1 *Mycobacterium avium* subsp. *paratuberculosis* isolated from wild red deer (*Cervus elaphus*) in northern Italy
2

3 Alessia Galiero[1]*, Simone Leo[1], Chiara Garbarino[1], Simone Russo[1], Stefano Giacomelli[2], Alessandro
4 Bianchi[3], Karin Trevisiol[4], Ilda Idrizi[4], Gezim Daka[4], Filippo Fratini[5], Barbara Turchi[5], Domenico Cerri[5],
5 Norma Arrigoni[1], Matteo Ricchi[1]
6
7
8 [1] Istituto Zooprofilattico Sperimentale dell'Emilia Romagna e della Lombardia, Sezione di Piacenza-Gariga,
9 Strada della Faggiola 1, 29027, National Reference Centre for Paratuberculosis, Gariga di Podenzano (PC),
10 Italy

11 [2] Istituto Zooprofilattico Sperimentale dell'Emilia Romagna e della Lombardia, Sezione di Brescia, Via
12 Bianchi 7/9, 25124, Brescia, Italy

13 [3] Istituto Zooprofilattico Sperimentale dell'Emilia Romagna e della Lombardia, Sezione di Sondrio, Via
14 Bormio 30, 23100, Sondrio, Italy

15 [4] Istituto Zooprofilattico Sperimentale delle Venezie, Sezione di Bolzano, Via Laura Conti 4, 39100,
16 Bolzano, Italy

17 [5] Department of Veterinary Sciences, University of Pisa, Viale delle Piagge, 2, 56124 Pisa, Italy

18

19 *Corresponding author: alessia.galiero@izsler.it

20

21 **ABSTRACT**

22 Paratuberculosis (or Johne's disease) is an infectious disease which affects mainly ruminants and it is caused
23 by Mycobacterium avium subsp. paratuberculosis (MAP). During a culling program (years 2011–2015) aimed
24 at controlling the red deer (Cervus elaphus) population in Stelvio National Park (Italian Alps), where para-
25 tuberculosis was already described in this species, 382 tissue samples from the Lombardy Region and 102
26 fecal specimens from the Autonomous Province of Bolzano were analyzed by PCR. Of these, 77 samples
27 (20.16%) from the Lombardy area and 19 specimens (18.63%) from the Bolzano area resulted PCR positive.
28 The cultural test was carried out on PCR positive samples (n = 96), enabling the isolation of 19 MAP field
29 strains which were genotyped using MIRU-VNTR typing and Short Sequence repeats (SSRs).
30 Our results suggest that all isolates share an identical VNTR profile corresponding to the INMV1 genotype.
31 The only variation was on the locus SSR2, but the utility of this last locus has already been questioned because
32 of its instability. Overall, these data suggest a common clonal origin and host adaptation during the diffusion
33 of paratuberculosis in this population. Finally, this profile is the same as that which has already been described

34 in the cattle population in Northern Italy, suggesting a possible inter-species disease transmission pattern from
35 wildlife to domestic ruminants and vice versa

