1 **Tuberculosis outbreak caused by *Mycobacterium caprae* in a rabbit**

2 **farm in Spain**

3 **Running title:** *Mycobacterium caprae* **outbreak in farmed rabbits**

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26 **Summary**

27 Animal tuberculosis remains a great source of socioeconomic and health concern

28 worldwide. Its main causative agents, *Mycobacterium bovis* and *Mycobacterium*

29 *caprae*, have been isolated from many different domestic and wild animals. Naturally

30 occurring tuberculosis is extremely rare in rabbits and implication of *M. caprae* has

31 never been reported earlier. This study describes a severe tuberculosis outbreak caused

32 by *M. caprae* in a Spanish farm of rabbits raised for meat for human consumption. The

33 disease was first identified in a cachectic dam and then it was confirmed in ten does

34 with similar clinical signs. Subsequently, a depopulation operation was ordered for

35 public health, animal welfare and environmental reasons. To broaden knowledge of

36 spontaneous tuberculosis in rabbits, a study focused on pathological, epidemiological

37 and diagnostic aspects was carried out on 51 does and 16 kittens after receiving the

38 necessary authorizations. These animals were subjected to a modified intradermal test.

39 After being euthanized, rabbits were examined for the presence of visible tuberculosis-

40 compatible lesions. Lung, kidney, cecal appendix and *sacculus rotundus* samples

41 underwent microbiological and anatomopathological analysis. Infection was revealed by

42 at least one of the methods used in 71% of dams and in 44% of kittens. The intradermal

43 test was shown to be a good indicator of infection. Lung was the tissue for which more

44 animals were positive but renal and intestinal tissues were also affected in many cases.

45 Apparently, *M. caprae* spread mainly through the aerogenous route. Infection was

46 pathologically characterized by the absence of evident fibrous capsules surrounding

47 granulomas. A spoligotype (SB0415) frequently found in this area was considered

48 responsible for the outbreak but the source could not be established. Regardless of the

49 exceptional nature of animal tuberculosis in this host, rabbit industry might not escape

50   from its effects and therefore, current biosafety and surveillance strategies should also

51   consider this disease.

52   **Keywords**: tuberculosis, outbreak, *Mycobacterium caprae*, rabbit, zoonoses.

53

54 **1. Introduction**

55 Animal tuberculosis (TB) is a globally distributed zoonotic chronic disease listed in the

56 Terrestrial Animal Health Code (TAHC) of the World Organisation for Animal Health

57 (OIE). The disease is controlled in most developed countries but it remains a significant

58 source of concern due to its huge socioeconomic impact caused through the loss of

59 productivity and the costs of disease control strategies as well as to the threat it poses to

60 human health through zoonotic infection (Malone and Gordon, 2017). TB eradication

61 has not been achieved in different countries due to factors including the low sensitivity

62 of diagnostic methods, poor implementation of control measures, persistence of bacteria

63 in the environment and the existence of domestic and wild maintenance hosts. The

64 disease is caused by members of the *Mycobacterium tuberculosis* Complex (MTBC),

65 primarily *M. bovis* and *M. caprae*, species considered to have the widest host range

66 within the complex (Biet et al., 2005; Pesciaroli et al., 2014; Gortazar et al., 2015;

67 Malone and Gordon, 2017). *M. caprae* isolates have been retrieved from cattle, goat,

68 sheep, deer, bison, pig, wild boar, fox and humans in various countries including Spain,

69 Germany, Poland, Austria, Czech Republic, Switzerland, Tunisia and Nigeria and also

70 from captive animals of different origins (Amato et al., 2017; Ghielmetti et al., 2017;

71 Krajewska-Wędzina et al., 2017; Malone and Gordon, 2017; Rettinger et al., 2017;

72 Djemal et al., 2018; Krzysiak et al., 2018; Ulmann et al., 2018; Yoshida et al., 2018;

73 Ahmad et al., 2019).

74 Although rabbits (*Oryctolagus cuniculus*) have been extensively and successfully used

75 as models for the study of different types of TB, natural infection with mycobacteria

76 seems to be very rare and almost limited to non-tuberculous mycobacteria like *M. avium*

77 subspecies (Arrazuria et al., 2017). Reports on naturally occurring MTBC infection in

78 rabbits are restricted to one wild animal from New Zealand with generalized TB due to

79   *M. bovis* (Gill and Jackson, 1993), isolation of *M. bovis* from several farmed rabbits

80   bred for fur (A. S. Griffith, 1939) and one non-confirmed *M. bovis* culture obtained

81   from a skin lesion on the neck of a rabbit in Ireland (Delahay et al., 2002). Several

82   studies searched for MTBC in wild rabbits but failed to detect it (Little et al., 1982;

83   Coleman and Cooke, 2001; Matos et al., 2016; Arrazuria et al., 2017). As a result of

84   lacking evidence of natural TB in rabbits, some authors suggested that rabbits could

85   exhibit some resistance to *M. bovis* infection or have some kind of behavior-associated

86   protection from getting infected (Arrazuria et al., 2017). Resistance to *M. tuberculosis*

87   infection is even more evident (Good et al., 2018). According to Coleman et al. these

88   lagomorphs can be considered dead-end hosts not able of transmitting *M. bovis* to other

89   rabbits or animal populations (Coleman and Cooke, 2001).

90   To the best of our knowledge, natural infection with *M. caprae* has never been reported

91   in rabbits thus far. Here we describe an outbreak of TB caused by *M. caprae* in a farm

92   of rabbits raised for meat for human consumption. Due to the severity and significance

93   of the outbreak a depopulation operation was ordered by competent authorities for

94   public health, animal welfare and environmental reasons. We took advantage of this

95   opportunity to broaden knowledge of pathological, epidemiological and diagnostic

96   aspects of spontaneous tuberculous infection in farmed rabbits.

