
310 relation to the general expected achievement and sensitivity of the SC manual process. This can  
311 indicate a lack of accuracy of the robotic system in comparison with the SC manual procedure.  
312 However, the Tri-Reagent extraction procedure, even though it is a manual method, it is a  
313 simple and rapid system that offers high sensitivity, completing an extraction of 24 samples in  
314 less than an hour. The different stages of the detected sensitivity (58.33% in NVL, 75% in the  
315 samples with initial lesions and 80.64% for the widespread lesions, by using the conventional  
316 extraction method), in relation to the pathological state of the animal, were previously described  
317 (Norby et al., 2004) for other diagnostic techniques and this fact could be useful when applying  
318 the molecular technique, being, in this particular case, useful in animals with lesions compatible  
319 with TB. The correlation of the different detected sensitivities with the Ct, is a clear reflection of  
320 the amount of bacteria which are present in each kind of samples and they also indicate the  
321 possibilities of the system for each kind of sample.

322 So, this approach offers three advantages: (i) the assay is fast (within two days for a minimum  
323 of 48 samples), (ii) the assay has a moderate sensibility and is extremely specific, as shown in

324 samples NVL and AFB negative and (iii) it allows the detection of several mycobacterium  
325 species of veterinary interest (such as *M. bovis* and *M. avium subs. paratuberculosis*).  
326 The addition of the IPC measured by a parallel real-time PCR assay completed the quality  
327 assurance of the diagnostic assay, as it allows the monitoring of both the sample manipulation  
328 procedures and the presence of PCR inhibitors. Indeed, the DNA extraction procedure  
329 significantly affected the recovery rate of the nucleic acids, introducing a statistically significant  
330 bias of underestimation. Moreover, whereas the detection and amplification profile of the IPC  
331 reveals the presence of PCR inhibitors, the measurement of the sample recovery rate establishes  
332 the precise cutoff of sensitivity for each negative sample.  
333 In conclusion, our multiplex real time PCR system represents a fully controlled, fast, high-  
334 throughput diagnostic tool for the rapid detection of *Mycobacterium* presence directly in animal  
335 clinical specimens, which could be an useful tool in the context of bovine tuberculosis abattoir  
336 surveillance programs and granuloma submission programs.

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449 **Figure legends**

450

451 Figure 1. Ct value versus DNA extraction system/pathological status groups plot.

452 Extraction system: Sol= Guanidinium thiocyanate DNA purification, SC= DNA

453 selective capture systems; Pathological status: NVL= non-visible lesions, IN= Early

454 infection, Gen= Generalized condition.

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Table 1. Reference strains used in this study and results of real-time PCR

Strain	Conventional	
	PCR results <sup>1</sup>	Q-PCR results <sup>2</sup>
<i>Mycobacterium. bovis</i> CIP 105050	+/+	+/-/-
<i>Mycobacterium avium</i> subsp <i>avium</i> CIP 104244	+/-	-/+/-
<i>Mycobacterium avium</i> subsp <i>avium</i> CIP 105415	+/-	-/+/-
<i>Mycobacterium avium</i> subsp <i>silvaticum</i> CIP 103317	+/-	-/+/-
<i>Mycobacterium avium</i> subsp <i>paratuberculosis</i> CIP 103963	+/-	-/+/-
<i>Mycobacterium avium</i> subsp <i>paratuberculosis</i> CIP 103964	+/-	-/+/-
<i>Mycobacterium avium</i> subsp <i>paratuberculosis</i> CIP 103965	+/-	-/+/-
<i>Mycobacterium gordonae</i> CIP 104529	+/-	-/-/+
<i>Mycobacterium gordonae</i> CIP 64.27	+/-	-/-/+
<i>Mycobacterium kansasii</i> CIP 104589	+/-	-/-/+
<i>Mycobacterium. fortuitum</i> CIP104534	+/-	-/-/-
<i>Mycobacterium fortuitum</i> subsp <i>fortuitum</i> CIP 104595	+/-	-/-/-
<i>Mycobacterium abscessus</i> CIP 104536	+/-	-/-/-
<i>Mycobacterium flavescens</i> CIP 104533	+/-	-/-/-
<i>Mycobacterium scrofulaceum</i> CIP 105416	+/-	-/-/-
<i>Actinobacillus pleuropneumoniae</i> CIP 100916	-/-	-/-/-
<i>Actinomyces bovis</i> CIP 103258	-/-	-/-/-
<i>Arcanobacterium haemolyticum</i> CIP 103370	-/-	-/-/-
<i>Bordetella bronchiseptica</i> CIP 102314	-/-	-/-/-
<i>Corynebacterium bovis</i> CIP 54.80	-/-	-/-/-
<i>Haemophilus influenzae</i> CIP 100032	-/-	-/-/-
<i>Streptococcus bovis</i> CIP 102302	-/-	-/-/-

<sup>1</sup> Molecular test based in 16S rRNA genus specific and MPB70 MTC specific markers (Liebana et al., 1996).

<sup>2</sup> Multiplex Q-PCR for MTC, MAC and NTM.

Table 2. Oligonucleotide primers and probes.

Oligonucleotide	Sequence (5' → 3')	Target/sequence
Forward primers		
CMTB-224F	ACAAAGTTGGCCACCAACACA	CMTB / ITS
CMA-228F	CAAATGATTGCCAGACACACTATTG	CMA / ITS
GENMYC-243F	ACTATTGGGTCCTGAGGCAACA	<i>Genus<sup>a</sup></i> / ITS
Reverse primer		
UNIMYC-319R	AAACACCACACCCCACCACC	Universal R / ITS
MGB Probes		
MGB-CMTB-283	FAM-TGTTGTCCCACCGCCT	CMTB / ITS
MGB-CMA-258	TAMRA-TGAGACAACACTCGGTC	CMA / ITS
MGB-GENMYC-285	NED-TGTCCCACCATCTTG	<i>Genus<sup>a</sup></i> / ITS
Capture probes		
Ec16S.1390	Biotin-aaaaaTTGTACACACCGCCCGTCA	<i>Genus</i> / 16s rDNA
Mb23S.44n	Biotin- aaaaaTCTCGATGCCAAGGCATCCACC	<i>Genus</i> / 23s rDNA

Table 3. Clinical sensibility (%) and *Ct* value in by extraction system and pathological group. .

Pathological status		NVL	Early infection	Generalized condition	Overall sensibility
N° of samples/status		50	44	31	
Culture sensibility		72% (36/50)	75% (33/44)	90% (28/31)	77.6% (97/125)
Q-PCR					
		NVL Culture negative	NVL culture positive		
Conventional extraction sensibility	0 (0/14)	61.11% (22/36) <sup>1</sup> 30.62 (±1.12) <sup>2</sup>	79.74% (35/44) 27.62 (±4.17)	80.64% (25/31) 22.59 (±5.7)	65.6% (82/125)
SC extraction sensibility	0 (0/14)	27.77% (10/36) 31.48 (±3.38)	45.45% (20/44) 30.03 (±2.3)	74.19% (23/31) 26.18 (±6.31)	42.4% (53/125)

<sup>1</sup> Sensibility (number of positive samples/number of samples by lesion type).

<sup>2</sup> *Ct* value ± *Std. Dev.*

Figure 1. Ct value versus DNA extraction system/pathological status groups plot.

Extraction system: Sol= Guanidinium thiocyanate DNA purification, SC= DNA selective capture systems; Pathological status: NVL= non-visible lesions, IN= Early infection, Gen= Generalized condition.

