1 **Polyclonal infection as a new scenario in *Mycobacterium caprae* epidemiology**

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21 **Highlights**

- 22 • *M. caprae* ($n=55$) genotyping over 12-year period revealed SB0157 and 24 MIRU-VNTR types.
- 23 • Fine-scale discrimination of isolates supports clonal structure and expansion of founder.
- 24 • Adaptive genotypic divergence of *M. caprae* paves the way for sympatric speciation.
- 25 • Unprecedented intra-host microevolution was detected at MIRU4 in over 20% animals.
- 26 • Studies needed to evaluate functional implications of in vivo *M. caprae* parallel evolution.

27

28 **Abstract**

29 Portugal is one of the European Union (EU) countries with an ongoing eradication program for
30 tuberculosis (TB) in cattle, which does not include systematic goat testing. However, surveillance in
31 small ruminants is increasingly important, since goat and sheep can harbour *Mycobacterium caprae*
32 and be an infection source to cattle with impact in the success of bovine TB control. Furthermore, the
33 information regarding the epidemiology and biology of *M. caprae* is quite limited comparing to the
34 cognate bovine-adapted ecotype, *M. bovis*.

35 In this work, we applied spoligotyping and MIRU-VNTR (M*ycobacterial* I*nterspersed* R*epetitive*
36 U*nts-Variable* N*umber of Tandem* R*epeats*) to *M. caprae* obtained between 2003 and 2014 from 55
37 animal hosts, including goat (n=29), cattle (n=21), sheep (n=1) and wild boar (n=4) from Portugal.
38 The molecular analysis revealed a unique spoligotyping pattern (SB0157) and 24 MIRU types.
39 Genotyping of serial *M. caprae* from herds with recurrent outbreaks enabled fine-scale discrimination
40 of epidemiologically related isolates, supporting a clonal structure in Portugal and denoting the
41 emergence of clonal diversity at the herd level, more apparent for MIRU4. Results suggest a founder
42 effect and adaptive genotypic divergence, paving the way for sympatric speciation.

43 Double allele findings at MIRU4 in over 20% of infected animals indicates that co-infection and *in*
44 *vivo* microevolution are frequent events in the goat-adapted ecotype. While polyclonal infection
45 appears to be common in *M. caprae* epidemiology, the significance of subtle genotypic variations
46 remains to be disclosed at the functional level, namely at the interface with the host, to expand
47 knowledge on the epidemiological scenario of this neglected ecotype.

48
49 **Keywords:** *Mycobacterium caprae*; animal tuberculosis; spoligotyping; VNTR; clonal diversity;
50 polyclonal infection

51 **1. Introduction**

52 *Mycobacterium caprae* (*M. caprae*) is a member of *Mycobacterium tuberculosis* complex (MTBC)
53 (Aranaz et al., 2003), a group of eleven host-adapted ecotypes, responsible for causing tuberculosis
54 (TB) in different mammal species (Hershberg et al., 2008; Smith et al., 2006; Dippenaar et al., 2015;
55 Clarke et al., 2016).

56 *M. caprae* was originally described as the causative agent of TB in goats (Aranaz et al., 2003).
57 However, this pathogen has been isolated from other domestic (cattle, sheep, swine) and wildlife (red
58 deer, wild boar and red fox) species, suggesting that its host specificity is apparent and that cross-
59 species transmission is more relevant than previously acknowledged (Aranaz et al., 2003, Rodríguez
60 et al., 2011). With few exceptions, *M. caprae* has mainly been reported in European countries, mostly
61 from the central and south continental regions, where goat is often managed as a species of economic
62 interest to the traditional cheese, meat and wool industry. In Portugal, *M. caprae* represents ca. 4%
63 of TB breakdowns (Matos et al., 2010, Cunha et al., 2012). In bordering country Spain, it represents
64 7.4% (Rodríguez et al., 2011), while in Italy it is responsible for over 10% of TB outbreaks in cattle
65 herds (Chiari et al., 2014). There have been some studies reporting *M. caprae* infection in red deer
66 (*Cervus elaphus*) (Rodríguez et al., 2011; Schoepf et al., 2012) and wild boar (*Sus scrofa*) (Rodríguez
67 et al., 2011; Cunha et al., 2012; García-Jiménez et al., 2012), mainly from Spain and countries with
68 Alpine wildlife habitats.

69 *M. caprae* has also been linked with TB in humans, with cases reported in Spain, Germany and
70 Croatia (Cvetnic et al., 2007, Kubica et al., 2003, Rodríguez et al., 2009). As with *M. bovis*, the
71 consumption of unpasteurized dairy products and direct close contact with diseased animals have
72 been attributed as potential sources of infection (Cvetnic et al., 2007).

73 Portugal, Spain, the United Kingdom, Austria, Ireland and Italy, are the European Union (EU)
74 countries with an ongoing EU-financed TB eradication program in cattle herds. At present, EU
75 regulations require TB surveillance and testing in dairy non-bovine species (Regulation 853/ 2004 of
76 the European Council, section IX, Chapter I), however not all of the aforementioned countries

77 currently have a specific eradication program for TB in goats. Austria have included in their
78 surveillance schemes goat flocks that are in close contact with cattle, while in Spain these schemes are
79 only applied to regions with a high density of goat flocks, such as Castilla y León, Murcia, Andalucía
80 and Islas Canarias (Bezós *et al.*, 2014).

81 TB surveillance in small ruminants is essential due to the risk of raw milk contamination and the
82 potential subsequent public health effects. Both sheep and goat can harbor *M. caprae* or *M. bovis*
83 [Matos *et al.*, 2010; Muñoz-Mendoza *et al.* (2012, 2016)] and may be a source of infection to other
84 small ruminants and also to cattle when managed in mixed species farms, jeopardizing the
85 accomplishments of costly bovine TB eradication programs. The detection of infected animals is thus
86 key for the effectiveness of control programs, however there is a lack of standard criteria to implement
87 the intradermal tuberculin testing in goats, and certain intrinsic methodological aspects, as the site of
88 injection or the interpretation criteria of test results, vary across and within countries (Pesciaroli *et*
89 *al.*, 2014). This situation leads to the application of these tests following the standards developed for
90 cattle, which may misguide result interpretation and mislead infection management (Bezós *et al.*,
91 2011; Bezós *et al.*, 2012). Moreover, TB-compatible lesions in small ruminants are more rarely
92 detected at slaughterhouses due to lower awareness and less exhaustive meat inspection procedures
93 than those commonly carried out for cattle (Bezós *et al.*, 2012a).

94 Despite the relevance of *M. caprae* as a TB-causing agent in goats, sheep, as well as cattle, diagnosis
95 accuracy of related infections and population structure of the microorganism in domestic and wild
96 animals have been poorly studied, in contrast to *M. bovis*. However, the fine discrimination of *M.*
97 *caprae* isolates is key to understand the infection source and transmission pathways, as well as to
98 apprehend the intrinsic biology of this pathogen. This knowledge may contribute to re-define or adjust
99 TB eradication programs since, despite all efforts, the disease endures in the Mediterranean and is a
100 re-emergent problem in central Europe (Ghielmetti *et al.*, 2017, Gormely *et al.*, 2014). In this context,
101 spoligotyping and MIRU-VNTR (M*ycobacterial* I*nterspersed* R*epetitive* U*nit*-V*ariable* N*umber* o*f*
102 T*andem* R*epeats*) typing have become common genotyping tools to characterize both *M. bovis* and

103 *M. caprae* isolates. While commonly used alone in the recent past for epidemiological surveillance,
104 spoligotyping has limitations due to homoplasmy and the inability to distinguish mixed infections or
105 clonal variants, since a unique spoligotype profile would be generated due to overlapping
106 hybridization patterns. In contrast, MIRU-VNTR typing examines the copy number of tandem
107 repetitions in polymorphic *loci*, potentially enabling the identification of concomitant allelic profiles,
108 and, thus, assessment of mixed infections in the case of clearly distinct fingerprints.