36 Keyword: paratuberculosis; red deer; *Cervus elaphus*; culture; PCR; genotype

37

38 1. INTRODUCTION

39 Paratuberculosis (or Johne's disease) is an infectious disease which affects ruminants and is caused by
40 *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Anonymous, 2014).
41 In Italian wildlife it has been detected in roe deer (Robino et al., 2008), alpine ibex (Ferroglio et al., 2000),
42 wild boar (Zanetti et al., 2008) and red deer in many areas including that investigated in the present study
43 (Ferroglio et al., 2000; Fraquelli et al., 2005; Nebbia et al., 2000; Robino et al., 2008).
44 Infection is usually acquired through fecal-oral and congenital routes (Mackintosh et al., 2004) (Thompson et
45 al., 2007; van Kooten et al., 2006). Notably, the latter commonly occurs in red deer (van Kooten et al.,
46 2006). In this regard, MAP was isolated from 90% of fetuses born from clinically affected hinds (van Kooten
47 et al., 2006) and from 78% of fetuses born from subclinically infected red deer (Thompson et al., 2007).
48 There is also the possibility of inter-species transmission through the ingestion of grass contaminated by
49 infected feces from domestic and wildlife ruminants and non-ruminants, especially wild rabbits (Carta et al.,
50 2013; Daniels et al., 2003a; Daniels et al., 2003b). In this regard, a difference in the MAP load excreted by
51 feces among the species has been described: cattle and sheep can excrete up to 10^8 MAP CFU/g of feces
52 (Cocito et al., 1994; Whittington et al., 2000), while wild rabbits shed up to 7.6 x10^5 MAP CFU/g of feces
53 (Daniels et al., 2003a).
54 In deer, signs of illness include weight loss, poor body condition and diarrhea with fecal staining around the
55 perineum and hindquarters. Notably, during the differential diagnosis, also yersiniosis, abomasal parasitism,
56 avian tuberculosis and chronic malignant catarrhal fever should be taken into account (Mackintosh, 1998).
57 During post-mortem examination, clinically affected deer frequently show enlargement of mesenteric lymph
58 nodes, often with caseous lesions, prominent lymphatic drainage vessels from the jejuneum to adjacent
59 lymph nodes and omentum’s oedema; in addition, unlike cattle and sheep, ileum does not always show
60 thickening. Microscopic lesions are characterized by the presence of acid-fast laden macrophages in affected
61 lymph nodes, often with caseation and foci of calcification. In addition, the ileocecal valve often shows loss

62 of villous structure and contains mixed cellular infiltrate and numerous acid-fast organisms; notably,
63 analogous lesions are detectable in ileum and jejunum (Mackintosh et al., 2004).
64 In order to improve the knowledge about the wildlife and its role in the transmission of the disease to cattle,
65 our study was aimed at sub-typing MAP field strains isolated from the wild red deer population which
66 inhabits Stelvio National Park (Italy), by a combination of MIRU-VNTR and Short Sequence Repeats
67 sequencing (SSRs), as previously described (Ricchi et al., 2011).
68
69 **2. MATERIALS AND METHODS**
70 **2.1 Sampling**
71 The sampling plan was designed to include all the subjects culled during a program aimed at controlling the
72 red deer population in Stelvio National Park from 2011 to 2015, in Lombardy Region (n= 382 tissues) and in
73 the area of the Autonomous Province of Bolzano (n=102 feces and tissues) (Figure 1). The animal age was
74 estimated based on the teeth eruption patterns: deer until two years old were classified as yearlings, while
75 those with more than two years as adults.
76
77 **2.2 Post-mortem examination**
78 The post-mortem inspection was carried out on all subjects: signs of illness including weight loss, poor body
79 condition and diarrhea and any visible lesion in mesenteric lymph nodes and gastrointestinal tract were
80 recorded. With reference to this, macroscopic lesions were graded according to the classification described
81 by Fraquelli (Fraquelli et al., 2005).
82
83 **2.3 qPCR and cultural assay**
84 **Lombardy area**
85 DNA extraction and quantitative PCR targeting IS900 (Ricchi et al., 2014) was carried out by examining
86 tissue samples (pool consisting of ileal mucosa, ileocecal valve and mesenteric lymph nodes). Positive
87 samples were then cultured in VersaTREK® system and on Herrold's egg yolk medium (HEYM)
88 supplemented with mycobactin J (ID.VET, Montpellier, France) as reported in the OIE manual (Anonymous,
89 2014; Savi et al., 2015).

The Autonomous Province of Bolzano area

DNA extraction and quantitative PCR targeting IS900 were performed on fecal specimens (n=102) according to the protocols described by (Pozzato et al., 2011b) and by (Pozzato et al., 2011a), respectively. From fecal PCR positive animals, a pool of tissue samples consisting of ileal mucosa, ileocecal valve and mesenteric lymph nodes was cultured on Herrold's egg yolk medium (HEYM) supplemented with mycobactin J (ID.VET, Montpellier, France) and on Middlebrook 7H11 medium (M7H11) supplemented with mycobactin J (ID.VET, Montpellier, France), according to the protocol described by Whittington (Whittington et al., 1999) which was slightly modified according to (Galiero et al., 2017).