97   **2. Materials and methods**

98   **2.1. Farm and animals**

99   The farm raised rabbits (commercial New Zealand White and California hybrids) for

100   meat destined for human consumption at a commercial scale. The enclosure where the

101   TB outbreak was declared contained 1,000 breeding does (at least 24 weeks of age) and

102   the offspring, consisting of 8,000-10,000 kittens (up to 5 weeks of age). This building is

6

103   544 m^2 (34 m × 16 m) and has two floors. Animals were distributed in 5 rows of flat-

104   deck cages per floor. The farm was managed by the owner and one employee with

105   occasional support from a third worker. Management practices are detailed as follows:

106   Animals are housed in individual cages with their offspring. Contact between animals

107   from different cages is limited and can only happen between individuals housed in

108   immediately adjacent cages. The first insemination of does is at the age of 19-24 weeks.

109   Does have an annual mean of 7 litters with an average of 10 kittens per litter. The

110   offspring live together with dams until weaning (5 weeks after birth) and then they are

111   moved to another enclosure and fed until being slaughtered at 9 weeks of age. At

112   slaughter, official veterinarians had not detected TB-compatible lesions in these

113   animals. Replacement is done by animal purchase and self-breeding. Older dams used

114   for self-breeding and sires for semen production are kept in different enclosures in the

115   farm. Water for rabbits is supplied directly through the drinking water distribution

116   system. Animals are fed with commercial pellets indicated for breeding, rearing and/or

117   growing. Cages are cleaned when a dam is replaced, before housing a new doe in the

118   cage. There was no known direct contact between farmed rabbits and any animals from

119   the outside. In spite of this, both livestock from neighboring farms and wildlife are

120   present in the area.

121   **2.2. Outbreak description and management**

122   Clinical signs as progressive loss of weight and condition appeared in does aged 1-1.5

123   years (having given birth 3 to 5 times) in most cases. Once farm veterinarians were

124   informed of a weekly mortality rate of 1% consisting of animals showing these signs,

125   samples of one dam were submitted to the laboratory and *M. caprae* infection was

126   identified.

127   Subsequently, ten animals with similar signs were euthanized, necropsied, thoroughly

128   examined and samples underwent histopathological and microbiological analysis for TB

129   diagnosis. Disseminated tuberculous infection by *M. caprae* was confirmed in all 10

130   does.

131   After being notified, the competent authority ordered a depopulation operation that was

132   carried out in agreement with the Regulation (EC) No 1099/2009 of the Council of the

133   European Union. In order to study the significance of the outbreak and explore options

134   for *in vivo* identification of infected rabbits, an intradermal test (IDT) with bovine

135   purified protein derivative (bPPD) was performed just before the depopulation action on

136   51 dams and 16 kittens selected randomly. During the depopulation operation, IDT

137   results were obtained and tuberculinized animals were weighed, necropsied and

138   screened for the presence of TB-compatible gross lesions. Lung, kidney, *sacculus*

139   *rotundus* and cecal appendix samples were analyzed by histopathology and culture to

140   study the level of dissemination as well as the possible entry and shedding routes of

141   bacteria. This time, *sacculus rotundus* and cecal appendix were pooled for culture.

142   The authors confirm that the ethical policies of the journal, as noted on the journal's

143   author guidelines page, have been adhered to. Animals used in this study belonged to a

144   registered commercial farm and were submitted only to procedures that according to

145   European (Directive 2010/63/EU) and Spanish (Real Decreto 53/2013) legislation on

146   experimental animals are exempt from its application. The animals were slaughtered in

147   agreement to a depopulation operation ordered and approved by the competent

148   authority, the Department of Agriculture, Livestock and Environment of the

149   Government of Aragón, under supervision by official veterinarians and in compliance

150   with the best practices in the field and the methods permitted under the Regulation (EC)

151   No 1099/2009 of the Council of the European Union.

152   **2.3. Intradermal test (IDT)**

153   Owing to the difficulty in using tuberculin test protocols used with rabbits in

154   experimental conditions, an alternative one to be applied under field conditions was

155   designed and used (see discussion). The procedure for the IDT was checked in 49

156   rabbits from a TB-free farm prior to being used with the rabbits from the TB-affected

157   farm. One hundred μl (2,500 IU) of bPPD (CZ Veterinaria, Pontevedra, Spain) were

158   inoculated in the inner lower side of the ear pinna using a Dermojet syringe (Akra

159   Dermojet, Pau, France). To have a reference of the inoculation site, a circle surrounding

160   the perimeter of the head of the Dermojet syringe (17 mm in diameter) was drawn with

161   a marker just immediately before injection. Intradermal reactions were read 48-72 hours

162   after inoculation by ear inspection and palpation. Three types of reactions were

163   observed: a) no skin induration and no visible reaction, neither to the naked eye nor

164   against a backlight, b) no skin thickness increase and appearance of a very slight

165   reaction barely visible or not visible to the naked eye that could appear covering the half

166   of the reference circle or less if examined against a backlight, and c) increased skin

167   thickness and presence of a reaction readily visible to the naked eye that completely

168   covered or was bigger than the reference circle when examined against a backlight.

169   Reaction types a) and b) were interpreted as negative and type c) as positive. Most of

170   the 49 rabbits from the TB-free farm that were used as a negative reference to validate

171   this IDT procedure had a-type reactions and a few of them b-type reactions and were all

172   deemed negative to the test. None of these rabbits showed clinical signs or lesions

173   compatible with TB.

174   **2.4. Gross pathology and histopathology**

175   After necropsy, tissues were macroscopically inspected in search of TB-compatible

176   lesions. Samples (see Tables 1 and 2) were collected and fixed in 10% buffered

177   formalin and subsequently dehydrated through a graded alcohol series before being

178   embedded in paraffin wax. Sections, 3-5 μm thick, were stained with Carazzi's

179   hematoxylin and eosin (HE), for histopathological studies, and Ziehl-Neelsen (ZN)

180   method for acid-fast bacilli (AFB) detection. The amount of AFB was classified

181   semiquantitatively as 0 (no detectable bacilli), 1 (scant AFB), 2 (moderate, easily

182   detectable AFB) or 3 (high load of AFB).