109 Considering: 1) the circulation of *M. caprae* within TB maintenance community (goat, sheep, cattle),
110 affecting species of economic interest in continental Europe, 2) its emerging importance to cases of
111 zoonotic TB in humans, and 3) the lack of information regarding its epidemiology, population
112 structure and genome diversification, our work envisaged the systematic molecular analysis of *M.*
113 *caprae* isolates, both at the country and herd levels, in order: (i) to assess the reliability or limitations
114 of spoligotyping and MIRU-VNTR for epidemiological investigation when *M. caprae* is involved;
115 (ii) to enable fine-scale spatiotemporal discrimination of *M. caprae* isolates; (iii) to evaluate the
116 occurrence of mixed infections; (iv) to appraise the emergence of clonal diversification. The
117 genotyping data described and analysed as a whole in this report were obtained on several instances
118 during the period 2003-2014, as indicated below.

119 **2. Materials and Methods**

120 **2.1. Bacterial isolates origin and molecular identification**

121 The *M. caprae* isolates under analysis in this study were obtained between 2003 and 2014 from tissue
122 samples of 55 animals, namely goat (*Capra aegagrus hircus*) (n=29), cattle (*Bos taurus*) (n=21),
123 sheep (*Ovis aries*) (n=1) and wild boar (*Sus scrofa*) (n=4) (Supplementary Table 1).

124 This 12-year molecular analysis concerns three periods: period 1 [(2003-2006), including genotyping
125 results from Duarte *et al.* (2010)], period 2 [(2007-2008), including genotyping results from Cunha
126 *et al.* (2012)], and period 3 (2009-2014, original data from this study). Data from Duarte *et al.* (2010),
127 includes ten *M. caprae* isolates from seven goats from Beja, two bovines from Braga and one wild
128 boar from Castelo Branco. Information from Cunha *et al.* (2012) increased previous data, adding nine

129 *M. caprae* isolates from five goats and one bovine from Bragança, two bovines from Aveiro and wild
130 boar from Castelo Branco.

131 The animal samples (lung and mediastinal, mesenteric, pre-scapular, popliteal, and/or
132 retropharyngeal lymph nodes) originated from sanitary slaughter after positive intradermal testing or
133 from routine meat inspection. Tissue samples from each animal were pooled and processed in a
134 biosecurity level 3 facility at INIAV IP (National Institute for Agrarian and Veterinary Research,
135 Portugal), according to the OIE Manual for Terrestrial Animals, with the technical specificities
136 detailed in Cunha *et al.* (2012). Smears from homogenate sediment were prepared and stained by the
137 Ziehl-Neelsen (ZN) technique to visualize acid-fast bacilli. Inoculation of homogenates on
138 Stonebrink (Biogerm, Portugal) and Löwenstein-Jensen pyruvate (Biogerm, Portugal) solid media
139 followed the guidelines for the diagnosis of bovine tuberculosis (OIE, 2008). To exclude cross
140 contamination, a physiological saline blank per each processed batch was also inoculated into solid
141 medium. Cultures were incubated for a minimum of 12 weeks at 37°C and slopes were examined for
142 macroscopic growth every week during the incubation period. One to three colonies grown on
143 selective slants were picked randomly and stained by ZN. Bacteria confirmed for acid-fast character
144 were subsequently characterized. Cell cultures growing on slopes were suspended in TE buffer, heat
145 killed, centrifuged and the crude supernatant containing nucleic acids was stored at -20°C. Species
146 identification of presumptive mycobacterial isolates was performed by restriction fragment length
147 polymorphism analysis of PCR- amplified *gyrB* gene (PCR-REA) as described in Cunha *et al.* (2012)
148 or using commercially available systems [GenoType Mycobacterium (Hain diagnostics, Germany)]
149 according to manufacturer instructions. In all PCR batches, in addition to samples, positive controls
150 (*M. tuberculosis* H37RV and *M. bovis* BCG) and a negative control (water) were included.

151 **2.2. Study area**

152 Twelve of the 18 districts (administrative level sampling unit) of mainland Portugal were represented
153 in this study (Supplementary Fig. 1). Bragança (North-East) is the district with more *M. caprae*

154 isolates ($n = 21$) and sampled host species (sheep, goat, cattle). Two *M. caprae* isolates were from
155 cattle of the Azores islands.

156 **2.3. Epidemiological information of infected herds**

157 Basic life history traits, as gender and age, were only available for some of the domestic animals
158 under analysis.

159 Epidemiological information on livestock, specifically previous history of TB in the herd, animal
160 movement history and animal management conditions (herd type and dimension) was also only
161 accessible for a subset of hosts.

162 **2.4. Anatomical localization and histological characteristics of TB-compatible lesions**

163 For histopathological examinations, animal samples were fixed in 10% buffered formalin and
164 paraffin- embedded by standard procedures. Five micrometer-thick sections were stained with
165 haematoxylin and eosin and examined using light microscopy. Microscopic lesions (data available
166 for 20 animals only, from 2008 onwards) were histologically classified into three types according to
167 lesion progression: type I- “caseous granulomatous lesions”, type II- “caseous granulomatous lesions
168 with calcification” and type III- “caseous granulomatous lesions with cavitation”.

169 **2.5. Spoligotyping and MIRU-VNTR analyses of *M. caprae* isolates**

170 Spoligotyping was performed according to Kamerbeek *et al.* (1997), with the modifications described
171 by Botelho *et al.* (2015). The spoligotyping pattern was converted into a binary code of 43 digits that
172 was checked against the international database Mbovis.org (<http://www.mbovis.org/index.php>).

173 Variable number tandem repeat analysis was performed using a previously established 8-*loci* MIRU-
174 VNTR set, whose allelic diversity was shown to be appropriate to study the epidemiological scenario
175 in Portugal (Duarte *et al.*, 2010): VNTR3232, ETR-A, ETR-B, ETR-C, QUB11a, QUB11b, MIRU26
176 and MIRU4. These *loci* were amplified by PCR using the primers and cycling conditions previously
177 described by Cunha *et al.* (2012). Positive controls (DNA extracted from *M. tuberculosis* H37Rv and
178 *M. bovis* BCG) and a negative control (water) were included in all PCR assays.