183   **2.5. Sample culture and identification of isolates**

184   One g of sample (see Tables 1 and 2) was homogenized in 10 ml of sterile distilled

185   water. The homogenate was decontaminated and processed for culture in BD BBL™

186   Mycobacteria growth indicator tubes (MGIT™) (Becton Dickinson, Franklin Lakes, NJ,

187   USA) supplemented with BACTEC™ MGIT™ growth supplement and PANTA™

188   antibiotic mixture according to manufacturer's instructions. Inoculated tubes were

189   introduced in a BACTEC™ MGIT™ 960 System and an incubation protocol of 42 days

190   was run. DNA extracted from all positive cultures was tested by a tetraplex qPCR

191   (Sevilla et al., 2015) for simultaneous MTBC confirmation or detection of other

192   mycobacteria. For species identification of MTBC positive samples a panel of

193   singleplex PCR assays previously described (Sevilla et al., 2017) to detect the regions of

194   difference (RD) and spoligotyping (Kamerbeek et al., 1997) were used. Amplification

195   of RD 1, 4, 9, and 12 was carried out using the primers (RD1_F_5'-CCC TTT CTC

196   GTG TTT ATA CGT TTG A-3', RD1_R_5'-GCC ATA TCG TCC GGA GCT T-3',

197   RD4_F_5'-CCA CGA CTA TGA CTA GGA CAG CAA-3', RD4_R_5'-AAG AAC

198   TAT CAA TCG GGC AAG ATC-3', RD9_F_5'-TGC GGG CGG ACA ACT C-3',

199   RD9_R_5'-CAC TGC GGT CGG CAT TG-3', RD12_F_5'-CGT TGG AAC GCG

200   AAA TAC G-3', RD12_R_5'-CCA GGA TAT GGG CGC AAA T-3') reported earlier

201   by Halse et al. (Halse et al., 2011) in independent conventional singleplex PCR assays.

202   Reactions were performed in a total volume of 25 μl containing 1× HotStarTaq Master

203   Mix (Qiagen GmbH, Hilden, Germany), 300 nM of the appropriate primer pairs and 5

204   μl of DNA. Thermocycling conditions were as follows: 1 cycle at 95°C for 15 min,

205   followed by 40 cycles at 94°C for 30 s, 60°C for 30 s and 72ºC for 1 min, and a final

206   step of 72ºC for 10 min. The resulting RD signature was interpreted as follows: *M.*

207   *tuberculosis* is RD1+, RD4+, RD9+ and RD12+; *M. canettii* is RD1+, RD4+, RD9+ and

208   RD12−; *M. africanum* and *pinnipedii* are RD1+, RD4+, RD9− and RD12+; *M. microti*

209   is RD1−, RD4+, RD9− and RD12+; *M. caprae* is RD1+, RD4+, RD9− and RD12−; *M.*

210   *bovis* is RD1+, RD4−, RD9− and RD12−. The amplification of the direct repeat region

211   of *M. tuberculosis* was accomplished by using the primers DRa (biotin-5'-GGT TTT

212   GGG TCT GAC GAC-3') and DRb (5'-CCG AGA GGG GAC GGA AAC-3') in a 50

213   μl reaction containing 400 nM of each oligonucleotide, 0.5 U of *Tth* polymerase

214   (Biotools B&M Labs S. A., Madrid, Spain), 1× buffer (with $MgCl_2$), 200 mM of each

215   deoxynucleoside triphosphate (Invitrogen, Ltd., Paisley, United Kingdom) and

216   approximately 10 ng of template DNA. PCR tubes were heated at 96ºC for 3 min and

217   subjected to 30 cycles of 1 min at 96ºC, 1 min at 55ºC, and 30 s at 72ºC, with a final

218   extension step at 72ºC for 5 min. Biotin-labeled PCR products were hybridized to the 43

219   oligonucleotides corresponding to the spacer sequences immobilized on a membrane

220   using a miniblotter (Immunetics, Cambridge, MA, USA) (Kamerbeek et al., 1997).

221   After incubation with streptavidin-peroxidase (Roche Diagnostics GmbH, Mannheim,

222   Germany) the presence of spacers was revealed using the ECL detection reagent and

223   exposing the membrane to a Hyperfilm ECL (Amersham, GE Healthcare Ltd.,

224   Buckinghamshire, UK) in accordance with the instructions of the manufacturer. Isolates

225   confirmed as non-tuberculous mycobacteria by the tetraplex qPCR were identified using

226   GenoType Mycobacterium CM kit (Hain Lifescience, Nehren, Germany) following the

227   instructions of the manufacturer. Briefly, a total reaction mixture of 50 μl consisting of

228   10 μl AM-A reagent, 35 μl AM-B (provided with the kit) and 5 μl DNA was prepared

229   for each sample. Amplification thermal profile was as follows: 1 cycle of 15 min at

230   95°C, 10 cycles of 30 sec at 95°C and 2 min at 65°C, 20 cycles of 25 sec at 95°C, 40 sec

231   at 50°C and 40 sec at 70°C and a final extension cycle of 8 min at 70°C. Twenty μl of

232   PCR product were mixed with an equal volume of denaturation solution and hybridized

233   to pre-coated membrane strips in hybridization buffer using a TwinCubator (Hain

234   Lifescience). After washings, strips were incubated with 1 ml of diluted conjugate and

235   then with 1 ml of diluted substrate. Once bands were clearly visible, the reaction was

236   stopped with distilled water and the band pattern obtained was interpreted according to

237   the indications of the kit.