179 Amplicon size was estimated by electrophoresis in a 1.5% agarose gel, prepared in Tris Buffer EDTA
180 1x. All electrophoretic runs were conducted in the same gel cast and tank, maintaining the same
181 parameters (90V for a period of 3 hours). The number of repeats in each amplified locus was attributed
182 by correlation with the amplicon size according to previously published tables (Supply *et al.*, 2006).
183 Each MIRU-VNTR profile was nominated as a genotypic profile, and the profiles were grouped into
184 MIRU types following Cunha *et al.* (2012). The genotypic profiles presented are joint results from
185 this study (isolates from 2009 to 2014), Duarte *et al.* (2010) and Cunha *et al.* (2012) (Table 1).
186 Amplification of double alleles at MIRU4 locus was confirmed at least in three independent PCR and
187 electrophoresis experiments for each of the 12 *M. caprae* TB cases wherein this situation was
188 registered. Furthermore, the PCR amplification products corresponding to alleles 3 and 4 at MIRU4
189 from five randomly selected TB cases with two genotypic variants were run on agarose gel, the DNA
190 fragments were excised, purified (QIAquick gel extraction kit; Qiagen, Hilden, Germany) and
191 commercially sequenced (GATC Biotech, Germany). Original chromatogram files were inspected
192 and reviewed. The resulting Fast Adaptive Shrinkage Thresholding Algorithm (FASTA) sequences
193 were aligned in ClustalX 2.1, mapped against *M. bovis* AF2122 as reference, and the number of
194 tandem repetitions confirmed in silico for each amplification product. For the 12 cases where these
195 double alleles were detected, four additional *loci* (MIRU16, MIRU40, ETR-E and QUB26) were
196 added to the initial panel of MIRU-VNTR *loci* to eventually improve discrimination (12-*loci* MIRU-
197 VNTR). These *loci* were amplified by PCR using the primers described by Supply *et al.* (2001) and
198 Skuce *et al.* (2000), and the cycling conditions were the same as previously described for the other
199 *loci* of the panel.

200 The discriminatory power (D) of MIRU-VNTR typing and the individual allelic diversity (h) of the
201 *loci* panel tested was calculated as described by Hunter and Gaston (Hunter & Gaston, 1988), using
202 an online tool available on the website <http://insilico.ehu.es> (accessed on may 2019). The
203 discriminatory power of the technique was calculated for complete genotypic profiles only.

204 **2.6. Phylogenetic analysis**

205 In order to study the phylogenetic relationships among *M. caprae* isolates, a minimum spanning tree
206 (MST) was generated building on MIRU types and using the MIRU-VNTR_{plus} web application
207 available at <http://www.miru-vntrplus.org> (accessed on march 2019). The incomplete profiles (M117,
208 M117' and M119) were excluded from this analysis. The *loci* QUB11a and VNTR3232 are not
209 included in the standardized data sets of MIRU-VNTR_{plus} and therefore were arbitrarily assigned to
210 database fields of standard *loci*.

211 **2.7. Statistical analysis**

212 The Chi-square ($\alpha=0.05$) test was used whenever appropriate to test statistical significance of several
213 associations. Contingency tables and tests were performed in IBM SPSS Statistics (version 24.0.0).
214 Results were considered to be significant if $p<0.05$.

215 **3. Results**

216 **3.1. Epidemiological information of animals under analysis**

217 Regarding the basic life history traits of the animal population under analysis, 24 were female, nine
218 were male and for 22 gender was unknown; 60% ($n = 33$) were less than 12 years old (Supplementary
219 Table 1).

220 Information regarding herd type (multiple versus single species) was available for 41 out of 51
221 (80.4%) domestic animals. In a minority of cases (19.6%, $n = 8/41$), herds were shared by more than
222 one animal species (Supplementary Table 1).

223 Herd dimension was registered for 35 animals (68.6%) and classified into three types according to
224 size: type A, less than 50 individuals ($n = 8$; 23%); type B, between 50 and 150 ($n = 7$, 20%); and
225 type C, more than 150 animals ($n = 20$; 57%). The majority of goat herds were type C, whereas cattle
226 herds were types A or B. Information about herd management (intensive or extensive) was available
227 for 17 (33.3%) animals; eleven (64.7%) were in an extensive regime (Supplementary Table 1).

228 **3.2. Anatomical localization and histological characteristics of TB-compatible lesions**

229 In total, post-mortem macroscopic lesions compatible with TB were observed in 25 out of 55 screened
230 animals (45.5%), including 14 goats (48.3%), six bovines (28.6%), four wild boars and one sheep.
231 Moreover, for 20 of these animals (from 2008 onwards) a histological description was also available.
232 Type I lesions were present in five animals (7.3%), type II in nine (16.4%), and type III in six (10.9%)
233 (Supplementary Table 1).

234 Type I lesions were restricted to lung and mediastinal lymph nodes and type III lesions to lung.
235 However, type II lesions were located in more anatomical regions, including lung and mediastinic,
236 mesenteric and submaxillary lymph nodes (Supplementary Table 1).

237 **3.3. Spoligotyping and multilocus variable number of tandem repeat analysis of *M. caprae***
238 **strains**

239 Fifty-five culture-confirmed TB cases, confirmed by *gyrB*-REA and Genotype® as *M. caprae*, were
240 analysed by spoligotyping and MIRU-VNTR. Spoligotyping performed directly on *M. caprae* DNA

241 recovered from cultured microorganisms homogeneously generated SB0157 pattern, while 8-*loci*
242 MIRU-VNTR could discriminate 67 genotypic profiles grouped into 24 MIRU types that were
243 classified following the nomenclature adopted in Cunha *et al.* (2012) (Table 1). *M. caprae* strains
244 with double alleles were subdivided in two genotypic profiles (i.e. MIRU types), one for each allele,
245 that is why 67 genotypic profiles could be generated by 55 *M. caprae* isolates. Despite several
246 attempts, lack of amplification of MIRU4 in three isolates hampered completion of three allelic
247 profiles (M117, M117' and M119).

248 The overall discriminatory index of MIRU-VNTR was $D=0.8856$. The allelic diversity of individual
249 locus ranged from 0.0592 to 0.5509, in decreasing order: VNTR 3232 ($h=0.5509$), MIRU4
250 ($h=0.5441$), ETR-A ($h=0.5061$), QUB11b ($h=0.4885$), QUB11a ($h=0.4283$), MIRU26 ($h=0.2451$),
251 and ETR-B and ETR-C ($h=0.0592$).

252 **3.3. Detection of genotypic variants within the same host**

253 In 12 TB cases (21.8%), infection with clonal variants was suspected due to the concomitant detection
254 of two *M. caprae* genotypic profiles within the mycobacterial DNA recovered from the same animal.
255 Polyclonal infection was suggested by the presence of double alleles (allele 3 or 4, in all cases)
256 exclusively in MIRU4, while in each of the additional seven *loci* only single alleles were detected
257 (Table 1). All cases of recovered DNA displaying double alleles in one locus that were suggestive of
258 clonal microevolution from founder strain were retested on several occasions and the simultaneous
259 presence of alleles 3 and 4 confirmed. The PCR amplification products of the DNA fragments
260 corresponding to allele 4 and allele 3 of five randomly selected double allele findings were sequenced
261 and the corresponding sequences were aligned and mapped on the sequence of MIRU4 from *M. bovis*
262 AF2122. Two sequences of allele 3 were excluded due to poor quality. No nucleotide sequence
263 polymorphisms between the *M. bovis* reference strain and the field strains were found at this locus
264 (supplementary Figure 2). In all cases, the sequence of allele 4 differed exactly in one tandem
265 repetition (77 bp) when compared with the sequence of allele 3, supporting the co-existence of double
266 alleles.

267 The profiles of these MIRU4 genotypic variants were distinguished into separate MIRU types: M132
268 and M133 (concomitantly isolated from 10 animals, cattle and goat), M134 and M135 (one bovine),
269 and M136 and M137 (one goat) (Table 1).

270 In an attempt to further confirm that the genotypic profiles of the 12 cases with double alleles findings
271 at MIRU4 locus did not differ in any other locus, the panel of MIRU-VNTR was increased from eight
272 to twelve *loci*. The results obtained for MIRU16, MIRU40 and ETR-E were equal for all the cases
273 under analysis, generating alleles 5, 1 and 4, respectively. Discrimination was achieved when adding
274 QUB26 locus, with five DNA samples yielding allele 2 and seven generating allele 3. No double
275 alleles were detected in this additional panel of four loci, supporting the original suspicion for the co-
276 existence of clonal variants.

277 The genotypic variants with double allele findings were detected along the years [2008 (in one
278 bovine), 2009 (one bovine), 2010 (eight goats, one bovine), 2012 (one goat) and 2013 (one goat)] in
279 herds located in Bragança, Lisboa, Beja and Évora (Table 1). The proportion of animals putatively
280 harbouring *M. caprae* clonal variants was statistically associated ($p = 0.038$) with Bragança district.
281 The influence of host species or time period on co-infection with clonal populations was found not to
282 be statistically significant.

283 **3.4. Distribution of MIRU types by host**

284 The most common MIRU type was M132 (32.7% of infected animals), which was detected either in
285 animals) simultaneously harbouring two genotypic profiles ($n = 10$) but also as a single infecting
286 population ($n = 8$); following was M133 (detected only in animals with two *M. caprae* genotypic
287 profiles, $n = 10$), M137 (generated by one co-infecting genotypic profile, $n = 1$; and single infected,
288 $n = 8$) and M69 (detected only in single infected, $n = 6$); four MIRU types clustered two genotypic
289 profiles each (M70, M116, M118, M128), and the remaining types ($n = 15$) were singletons, although
290 it cannot be discarded that M117 and M117', for which MIRU4 allele could not be determined, might
291 represent a unique MIRU type (Table 1). For the three cases of incomplete genotypic profiles (M117,
292 M117' and M119), the panel of MIRU-VNTR was also increased from eight to twelve *loci*. The

293 results obtained for MIRU40 and QUB26 were equal for all the cases under analysis (alleles 2 and 5,
294 respectively). M117 and M117' yielded allele 5 and M119 the allele 4 in ETR-E while M119 had
295 allele 4. No double alleles were detected in this additional panel of genotyped loci.

296 The genotypic profiles from goats (n = 38) were grouped into 11 MIRU types, M132 being the most
297 common (31.6%); cattle exhibited the highest diversity, with 24 genotypic profiles grouping into 15
298 MIRU types, of which M132 was also the most frequent; the single infected sheep also harboured
299 M132; and the four wild boar *M. caprae* isolates generated three profiles. Four MIRU types were thus
300 common to several host species: M70 to wild boar and cattle, M133 to goat and cattle, M132 to goat,
301 cattle and sheep, and M137 was found in goat, cattle and wild boar (Table 1).

302 **3.5. Distribution of MIRU types by geographical location**

303 Among the 12 sampled districts of mainland Portugal, Bragança and Beja yielded more than half of
304 the *M. caprae* genotypic profiles under study and higher diversity of MIRU types (eight and seven,
305 respectively). Four MIRU types were common to different geographical locations: M70 is present in
306 Castelo Branco and Braga; M132 is widespread in the north (Bragança, Vila Real and Viana do
307 Castelo) and was also detected in Lisbon; M133 was frequent in Bragança herds and also found in
308 Lisbon; and M137 is dispersed from north to south, in a total of seven districts (Vila Real, Guarda,
309 Viseu, Castelo Branco Lisboa, Portalegre and Évora). Two singletons were isolated from cattle of the
310 Azores islands (Table 1).

311 **3.6. Clonal complexes defined by MIRU types**

312 A minimum spanning tree (MST) was built with the 64 complete genotypic profiles, grouped in 21
313 MIRU types, using the single locus variant (SLV) analysis, grouping the MIRU types into three clonal
314 complexes (CC, named CC1 to CC3), with the exception of M116, M118, M122, M120 and M140
315 that were not included in any of the CC (Fig. 1a).

316 Clonal complex 1 is dispersed from north to south of mainland Portugal, in a total of ten districts;
317 CC2 is present in six districts; and CC3 is restricted to Beja and Azores islands and exclusively found
318 in cattle. Beja is the single district with MIRU types from the three clonal complexes (Table 1).

319 A detailed inspection was performed for Bragança and Beja districts, as these were the districts with
 320 more TB breakdowns and *M. caprae* isolates, enabling a spatiotemporal analysis. For Bragança, MST
 321 drawn for complete MIRU types, grouped one of five haplotypes in CC1 and two in CC2 (Fig. 1b).
 322 In Beja, the isolates were grouped within one of the three clonal complexes (Fig. 1c): genotypic
 323 profiles from herd BJ1 are clustered in CC1; from BJ2, BJ3 and BJ4 herds in CC3; and from BJ5, in
 324 CC2. Moreover, a temporal separation was evidenced, with isolates from 2003 and 2004 grouping in
 325 CC1, and from 2009 and 2010 in CC2 or CC3 (Fig. 1c).
 326 The Chi-square test was used to compare the proportion of isolates, with complete genotypic profiles,
 327 grouped by clonal complexes, across host species, geographic location and year (Supplementary table
 328 2a to 2d). CC3 was found to be associated to cattle ($p<0.01$).
 329 Geographic location was analysed by district and NUTS II regions: North (Viana do Castelo, Braga,
 330 Porto, Vila Real and Bragança districts), Center (Aveiro, Viseu, Guarda, Castelo Branco, Leiria,
 331 Santarém and Coimbra districts), Alentejo (Portalegre, Évora, Beja and Setúbal districts) and Azores
 332 islands. The analysis of proportion of clonal complexes per NUTS indicated a significant association
 333 of CC1 with Alentejo, CC2 with North region and CC3 with Alentejo and Azores islands. When this
 334 analysis was stratified to the district level, a significant association was registered for CC1 with Évora,
 335 CC2 with Bragança and CC3 with Beja ($p<0.01$).
 336 Three time periods were analysed, considering the epidemiological situation of TB in Portugal:
 337 between 2003 and 2006, in which herd prevalence steadily decreased; between 2007 and 2010, in
 338 which herd prevalence raised more than 3-fold; and between 2011 and 2014, in which there was
 339 substantial reinforcement of ante-mortem and post-mortem vigilance in livestock and carcass
 340 examination of hunted big game became mandatory in specific regions. Statistical significant
 341 associations ($p<0.01$) between time period and clonal complex were confirmed in the case of CC1
 342 330 for the periods 2003-2006 and 2011-2014.

343 **3.7. Association between MIRU types and severity of TB lesions**

344 Histological description of TB compatible lesions was available for 20 animals, including three males
345 and 14 females. Males were less than 2 years old and presented type I or type II lesions. Females' age
346 ranged from 1 to 10 years old and the three types of lesion progression were documented
347 (Supplementary Table 1).