238   **3. Results**

239   **3.1. Outbreak confirmation on ten does showing clinical signs**

240   The main external signs observed in these 10 rabbits included cachexia (see Figure 1)

241   and weakness. Microbiological and pathological findings are summarized in Table 1. At

242   necropsy, gross lesions consisting in numerous whitish coalescent nodules with necrosis

243   of variable size were observed. Lungs, kidneys, *sacculus rotundus*, cecal appendix

244   (Figure 1), bronchial, mediastinal and mesenteric lymph nodes were the most affected

245   tissues, and to a lesser extent spleen, liver, pleura, peritoneum and diaphragm.

246   Moreover, the presence of purulent exudate in the tympanic bulla was noticeable. In

247   addition, necrotic foci were observed sporadically in stomach, small and gross intestine,

248   mandibular lymph nodes and skin. Microscopically, lesions of granulomatous

249   inflammation were observed with the presence of AFB. Macrophages and epithelioid

250   cells were more abundant than Langhans giant cells and, similarly, necrosis was

251   observed more frequently than mineralization. Small groups of macrophages were

252   observed, mainly in the liver and spleen, where the presence of AFB was unusual. All

253   ten animals showed disseminated *M. caprae* infection. All or almost all were culture

254   positive for lung, nasal turbinate, liver, kidney, spleen, *sacculus rotundus* and cecal

255   appendix.

256   **3.2. Outbreak investigation on the randomly selected 67 rabbits**

257   The results obtained for these 67 rabbits are summarized in Table 2. The proportion of

258   animals positive to histopathology, microbiology or IDT was 70.6% and 43.8%

259   amongst dams and kits, respectively. An overall proportion of 53.7% of IDT positive

260   animals was observed, 58.8% for adults and 37.5% for young. The tissue with more

261   positive animals was the lung (33) followed by the kidney (25) and the pool of *sacculus*

262   *rotundus* and cecal appendix (20). Infection appeared to be exclusively restricted to the

263   lungs in 4 dams and a kitten. Amongst animals with positive pathological or

264   microbiological results, involvement of lungs could not be proven in 3 dams and 2

265   kittens. All dams deemed IDT positive displayed a positive result in lungs except for

266   one doe negative to the rest of analyses and two that were positive only in other body

267   sites.

268   Gross lesions morphologically compatible with TB were characterized by circumscribed

269   whitish focus, 1 to 10 mm diameter, of necrotizing granulomatous inflammation

270   randomly distributed in affected organs (Table 2). These multifocal lesions tended to

271   coalesce in extensive necrotizing purulent lesions, mainly in lungs.

272   Microscopically (Figure 2) these irregular rounded lesions were composed by a central

273   core of caseous necrosis, with presence of neutrophils, many of them degenerated,

274   surrounded by a rim of epithelioid cells and macrophages and an outer layer of

275   lymphocytes and plasma cells. These multifocal to coalescing pyogranulomatous

276   lesions showed no evident connective capsule. The amount of Langhans giant cells and

277   the mineralization degree were scarce. In affected lung samples, granulomatous lesions

278   were observed adjacent to eroded bronchioles and presence of necrotic debris was noted

279   in the airways. Similarly, caseous material was identified into the lumen of renal

280   collector tubules of affected kidneys. ZN staining revealed free AFB in necrotic areas

281   and debris (even those in renal and respiratory lumina), and, to a lesser extent, in the

282   cytoplasm of macrophages of lesions. In general, the amount of AFB was higher in lung

283   sections than in the other studied organs.

284   All MTBC strains were identified as *M. caprae* (RD1+, RD4+, RD9− and RD12−)

285   spoligotype SB0415. In addition, two kittens yielded isolates other than MTBC. *M.*

286   *fortuitum* was isolated from the kidneys and the *sacculus rotundus* in one case and *M.*

287   *gordonae* from the kidney in another one.

288   Rabbits fell into different groups according to the infection status as assessed by

289   immune response, microbiological and pathological indicators (Table 2): Among IDT

290   negative does, 15 were apparently free of tuberculous infection (A group), 2 had

291   confirmed infection (B group) and 4 of them showed cachexia and disseminated

292   infection (C group). With regard to IDT positive does, there was one animal positive

293   only to the IDT (D group), two for which only extrapulmonary infection could be

294   demonstrated (E group) and subgroups F0 (n=9), F1 (n=9), F2 (n=4) and F3 (n=5)

295   encompassing does with pulmonary infection that were scored according to the

296   abundance of AFB seen in ZN (0 to 3). Kittens could be classified in groups consisting

297   of individuals negative to all analyses (n=8; G group), IDT negative kittens from which

298   mycobacteria other than MTBC was isolated (n=1; H group), IDT negative kittens with

299   confirmed infection in lungs (n=1; I group), kittens positive only to IDT (n=4; J group)

300   and IDT-positive kittens with necrotizing lesions in liver (n=2; K group). Only one

301   kitten (I group) showed severe pathological changes. No statistically significant

302   associations were identified between pathological status, culture results, ZN score and

303   IDT results.

4. Discussion

305   The occurrence of natural TB in rabbits is extremely rare and as far as we know natural

306   *M. caprae* infection has not been documented to date in this host. This mammal species

307   has been considered a dead-end host unable to spread *M. bovis* infection within its

308   population or to other animal populations (Coleman and Cooke, 2001).

309   Notwithstanding, here we report a severe and widespread outbreak of TB caused by *M.*

310   *caprae* in a commercial rabbit farm, where, more than 65% of animals from a random

311   sample obtained in the enclosure destined for breeding mothers were infected.

312   Naturally occurring tuberculous infection in rabbits is anecdotic irrespective of being a

313   highly susceptible host as has been demonstrated in experimental challenge studies,

314   especially with *M. bovis* (Coleman and Cooke, 2001; Delahay et al., 2002; Arrazuria et

315   al., 2017). It has been shown that wild rabbits are almost free of the disease, despite

316   studies carried out on areas with high animal TB prevalence. There is a single report of

317   a wild rabbit with confirmed *M. bovis* infection in New Zealand and another one that

318   was not confirmed by molecular methods in Ireland (Delahay et al., 2002). As a result,

319   it has been suggested that rabbits might have some type of resistance to infection,

320   perhaps through a limited exposure to MTBC bacilli that can be associated with its

321   living and grazing behavior (Arrazuria et al., 2017). In line with this and despite it being

322   rare as well, *M. bovis* was also detected in different rabbits from a fur farm (A. S.