348 *M. caprae* strains isolated from animals with type I lesions belong to M118, M128 or M132 MIRU
349 types and CC2 or CC3; animals with type III lesions exhibited CC1 or CC2 *M. caprae* isolates with
350 M132, M136 or M137 MIRU types. A wider strain variability was registered in animals with type II
351 lesions: M122, M131, M132, M133, M137 or M139, distributed across the three clonal complexes.
352 MIRU types M132 and M137 were common to lesion types II and III (Table 1; Supplementary Table
353 1).

354 Heterogenous outbreaks were common in herds with recurrent infection along two or more years.

355 **3.8. Epidemiological tracing within and between herds**

356 Although information about goat and sheep movement was not available, it was possible to obtain
357 data concerning cattle movement and previous history of TB in infected herds, enabling the
358 assessment of the transmission routes based on *M. caprae* genotypic profiles.

359 For eight of the 21 cattle under study, no movements were recorded prior to slaughter. For most of
360 the remaining animals, the documented movements occurred within the same district.
361 Epidemiological relationships were established among four infected cattle (B7, B8, B9, B10, B12)
362 that circulated up to a 10-year timespan in five herds (BJ2-BJ7) from Beja, sometimes in overlapping
363 periods (Supplementary Fig. 3). It is worthwhile noticing that the MIRU types generated by those
364 cattle isolates were M128, M129, and M131, which are SLVs within CC3, and M134 and M135
365 (included in CC2), which belong to the same animal.

366 Eventhough no records of animal movements were available for goat, M132 was confirmed in at least
367 four herds from Bragança. It cannot be excluded that this strain additionally infected other farms in
368 the region, since for seven animals herd identification was not available (Table 1). Moreover, two
369 cattle from different herds in the same region also harboured M132.

370 4. Discussion

371 Spoligotyping and MIRU-VNTR typing data have been used over the years to study the structure and
372 diversity of *M. bovis* population in Portugal, which is the prevalent ecotype of animal TB in Portugal.
373 Although the number of herds which were sampled several times over this study period is limited
374 (3/28) and information about animal movement was only available for some cattle herds, by assessing
375 the genetic relationships among isolates covering a 12-year surveillance period, the present study was
376 an opportunity to refine the understanding of the epidemiology of *M. caprae* infections, which are
377 much less common across Europe.

378 Irrespective of the host species and geographical region (mainland Portugal or Azores islands), all *M.*
379 *caprae* isolates homogeneously generated spoligotyping profile SB0157. This observation is
380 consistent with previous studies (Duarte *et al.*, 2010; Cunha *et al.*, 2012), confirming that SB0157
381 remains the sole *M. caprae* spoligotyping pattern ever recorded in Portugal and continuing to be
382 dominant in Iberia, since it is also the most commonly detected profile in bordering Spain (Rodríguez
383 *et al.*, 2011). However, in Spain 15 different spoligotyping patterns for *M. caprae* strains were already
384 reported (Rodríguez *et al.*, 2011; García-Jiménez *et al.*, 2016). Also in other countries where *M.*
385 *caprae* is present, usually several spoligotypes are detected, which makes the epidemiological
386 situation in Portugal quite singular.

387 Since spoligotyping did not discriminate infecting strains, MIRU-VNTR typing was thereafter
388 performed to assess intra-herd and intra- and inter-host specific genotypes, further discriminating the
389 *M. caprae* population into 24 MIRU types. Regrettably, the lack of amplification of MIRU4 in three
390 *M. caprae* strains hampered completion of three allelic profiles not included in the MST analyses.
391 Lack of amplification of MIRU4, but also of VNTR3232, MIRU16 and MIRU23, has been reported
392 by other colleagues, when applying MIRU-VNTR typing for molecular characterization of *M. caprae*
393 strains (Navarro *et al.*, 2014; Prodinger *et al.*, 2005). This situation might have been due to either
394 variability in the primer regions or the absence of the locus altogether. Repeated but failed attempts
395 to amplify this locus suggest that the latter hypothesis is plausible. The overall discriminatory index

(D = 0.8856) attained with MIRU-VNTR analyses indicated a good resolution of the selected eight *loci* panel to characterize this *M. caprae* population. ETR-B and ETR-C ($h = 0.0592$) were the least polymorphic, which is in agreement with previous data from animal TB cases in Portugal. In contrast, earlier studies from the country (Duarte *et al.*, 2010, Cunha *et al.*, 2012) pointed out QUB11a, followed by ETR-A and MIRU26, as the most polymorphic, while in this work VNTR3232 revealed as the most discriminatory for *M. caprae* ecotype. Down-sizing the allele diversity analysis for the three administrative regions with more *M. caprae* infection cases (Évora, Bragança, and Beja), discriminatory power of MIRU-VNTR ranged from 0.7 to 0.795, respectively. In Évora, discrimination of the three MIRU types recorded from 2010 to 2013, was achieved with *loci* QUB11a and MIRU4 (one repeat difference in each). The number of occurrences of a given allele for the selected *loci* panel was equal or superior to 24 (out of 30) in the case of Bragança herds, the exception being MIRU4, for which a superior diversity was found related to the discovery of intra-host genotypic variants. Even with recurrent breakdowns from 2007 to 2014, an increase in allelic diversity was, in general, not detected over time in Bragança. These results indicate that, within these regions, breakdowns were homogeneous and epidemiologically related with persistence of a clone in the region, as the genotypic profiles recorded in Évora were collapsed into CC1 and the majority recorded in Bragança belong to CC2. In Beja, a progressive expansion of allelic diversity, related to one to four repetition losses, could be registered from 2003 to 2010 and a temporal division was clear, with *M. caprae* strains from 2003 and 2004 grouping in CC1 and from 2009 onwards in CC2 and CC3. Results for this region suggest multiple sources of infection, together with microevolution of a persistent, recrudescence, clone.

A comprehensive analysis of multilocus genotypes suggests that, even though 24 MIRU types could be distinguished from the 67 genotypic profiles obtained from 55 *M. caprae* strains, most genotypes (16 out of 21) developed from single or double locus variations upon basal types (Fig. 1a), suggesting a clonal structure and supporting that the genetic heterogeneity of *M. caprae* outbreaks under scrutiny is apparent and consistent with the expansion of a founder strain. Single locus variant analysis

evidenced three complexes, with significant statistical association of CC1 to Alentejo, CC2 with Northern region and CC3 with Alentejo and Azores. Analysis of the available epidemiological information, including animal movement that confirmed regular cattle contacts across farms in Alentejo, suggest epidemiological linkage of several outbreaks (re)occurring along a temporal gradient. Our assumptions particularly gain strength in Bragança district wherein most outbreaks and recurrent breakdowns at single herds were genotypically related to a clonal population and M132 was recorded in closely located goat and sheep herds in different time periods. Moreover, two genotypic profiles (M132 and M133) were recorded in the same year in nine animals. Mounting to this complex scenario, numerous *M. caprae* infected hosts were concomitantly infected with two genotypic profiles, whose MIRU types were only distinguished by differences in MIRU4 locus. The spatiotemporal occurrence and frequency of these clonal variants was remarkably high, with 22% of the screened population being affected, and with M132 and M133 as the most frequent genotypic variants. The topology of the MST also leads us to conjecture the presence of an archetypical founder clone circulating in mainland Portugal, with spatiotemporal information reinforcing the notion of this clone possibly undergoing sympatric speciation, mirrored by microevolutionary events associated to the loss of one to two tandem repeats in a single locus. The genotypes of these SLVs strains possibly reflect genomic adaptation events underlying the expansion of the founder strain to different animal species (e.g. CC3 was exclusively found in cattle) and epidemiological scenarios, highlighting the complex transmission dynamics of a multi-host system that sustains *M. caprae* maintenance and spill-over in this study area.