323   Griffith, 1939). This indicates that infection could have spread within the farm due to

324   more susceptible individuals resulting from non-natural living conditions. Previous

325   research showed that *M. bovis* infection can be disseminated through the aerogenous

326   route in a laboratory rabbit colony with frequent involvement of lungs, pleura and

327   intestines (Lurie, 1944).

328   The pathological changes observed in affected animals were similar to those described

329   for infections caused by *M*. *bovis* in one reported naturally occuring case (Gill and

330   Jackson, 1993) and in experimental studies (Converse et al., 1998; A M Dannenberg,

331   2001). Nevertheless, unlike previous reports, a characteristic feature identified in our

332   case was the scarce development or absence of a fibrous capsule surrounding the

333   granulomatous core of lesions. It is not clear if this particularity could be due to the host

334   species, the mycobacterial species or even to the genotype of the strain involved, as

335   different outcomes have been reported (García-Jiménez et al., 2013; Bezos et al., 2015).

336   The absence of fibrosis in lungs has been previously observed in a study of spontaneous

337   latency in a rabbit model of pulmonary TB with *M*. *tuberculosis* CDC1551 strain

338   (Subbian et al., 2012). This trait might be associated to a less efficient ability to control

339   the tuberculous infection and contribute to an extensive spread in the affected organ, to

340   other body locations as well as to the environment. In badgers, *M*. *bovis* infection with

341   extensive macroscopic lesions, histologically characterized by poor fibrous capsule

342   formation to contain the granulomas, presence of abundant bacilli and ulceration into

343   the lumina of airways, leads to aerogenous bacterial shedding (Gavier-Widen et al.,

344   2001). García-Jiménez *et al*. showed that lesions caused by *M*. *caprae* seem to be more

345   prone to the excretion of bacilli in hunted wild boar if compared with *M*. *bovis*, turning

346   *M*. *caprae*-infected animals into a high-risk source of new infections (García-Jiménez et

347   al., 2013). We observed coalescing granulomas with extensive necrosis and poor

348   mineralization in the lungs of affected rabbits and cavity formation in some cases,

349   findings similar to those reported in an experimental rabbit model study of chronic lung

350   cavitary TB using the strain HN878 of *M*. *tuberculosis* (Subbian et al., 2011). In our

351   study, lungs were the organ more frequently affected and showed higher AFB burden.

352   Our findings suggest that *M. caprae* spread in the farm principally through the

353   aerogenous route with bacilli entering the respiratory tract and disseminating to other

354   body sites after primary infection. The fact that does were housed in individual cages

355   supports this idea, but other routes should not be ruled out. Bacterial shedding also

356   occurred through urine and feces, as indicated the detection of bacteria in kidneys

357   (including renal lumina), *sacculus rotundus*, cecal appendix and feces of many dams.

358   Viable *M. caprae* was also found in exudates obtained from the nasal turbinate and the

359   tympanic bullae of many dams with extensive TB (Table 1). This extrapulmonary

360   dissemination could result from oropharyngeal exposure or ingestion of mycobacteria

361   shed in respiratory secretions (self-reinoculation) or present in contaminated food, water

362   or other vehicles. Infection was detected without the involvement of respiratory tract in

363   3 dams, which lead us to think that bacterial entry through the oral route also occurred.

364   Although milk of breeding does could not be analyzed, it is likely that some kittens

365   were exposed to the bacteria through consumption of milk from heavily infected

366   mothers. In this study, uterus sampling and analysis has not been performed and

367   therefore vertical transmission has not been assessed but it should not be neglected as

368   this transmission route has been suggested for *M. bovis* from dams to calves (Ozyigit et

369   al., 2007). Caecotrophy is a particularity of rabbits' digestive physiology that should be

370   also considered (Arrazuria et al., 2017) because it can contribute to the maintenance and

371   dissemination of the bacilli.

372   In spite of the uncommonness of natural MTBC infection in rabbits, we wanted to

373   identify suitable *in vivo* methods able to detect infected individuals and estimate the

374   level of spread of the infection in this type of situations that could also be applied to

375   other situations. Tuberculin IDT has been frequently used to study different aspects of

376   TB development using the rabbit model with *M. tuberculosis*-derived Koch's Old

377   Tuberculin (Converse et al., 1998; Manabe et al., 2003; Arthur M. Dannenberg, 2009;

378   Good et al., 2018). However, these IDT protocols are hardly applicable to rabbits under

379   field conditions. Before the order of farm depopulation was executed, we tried to set up

380   and evaluate a simple IDT protocol using bPPD that could serve as a tool for rabbit *in*

381   *vivo* TB screening in the field. The method would need further evaluation and validation

382   analysis with more animals to set sensitivity and specificity values including rabbits

383   infected with different mycobacteria but it was proven to be feasible and reliable in our

384   outbreak conditions and specific when applied to rabbits free of TB.