The occurrence of double bands in MIRU4, as observed in 12 *M. caprae* infected animals, hint to co-infection with different strains, although it could not be determined if it was acquired on a single timepoint or on separate occasions, or if it was due to a microevolutionary event on founder strain. However, simultaneous infection of nine animals with two genotypes each seems highly unlikely as does transmission of co-infecting strains. Results rather support the idea of microevolution of an infecting strain inside the host. Application of 12-loci MIRU-VNTR to mycobacterial DNA recovered

448 from these animals showed that, in each animal, the two genotypic profiles only differ in one marker,
449 while DNA sequencing showed that differences between alleles are exclusively related to an INDEL
450 of a tandem repetition, reinforcing the notion of microevolution pressure upon MIRU4. Polyclonal
451 infection markedly differs from co-infection with different strains acquired on a single timepoint or
452 on separate occasions, since these would clearly yield distinct DNA fingerprints. So, our observations
453 are afore consistent with the initial infecting strain undergoing genetic changes, possibly to face
454 challenges at the interface with the host, resulting in the expansion of genotypes and possibly
455 phenotypes. Whether this clonal expansion has influenced virulence or immune evasion is yet
456 unknown, but given the frequency that we registered this phenomenon, we are persuaded by the idea
457 of functional implications arising from microbial genome modulation. No clear trend of virulence
458 increase (using lesion severity of infected animals as proxy) could be inferred in farms with long term
459 and recurrent infections, since our sampling size is limited to assess these trends.

460 The proportion of animals harbouring clonal *M. caprae* isolates was significantly associated with
461 Bragança district but the influence of host species or time span of herd infection was found not to be
462 statistically significant.

463 Reports on the emergence of clonal variants in *M. tuberculosis* infection are sporadic in the literature
464 (Al-Hajoj *et al.*, 2010, Shamputa *et al.*, 2006) and similar studies on animal-adapted ecotypes are
465 scarcer, with the majority being restricted to *M. bovis* (Gormley *et al.* 2014, Navarro *et al.*, 2016,
466 Ghielmetti *et al.*, 2017). Here we confirm that parallel evolution related to tandem repeat repeat loss
467 (or gain) is not restricted to *M. bovis*, being a frequent event in the goat host and occurring both in
468 chronically infected- and also on occasionally-infected herds. Whether intra-host parallel evolution
469 also occurs in other non-SB157 *M. caprae* isolates or is confined to this specific lineage circulating
470 in Portugal is unknown.

471 MIRU4 is found within the intergenic region of an operon encoding in *M. bovis* and *M. tuberculosis*
472 a mycobacterial two component system (Supply *et al.*, 1997). To elaborate on the potential genomic
473 impact resulting from pressure upon MIRU4, we mapped this locus on *M. caprae* Allgaeu and also

474 on *M. bovis* AF2122 and *M. tuberculosis* H37Rv genomes (NZ_CP016401.1, NC_002945.4 and
475 NC_000962.3, respectively). In all these, MIRU4 was located in a region with homology with the
476 hydroxyl terminal moiety encoding the ATP- binding site of *senX3*, a putative two component sensor
477 histidine kinase that is involved in the phosphorylation of *regX3*. MIRU4 region also overlaps the
478 5' region, within the phosphoacceptor site, of a probable DNA-binding response regulator presumed
479 to be involved in signal transduction and apparently common to all MTBC members. Since bacterial
480 two-component system are important in signal transduction, allowing microorganisms to adapt
481 physiological responses to changes in the surrounding environment, it is most likely that any
482 nucleotide loss or gain in these regions could trigger downstream effects, either at the transcriptional
483 and/or translational level, with functional implications.

484 *In vivo* microevolution and co-infection has probably gone underdetected in *M. caprae* infections
485 since first line molecular epidemiology studies are mainly based on spoligotyping and the procedure
486 of genotyping isolates from DNA recovered from a pool of tissues of a single animal is common,
487 originating overlapping fingerprints that cannot be distinguished in the case of co-infection. In this
488 matter, MIRU-VNTR typing may be more robust to differentiate both mixed or clonal populations,
489 although homoplasmy has also been reported for MIRU-VNTR.

490 Results from this work do reinforce the complexity of *M. caprae* epidemiology. More in-depth
491 genomic analyses enabled by last generation high-throughput sequencing platforms may provide
492 further insight into ecological processes underlying transmission and host adaptation. Functional
493 studies in *M. caprae* are also demanded to enable integration of evolutionary, ecological, and
494 functional perspectives able to inspire effective surveillance and control measures in goat herds,
495 including development of relevant legislation, as currently available for *M. bovis* and cattle infections.

496

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516

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635

Table 1. Systematic MIRU-VNTR typing of *M. caprae* isolates.

Geographic location	Herd	Host (ID)	MIRU-VNTR allelic profile								MIRU-VNTR type	Clonal Complex	No genotypic profiles	Nature of outbreak	Year isolation	Source
			VNTR 3232	ETR-A	ETR-B	ETR-C	QUB 11a	QUB 11b	MIRU 4	MIRU 26						
BG	BG2	B3	5	4	3	4	7	2	4	6	M120	-	1		2007	Cunha <i>et al.</i> , 2012
	BG1	G8, G10	5	5	3	4	7	4	4	6	M116	-	2	Heterogeneous (7 genotypes)	2008	Cunha <i>et al.</i> , 2012
		G9	5	5	3	4	7	4	-	6	M117	-	1			
		G11	5	5	3	4	7	4	-	6	M117'	-	1			
		G12	5	3	5	4	7	4	-	2	M119	-	1			
		G13	4	5	3	4	7	4	4	5	M132	2	1		2010	This study
			4	5	3	4	7	4	3	5	M133	2	1			
		G15	4	5	3	4	7	4	4	5	M132	2	1			
	BG3	G21	4	5	3	4	7	4	4	5	M132	2	1		2010	This study
			4	5	3	4	7	4	3	5	M133	2	1			
	BG4	B14	4	5	3	4	7	4	4	5	M132	2	1		2010	This study
			4	5	3	4	7	4	3	5	M133	2	1			
	BG5	S1	4	5	3	4	7	4	4	5	M132	2	1		2010	This study
	N/A	G14	4	5	3	4	7	4	4	5	M132	2	1		2010	This study
			4	5	3	4	7	4	3	5	M133	2	1			
	N/A	G16	4	5	3	4	7	4	4	5	M132	2	1		2010	This study
			4	5	3	4	7	4	3	5	M133	2	1			
	N/A	G18	4	5	3	4	7	4	4	5	M132	2	1		2010	This study
			4	5	3	4	7	4	3	5	M133	2	1			
	N/A	G19	4	5	3	4	7	4	4	5	M132	2	1		2010	This study
			4	5	3	4	7	4	3	5	M133	2	1			
	N/A	G20	4	5	3	4	7	4	4	5	M132	2	1		2010	This study
			4	5	3	4	7	4	3	5	M133	2	1			

	N/A	G22	4 4	5 5	3 3	4 4	7 7	4 4	4 3	5 5	M132 M133	2 2	1 1		2010	This study
	N/A	G17	4	5	3	4	7	4	4	5	M132	2	1		2010	This study
	BG8	G24	4	4	3	4	7	2	5	5	M138	1	1		2012	This study
	BG6	G29	4	5	3	4	7	4	4	5	M132	2	1		2014	This study
	BG7	B21	4	5	3	4	7	4	4	5	M132	2	1		2014	This study
BR	BR1	B1	8	5	3	4	7	4	4	5	M72	2	1		2004	Duarte <i>et al.</i> , 2010
	BR2	B2	8	4	3	4	11	2	4	5	M70	1	1		2006	
VC	VC	B18, B20	4	5	3	4	7	4	4	5	M132	2	2	Homogeneous	2013	This study
VR	VR1	B17	4	4	3	4	7	2	4	5	M137	1	1		2011	This study
	VR2	G28	4	5	3	4	7	4	4	5	M132	2	1		2014	
VS	VS	G25	4	4	3	4	7	2	4	5	M137	1	1		2012	This study
GD	GD	B19	4	4	3	4	7	2	4	5	M137	1	1		2013	This study
AV	AV	B4, B6	8	4	3	4	7	2	0'	5	M118	-	2	Homogeneous	2008	Cunha <i>et al.</i> , 2012
LS	LS1	B5	4 4	5 5	3 3	4 4	7 7	4 4	4 3	5 5	M132 M133	2 2	1 1		2008	This study
	LS2	B15	4	4	3	4	7	2	4	5	M137	1	1		2010	
BJ	BJ1	G1-G6	8	4	3	4	6	2	4	5	M69	1	6	Heterogeneous (2 genotypes)	2003/ 2004 2004	Duarte <i>et al.</i> , 2010
		G7	8	4	3	5	6	2	4	5	M71	1	1			
	BJ2	B7, B8	5	5	3	4	8	4	5	5	M128	3	2	Homogeneous	2009	This study
	BJ3	B9	5	5	3	4	8	4	3	5	M129	3	1		2009	This study
	BJ5	B10	4 4	5 5	3 3	4 4	7 7	4 4	3 4	4 4	M134 M135	2 2	1 1		2009	This study
	BJ4	B12	5	4	3	4	8	4	5	5	M131	3	1		2010	This study
EV	EV3	B13	4	4	3	4	6	2	4	5	M139	1	1		2010	This study

	EV1	G23	4	4	3	4	7	2	3	5	M136	1	1	Heterogeneous (3 genotypes)	2012	This study
		G26	4	4	3	4	7	2	4	5	M137	1	1		2013	
	EV2	G27	4	4	3	4	7	2	4	5	M137	1	1		2013	This study
AZ	AZ1	B11	5	4	3	4	8	2	5	5	M130	3	1		2009	This study
	AZ2	B16	2	4	3	4	1	2	4	5	M140	-	1		2010	This study
CB	N/A	WB1	8	4	3	4	11	2	4	5	M70	1	1	-	2005	Duarte <i>et al.</i> , 2010
		WB2	5	5	4	2	12	2	3	6	M122	-	1	-	2008	
		WB3	4	4	3	4	7	2	4	5	M137	1	1	-	2009	This study
PG	N/A	WB4	4	4	3	4	7	2	4	5	M137	1	1	-	2014	This study
Total	n=35 herds	n _(G) =29 n _(S) =1 n _(B) =21 n _(WB) =4									n= 24 MIRU types	n=3	n=67 genotypic profiles			

Legend: 0' means the absence of a 53 pb additional sequence in MIRU 4 *locus*; - indicates an undetermined allele; N/A – not available.

Host: G - goat, B - bovine, S - sheep and WB - wild boar; animals harbouring two genotypic profiles are marked in bold;

Geographic location: AV - Aveiro, AZ - Azores islands, BR - Braga, BJ - Beja, BG - Bragança, CB - Castelo Branco, EV - Évora, GD - Guarda, LS - Lisboa, PG - Portalegre, VC

- Viana do Castelo, VR - Vila Real and VS – Viseu.

Figure captions

Figure 1. Minimum spanning tree (MST) of (a) the complete *M. caprae* genotypic profiles (n=64); (b) the 14 genotypic profiles from Bragança district; (c) the 15 genotypic profiles from Beja district. MST was based only on complete MIRU-VNTR types, obtained with the single locus variant analysis, using the online application MIRU-VNTR_{plus}. Circle size is proportional to the number of genotypic profiles within each MIRU type; the numbers between two circles represent the number of differences between those MIRU types. Each clonal complex is identified in grey scale as indicated. M117, M117' and M119

are not represented once the number of repetitions for MIRU4 could not be determined. MIRU types of intra-host clonal variants are connected by double dashes.

Figure 2. Distribution and frequency of each clonal complex per geographic location (district as administrative region).

The pie charts show the proportion of *M. caprae* genotypic profiles grouped by clonal complex.

AV - Aveiro, AZ - Açores, BR - Braga, BJ - Beja, BG - Bragança, CB - Castelo Branco, CO - Coimbra, EV - Évora, FR - Faro, LR - Leiria, ST - Santarém, SB - Setúbal, GD - Guarda, LS - Lisboa, PG - Portalegre, PT - Porto, VC - Viana do Castelo, VR - Vila Real and VS – Viseu

Supplementary Figure 1. The geographic distribution and number of *M. caprae* strains from the 55 animals under analysis across the districts of mainland Portugal and Azores islands is evidenced in grey scale. Legend: AV - Aveiro, AZ - Açores, BR - Braga, BJ - Beja, BG - Bragança, CB - Castelo Branco, CO - Coimbra, EV - Évora, FR - Faro, LR - Leiria, ST - Santarém, SB - Setúbal, GD - Guarda, LS - Lisboa, PG - Portalegre, PT - Porto, VC - Viana do Castelo, VR - Vila Real and VS - Viseu.

Supplementary Figure 2. MIRU4 nucleotide sequence alignment of the reference strain *M. bovis* AF2122 and the sequences of allele 4 and allele 3 amplified from *M. caprae* DNA recovered from five different animals. The multiple sequence alignment was performed with ClustalX and each repeat motif of 77 bp is indicated above

Supplementary Figure 3. Cattle circulation in six herds (BJ2-BJ7) from the district of Beja (BJ). Different animals are represented by their ID codes (B#); arrows represent animal movement between herds; the time period (year) at which each animal or group of animals resided at a given herd is indicated. Light grey represents animal movement between herds and white represents the last stop (herd at time of death). The MIRU type of *M. caprae* isolate obtained at time of death is indicated for each animal. The shade of the grey box around each animal is representative of the respective clonal complex, as indicated. Attention to the movement of animal B7 that has two stays in herd BJ2, one in 2006 and another in 2008.

Supplementary Table 1. Available epidemiological information of *M. caprae* infected animals.