385   Animals could be categorized in different groups consistent with their immunological,

386   pathological and bacteriological status as showed in Table 2. There was only one doe

387   and 4 kittens with a positive IDT that could not be confirmed as *M. caprae*-infected,

388   indicating a high positive correlation between IDT and infection detection. In spite of

389   this, these cases could be interpreted as a lack of specificity of IDT. However,

390   considering the apparent high environmental bacterial burden inside the pavilion, it is

391   more likely that they were really exposed and thus they could represent animals that

392   were able to control the infection or in early stages of infection. There were 4 does with

393   cachexia and severe disseminated infection but negative to the IDT. In agreement with

394   the advanced TB seen in these animals, we are more inclined to think that this apparent

395   sensitivity limitation is due to their disability to produce any detectable cell-mediated

396   immune response (immunosuppression). This effect has been identified in other species

397   and diseases. In this case, the hypothesis could be supported by the results of a previous

398   experimental aerosol challenge with different *M. bovis* infectious doses (Converse et al.,

399   1998). Rabbits receiving high doses developed disseminated infection with abundant

400   gross lesions but showed small reaction sizes in response to *M. tuberculosis*-derived

401   tuberculin in comparison to the big size reactions observed in low dose rabbits with less

402   advanced disease.

403   Non-tuberculous mycobacteria were isolated from two rabbits. The rapidly growing *M.*

404   *fortuitum* was cultured from the kidneys and the intestines of one kitten. This ubiquitous

405   species is considered an opportunistic pathogen for humans (Griffith et al., 2007) and

406   animals (Bercovier and Vincent, 2001; Biet and Boschiroli, 2014). The environmental

407   slow grower *M. gordonae* was also recovered from the kidneys of another kitten. This

408   species is the most commonly isolated due to contamination when recovered from

409   human respiratory specimens, but it has also been described as a cause of pulmonary or

410   disseminated NTM disease in immunosuppressed individuals (Griffith et al., 2007).

411   Rabbits injected with an inoculum containing *M. fortuitum* into the subcutaneous fat of

412   the inguinal area developed a necrotizing suppurative granulomatous inflammation

413   (Lewis et al., 1994). In our study, histopathological analysis did not reveal lesions

414   attributable to mycobacterial infection in these two kittens. The incidental detection of

415   these mycobacteria in the kidneys and *sacculus rotundus* of these animals is of

416   unknown clinical relevance.

417   The source of *M. caprae* could not be determined, but the fact that a single spoligotype

418   has been identified suggests that a single strain was responsible for the outbreak. There

419   were some neighboring goat and sheep flocks. Since rabbits do not like eating the feed-

420   dust formed inside pellet bags, the farmer used to collect this dust in sacks and give it to

421   the owner of a sheep flock that also had some goats to feed his animals. Once finished,

422   the empty sacks were returned to the rabbit farm, which represented the most plausible

423   indirect contact we were able to identify. Another possibility is that infection entered

424   into the farm as a result of introducing materials contaminated by infected domestic or

425   wild animals grazing in or passing through the surrounding land. We aimed to

426   investigate these potential sources of *M. caprae*. The goats from the flock sharing

427   pellet-dust sacks were sold but they were IDT-negative. Unfortunately the information

428   we were able to collect regarding other possibilities is very scarce. A human origin for

429   the outbreak was also explored but no TB cases were described among people with

430   access to the farm. One thing is clear; SB0415 spoligotype is quite frequent in domestic

431   (especially goats and cattle) and wild (especially wild boar) animals in this geographic

432   area (unpublished data and information obtained from the official website of the

433   Spanish Database of Animal Mycobacteriosis MycoDB).

434   Public health concerns were also identified in the course of this study. Official

435   veterinarians did not detect any positive individual amongst 9-week-old rabbits

436   slaughtered for meat for human consumption before the outbreak was declared.

437   However, it is most likely that some of these animals already admitted into the food

438   market bore *M. caprae* infection owing to its detection in some of the kittens analyzed

439   in this study. Although most raw meats are intended for being consumed cooked, eating

440   undercooked meats is not unusual. Previous research made evident that cooked meat

441   can still pose a risk of human exposure to viable *M. bovis* (van der Merwe et al., 2009),

442   a finding surely applicable to *M. caprae* as well. Regardless of the exceptional nature of

443   natural MTBC infection in rabbits, this host species is also susceptible to it, including

444   that caused by *M. caprae*. As a consequence, rabbit meat could represent a source of

445   zoonotic TB and therefore, the rabbit industry should consider setting up more stringent

446   biosafety and control measures.

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456   **Conflict of Interest Statement**

457   The authors declare that the research was conducted in the absence of any conflict of

458   interest.

459   **References**

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604

Table 1: Gross pathology, histopathology, Ziehl-Neelsen and cultural results for the 10 does thoroughly analyzed to confirm the TB outbreak.

No.	Age (weeks)	Weight (Kg)	Nasal turbinate				Tympanic bulla				Lung				Liver				Kidney				Spleen				Stomach				*Sacculus rotundus*				Cecal appendix				Faeces
			GP	HP	ZN	Cul	GP	HP	ZN	Cul	GP	HP	ZN	Cul	GP	HP	ZN	Cul	GP	HP	ZN	Cul	GP	HP	ZN	Cul	GP	HP	ZN	Cul	GP	HP	ZN	Cul	GP	HP	ZN	Cul	Cul
1	126	2.770	-	-	0	+	+	nd	nd	-	+	+	3	+	-	+	1	+	+	+	3	+	-	+	1	+	+	+	2	+	-	+	1	+	-	+	2	+	-
2	45	3.050	-	-	0	+	+	nd	nd	-	+	+	3	+	-	+	0	+	+	+	3	+	+	+	3	+	-	nd	nd	nd	+	+	3	+	+	+	3	+	+
3	75	2.020	-	-	0	+	+	nd	nd	-	+	+	3	+	-	+	1	+	+	+	3	+	-	+	0	+	-	nd	nd	nd	+	+	1	+	+	+	3	+	+
4	181	2.000	-	-	0	+	+	nd	nd	+	+	+	3	+	-	+	1	+	+	+	3	+	-	+	0	+	+	+	3	nd	+	+	3	+	+	+	3	+	+
5	117	2.450	-	-	0	+	+	nd	nd	+	+	+	2	+	-	+	0	+	-	+	1	+	-	+	0	+	-	-	0	nd	+	+	3	+	+	+	3	+	+
6	61	nd	-	+	2	+	-	nd	nd	nd	+	+	1	+	-	-	0	+	+	+	2	+	-	+	0	+	-	-	0	nd	-	+	1	+	-	-	0	-	+
7	55	2.450	-	-	0	-	+	nd	nd	+	+	+	1	+	-	+	0	+	-	-	0	+	-	+	0	+	-	-	0	nd	-	+	0	+	-	+	1	+	+
8	47	2.750	-	+	2	+	+	nd	nd	+	+	+	3	+	+	+	2	+	+	+	2	+	+	+	3	+	+	+	1	nd	+	+	3	+	+	+	3	+	-
9	112	2.520	-	+	0	+	+	nd	nd	+	+	+	3	+	-	+	0	+	+	+	3	+	-	-	0	+	+	+	3	nd	+	+	3	+	+	+	3	+	-
10	197	3.100	-	-	0	+	-	nd	nd	nd	+	+	2	+	+	+	1	+	+	+	1	+	+	+	0	+	-	-	0	nd	+	+	2	+	+	+	3	+	-

Abbreviations: No., animal identification number; GP, gross pathology, HP, histopathology; ZN, Ziehl-Neelsen staining, 0= no detectable AFB, 1= scant AFB, 2= easily detectable AFB, 3= high AFB load; Cul, culture;

+, positive; –, negative; nd, not done.

611 **Table 2.** Intradermal test (IDT), anatomopathological and microbiological results

612 obtained for the randomly selected 51 does and 16 kittens that were analyzed to study

613 the significance of the TB outbreak. Grouping of rabbits: A. Does negative to all

614 analyses; B. IDT negative does with confirmed infection; C. IDT negative cachectic

615 does with disseminated infection (immunosuppressed); D. Does positive to IDT only; E.

616 IDT positive does with confirmed infection but negative lungs; F0. IDT positive does

617 with confirmed infection and positive lungs with no detectable AFB in ZN (ZN= 0); F1.

618 IDT positive does with confirmed infection and positive lungs with scant AFB in ZN

619 (ZN= 1); F2. IDT positive does with confirmed infection and positive lungs with easily

620 detectable AFB in ZN (ZN= 2); F3. IDT positive does with confirmed infection and

621 positive lungs with a high load of AFB in ZN (ZN= 3); G. Kittens negative to all

622 analyses; H. IDT negative kittens but positive to *Mycobacterium* sp. in culture; I. IDT

623 negative kitten with confirmed infection in lungs; J. kittens positive to IDT only; K.

624 IDT positive kitten with necrotizing lesions in liver.

N	Weight (Kg)	IDT	Lung GP	HP	ZN	Cul	Kidney GP	HP	ZN	Cul	*Sacculus rotundus* GP	HP	ZN	Cul†	Cecal appendix GP	HP	ZN	Liver GP	Spleen GP	Cecum GP	Colon GP	Small intestine GP	Rabbit group
15	3.90-4.85	−	−	−	0	−	−	−	0	−	−	−	0	−	−	−	0	−	−	−	−	−	A
1	4.30	−	−	−	0	−	−	−	0	+	−	−	0	−	−	−	0	−	−	−	−	−	B
1	4.95	−	−	+	0	+	−	−	0	−	−	−	0	−	−	−	0	−	−	−	−	−	B
1	2.40	−	+	+	1	+	+	+	3	+	+	+	2	+	+	−	0	−	+	+	+	+	C
1	2.45	−	+	+	1	+	+	+	3	+	−	−	0	+	+	+	3	−	−	−	−	−	C
1	2.60	−	+	+	1	+	+	+	1	+	+	IC	0	+	+	IC	0	−	+	−	−	−	C
1	2.80	−	+	+	2	+	+	+	2	+	+	+	0	+	+	+	1	+	+	−	−	−	C
1	3.90	+	−	−	0	−	−	−	0	−	−	−	0	−	−	−	0	−	−	−	−	−	D
1	3.65	+	−	−	0	−	−	−	0	−	+	IC	0	+	+	IC	0	−	−	−	−	−	E
1	4.20	+	−	IC	0	−	−	−	0	+	−	+	0	+	−	−	0	−	−	−	−	−	E
1	4.20	+	−	−	0	+	−	−	0	+	−	−	0	−	−	−	0	−	−	−	−	−	F0
2	4.30-4.35	+	+	−/IC	0	+	−	−	0	−	−	−	0	−	−	−	0	−	−	−	−	−	F0
1	4.35	+	+	−	0	+	−	−	0	−	−	−	0	+	−	−	0	+	−	−	−	−	F0
1	4.10	+	+	−	0	+	−	−	0	−	+	+	0	+	+	−	0	+	−	−	−	−	F0
1	3.85	+	+	+	0	+	−	−	0	−	+	+	1	+	−	−	0	−	−	−	−	−	F0
1	3.25	+	+	+	0	+	+	−	0	+	+	+	1	+	−	−	0	−	−	+	+	−	F0
1	3.75	+	+	+	0	+	−	−	0	−	+	+	1	+	+	+	0	+	−	−	−	−	F0
1	3.40	+	+	+	0	+	+	+	1	+	+	+	1	+	+	+	1	+	−	−	−	−	F0
1	3.35	+	+	+	1	+	−	−	0	−	−	−	0	−	−	−	0	−	−	−	−	−	F1
1	4.55	+	+	+	1	+	−	−	0	+	−	−	0	−	−	−	0	−	−	−	−	−	F1
1	4.95	+	+	+	1	+	+	−	0	+	−	−	0	+	−	−	0	−	+	−	−	−	F1
1	3.25	+	+	+	1	+	+	−	0	+	−	−	0	+	+	−	0	+	−	−	−	−	F1
1	4.25	+	+	+	1	+	+	+	0	+	−	−	0	−	−	−	0	+	−	−	−	−	F1
1	3.25	+	+	+	1	+	+	+	3	+	+	−	0	+	+	−	0	−	−	−	−	−	F1
1	4.65	+	+	+	1	+	+	+	1	+	+	−	0	+	+	+	1	+	+	−	+	−	F1
2	3.95	+	+	+	1	+	−	−	0	+	+	+	2/1	+	+	+/−	1/0	+	−	−	−	−	F1
1	4.00	+	+	+	2	+	−	−	0	−	−	−	0	−	−	−	0	+	−	−	−	−	F2
2	3.00-4.00	+	+	+	2	+	−	−	0	+	−	−	0	−	−	−	0	−/+	+/−	−	−	−	F2
1	4.55	+	+	+	2	+	+	+	1	+	−	−	0	+	+	+	0	+	−	−	−	−	F2
3	4.25-5.00	+	+	+	3	+	−	−	0	+	−	−	0	−	−	−	0	+/−	−	−	−	−	F3
1	3.40	+	+	+	3	+	+	+	1	+	+	+	1	+	+	+	1	+	−	+	+	−	F3
1	3.85	+	+	+	3	+	+	+	2	+	+	+	0	+	−	−	0	+	−	−	−	−	F3
8	2.15-3.00	−	−	−	0	−	−	−	0	−	−	−	0	−	−	−	0	−	−	−	−	−	G
1	1.80	−	−	−	0	−	−	−	0	Mf	−	−	0	Mf	−	−	0	−	−	−	−	−	H
1	2.25	−	+	+	3	+	−	−	0	−	−	−	0	−	−	−	0	−	−	−	−	−	I
4	1.85-2.50	+	−	−	0	−	−	−	0	−	−	−	0	−	−	−	0	−	−	−	−	−	J
2	1.75-2.35	+	−	−	0	−	−	−	0	Mg/−	−	−	0	−	−	−	0	+	−	−	−	−	K