ID	Species	Gender	Herd type	Herd management	Herd dimension	Lesion type	MIRU type	Geographic location	Herd
G1	Goat	N/A	Mixed	N/A	C	N/A	M69	BJ	BJ1
G2	Goat	N/A	Mixed	N/A	C	N/A	M69	BJ	BJ1
G3	Goat	N/A	Single	N/A	C	N/A	M69	BJ	BJ1
G4	Goat	N/A	Single	N/A	C	N/A	M69	BJ	BJ1
G5	Goat	N/A	Single	N/A	C	N/A	M69	BJ	BJ1
G6	Goat	N/A	Single	N/A	C	N/A	M69	BJ	BJ1
G7	Goat	N/A	Single	N/A	C	N/A	M71	BJ	BJ1
G8	Goat	N/A	Single	N/A	C	N/A	M116	BG	BG1
G9	Goat	N/A	Single	N/A	C	N/A	M117	BG	BG1
G10	Goat	N/A	Single	N/A	C	N/A	M116	BG	BG1
G11	Goat	N/A	Single	N/A	C	N/A	M117'	BG	BG1
G12	Goat	N/A	Single	N/A	C	N/A	M119	BG	BG1
G13	Goat	Male	Single	N/A	C	N/A	M132 and 133	BG	BG1
G14	Goat	Female	N/A	N/A	N/A	N/A	M132 and 133	BG	N/A
G15	Goat	Female	Single	N/A	N/A	N/A	M132	BG	BG1
G16	Goat	N/A	N/A	N/A	N/A	N/A	M132 and 133	BG	N/A
G17	Goat	N/A	N/A	N/A	N/A	N/A	M132	BG	N/A
G18	Goat	N/A	N/A	N/A	N/A	N/A	M132 and 133	BG	N/A
G19	Goat	N/A	N/A	N/A	N/A	N/A	M132 and 133	BG	N/A
G20	Goat	N/A	N/A	N/A	N/A	N/A	M132 and 133	BG	N/A
G21	Goat	Female	Mixed	Extensive	B	N/A	M132 and 133	BG	BG3
G22	Goat	N/A	N/A	N/A	N/A	N/A	M132 and 133	BG	N/A
G23	Goat	Female	Single	Intensive	C	III	M137 and 140	EV	EV1
G24	Goat	Female	Single	Extensive	C	I	M138	BG	BG8
G25	Goat	Female	Single	Extensive	C	III	M137	VS	VS
G26	Goat	Female	Single	Intensive	C	III	M137	EV	EV1

G27	Goat	Female	Single	Extensive	C	III	M137	EV	EV2
G28	Goat	Female	Single	Extensive	A	II	M132	VR	VR2
G29	Goat	Female	Single	Extensive	A	III	M132	BG	BG6
B1	Bovine	Female	Single	Intensive	N/A	N/A	M72	BR	BR1
B2	Bovine	Female	Single	Intensive	A	N/A	M70	BR	BR2
B3	Bovine	Female	Single	Extensive	A	N/A	M120	BG	BG2
B4	Bovine	Male	Mixed	Intensive	N/A	I	M118	AV	AV
B5	Bovine	Male	Single	N/A	N/A	II	M132 and 133	LS	LS1
B6	Bovine	Male	Mixed	Intensive	N/A	I	M118	AV	AV
B7	Bovine	Female	Single	N/A	B	I	M128	BJ	BJ2
B8	Bovine	Female	Single	N/A	B	I	M128	BJ	BJ2
B9	Bovine	Female	Single	N/A	B	N/A	M129	BJ	BJ3
B10	Bovine	Male	Single	N/A	B	N/A	M134 and 135	BJ	BJ5
B11	Bovine	Male	N/A	N/A	N/A	N/A	M130	AZ	AZ1
B12	Bovine	Female	Single	Extensive	B	II	M131	BJ	BJ4
B13	Bovine	Female	Single	Extensive	C	II	M139	EV	EV3
B14	Bovine	Female	Single	Extensive	A	N/A	M132 and 133	BG	BG4
B15	Bovine	Female	Mixed	N/A	B	II	M137	LS	LS2
B16	Bovine	Female	Mixed	N/A	N/A	N/A	M140	AZ	AZ2
B17	Bovine	Male	N/A	N/A	N/A	N/A	M137	VR	VR1
B18	Bovine	Male	N/A	N/A	N/A	N/A	M132	VC	VC
B19	Bovine	Female	Mixed	N/A	A	III	M137	GD	GD
B20	Bovine	Male	Single	N/A	A	N/A	M132	VC	VC
B21	Bovine	Female	Single	N/A	A	II	M132	BG	BG7
S1	Sheep	Female	Single	Extensive	C	N/A	M132	BG	BG5
WB1	Wild boar	N/A	N/A	N/A	N/A	N/A	M70	CB	N/A
WB2	Wild boar	N/A	N/A	N/A	N/A	II	M122	CB	N/A

WB3	Wild boar	N/A	N/A	N/A	N/A	II	M137	CB	N/A
WB4	Wild boar	N/A	N/A	N/A	N/A	II	M137	PG	N/A

Legend: N/A: not available

Herd dimension: type A, less than 50 animals; type B, between 50 and 150 animals; and type C, more than 150 animals.

Lesion type: type I “caseous granulomatous lesions”, type II “caseous granulomatous lesions with calcification” and type III “caseous granulomatous lesions with cavitation”.

Geographic location: AV - Aveiro, AZ - Azores islands, BR - Braga, BJ - Beja, BG - Bragança, CB - Castelo Branco, EV - Évora, GD - Guarda, LS - Lisboa, PG - Portalegre, VC - Viana do Castelo, VR - Vila Real and VS – Viseu.

Supplementary Table 2a. Data introduced in SPSS for statistical analysis (association between clonal complex and host species).

Clonal complex	Bovine	Caprine	Wild boar	Ovine
CC1	6	13	3	0
CC2	11	20	0	1
CC3	5	0	0	0
Without complex	2	2	1	0

Supplementary Table 2b. Data introduced in SPSS for statistical analysis (association between clonal complex and geographic location - NUTS).

Clonal complex	Alentejo	Azores	Center	North
CC1	13	0	3	6
CC2	2	0	4	26
CC3	4	1	0	0
Without complex	0	1	1	3

Supplementary Table 2c. Data introduced in SPSS for statistical analysis (association between clonal complex and geographic location - districts).

Clonal complex	AV	AZ	BR	BJ	BG	CB	EV	GD	LS	PG	VC	VR	VS
CC1	0	0	2	7	1	2	5	1	1	1	0	1	1
CC2	2	0	0	2	23	0	0	0	2	0	2	1	0
CC3	0	1	0	4	0	0	0	0	0	0	0	0	0
Without complex	0	1	0	0	3	1	0	0	0	0	0	0	0

Legend: AV - Aveiro, AZ - Açores, BR - Braga, BJ - Beja, BG - Bragança, CB - Castelo Branco, CO - Coimbra, EV - Évora, FR - Faro, LR - Leiria, ST - Santarém, SB - Setúbal, GD - Guarda, LS - Lisboa, PG - Portalegre, PT - Porto, VC - Viana do Castelo, VR - Vila Real and VS – Viseu

Supplementary Table 2d. Data introduced in SPSS for statistical analysis (association between clonal complex and time period).

Clonal complex	Period 1	Period 2	Period 3
CC1	10	3	9
CC2	0	27	5
CC3	0	5	0
Without complex	0	5	0

Legend: Time period: period 1 between 2003 and 2006, period 2 between 2007 and 2010, and period 3 between 2011 and 2014.