625 †*Sacculus rotundus* and cecal appendix were pooled for culture.

626 Abbreviations: N, number of individuals in the group; IDT, intradermal test; GP, gross pathology, HP, histopathology; ZN, Ziehl-

627 Neelsen staining, 0= no detectable AFB, 1= scant AFB, 2= easily detectable AFB, 3= high AFB load; Cul, culture; +, positive; −,

628 negative; IC, inconclusive; Mf, *M. fortuitum*; Mg, *M. gordonae*.

629

630   **Figure 1.** A) External appearance of a cachectic dam. B) Lungs with numerous whitish

631   nodules of variable sizes. C) Kidney affected with tuberculous nodules. D) *Sacculus*

632   *rotundus* (*) and cecal appendix (►) with tiny necrotic foci; ileon (arrow) is also

633   affected.

634

635   **Figure 2.** A) Multifocal to coalescing necrotizing pyogranulomatous lesion in lung with

636   no evident fibrous capsule or mineralization (HE; bar=500μm). B) Necrotic debris in

637   airways and bronchiolar wall erosion (arrow) (HE; bar=100μm). C) Extensive necrotic

638   areas surrounded by granulomatous infiltrate in the mucosa of cecal appendix (HE;

639   bar=500μm). D) High amount of AFB in necrotic material found in the lumen of a renal

640   tubule (ZN; bar=50μm)

641

642

643

Figure 1: A) External appearance of a cachectic dam. B) Lungs with numerous whitish nodules of variable sizes. C) Kidney affected with tuberculous nodules. D) Sacculus rotundus (□) and cecal appendix (►) with tiny necrotic foci; ileon (arrow) is also affected.
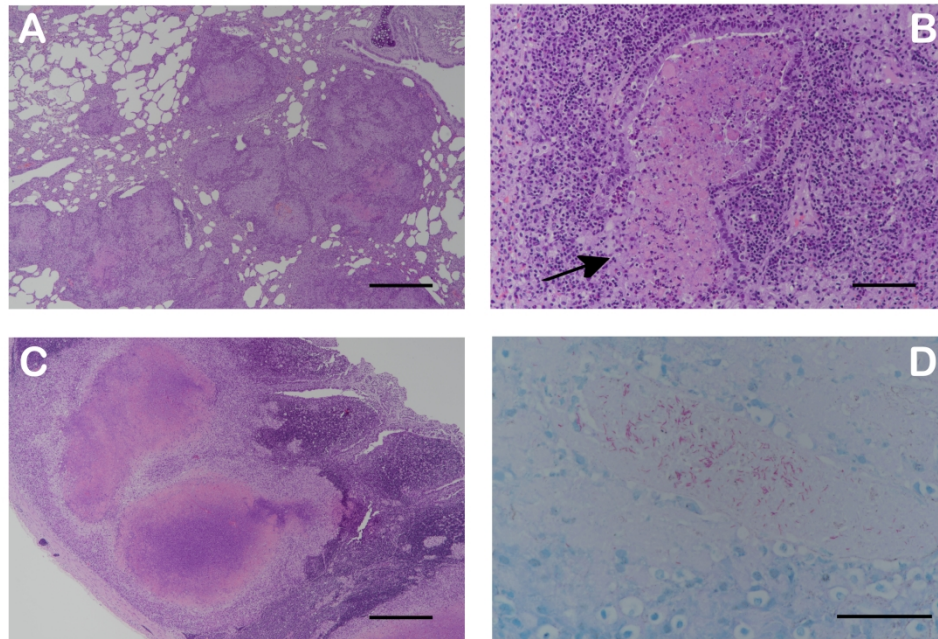
Figure 2: A) Multifocal to coalescing necrotizing pyogranulomatous lesion in lung with no evident fibrous capsule or mineralization (HE; bar=500µm). B) Necrotic debris in airways and bronchiolar wall erosion (arrow) (HE; bar=100µm). C) Extensive necrotic areas surrounded by granulomatous infiltrate in the mucosa of cecal appendix (HE; bar=500µm). D) High amount of AFB in necrotic material found in the lumen of a renal tubule (ZN; bar=50µm)