2.4 Typing and sub-typing of MAP field isolates

The confirmation of isolates was performed with f57-qPCR (Ricchi et al., 2014); subsequently, they were typed by DMC PCR for the assignment to type S or C (Collins et al., 2002) and subtyped according to the procedures described by (Ricchi et al., 2011). In particular, ten mini-satellite loci were analyzed: VNTR1067 and VNTR3527 (Overduin et al., 2004), VNTR25, VNTR47, VNTR292, VNTRX3, VNTR3, VNTR7, VNTR10 and VNTR32 (Thibault et al., 2007). Moreover, three micro-satellite loci, SSR1, SSR2 and SSR8 were also investigated (Amonsin et al., 2004). Because of technical reasons, for four and two strains it was not possible to obtain reproducible results for the loci SSR8 and SSR1, respectively.

2.5 Statistical analysis

In order to highlight an association between disease's prevalence and age, the statistical differences in positivity rates between two stratification groups of animals ($\leq$ 2 years versus $>$ 2 years) was tested with the chi-squared test using a free online software available at http://www.socscistatistics.com; differences were considered significant if *p-value* was < 0.05.

In addition, apparent prevalences of paratuberculosis in the investigated areas were calculated and compared (CI 95%) using the Wilson method by a free online software available at http://epitools.ausvet.com.au.

3. RESULTS AND DISCUSSION

118 In the Lombardy area, out of 382 investigated deer, 77 were PCR positive, giving an apparent prevalence of
119 20.16% (CI 95% 16.44 - 24.47), while in the Bolzano area, out of 102 fecal samples, 19 were PCR positive,
120 with an apparent prevalence of 18.63% (CI 95% 12.26 - 27.27); no statistical difference was observed between
121 the two areas. The slightly lower prevalence rate recorded in Bolzano area could be attributed to the different
122 diagnostic matrices. In fact, sub-clinically infected deer can only intermittently shed MAP and the
123 consequently low load in feces could lead to a false negative result despite the animal is truly affected
124 (Maroudam et al., 2015).
125 The results suggested that yearlings showed a higher risk of infection compared to adults, although the
126 comparison of the two age categories showed no statistic difference in the disease's prevalence between
127 yearlings (5/16, 31.25%) and adults (14/86, 16.28%) in Bolzano area (χ^2= 1.99; *p-value* = 0.157) and yearlings
128 (41/199, 20.60%) and adults (36/183, 19.67%) in Lombardy area (χ^2=0.051; *p-value* = 0.82). A possible
129 explanation for the greater number of positive subjects in the yearlings population rather than in older animals,
130 could be the quick progression from infection to clinical signs (Carta et al., 2013), and the subsequent increase
131 in the rate of mortality; in this regard, as suggested by (Fraquelli et al., 2005), in a deer's population exposed
132 to MAP, the lower disease's prevalence of the adults subjects could be attributed to the death of the younger
133 animals.
134 Pathological examination revealed most of the infected subjects did not show macroscopic lesions, while
135 lesions associated with MAP infection were recorded in few subjects (Table 1). The importance and extensions
136 of lesions, according to the scheme proposed by (Fraquelli et al., 2005) varied from limited affection of
137 lymphatic system to chronic enteritis with thickening of the wall and wrinkling of the mucosa associated with
138 severe enlargement of mesenteric lymph nodes (Table 1) (Figure 2, 3 and 4).
139 By DMC PCR, all MAP isolates resulted as type C, which is considered more virulent for red deer than type
140 S. In fact, in domestic deer, it has been demonstrated by cultural examination of tissue samples that oral
141 inoculation with type C strain determines a higher infection rate (100%) than type S strain (69%) (Mackintosh
142 et al., 2007; O'Brien et al., 2006). Moreover, type C strains induce a higher immunological response (O'Brien
143 et al., 2006), a lower weight gain, more serious histopathological lesions and higher seropositive rates (ELISA)
144 (Mackintosh et al., 2007) than type S. Comparing this finding to those of similar studies previously performed
145 in Northern Italy (Fraquelli et al., 2005; Nebbia et al., 2000; Robino et al., 2008), it can be stated that in our

146 country red deer population is infected with C type strains. Furthermore, from an epidemiological point of
147 view, the detection of MAP in wildlife red deer population points out to a possible inter-species disease
148 transmission from wildlife to domestic ruminants and *vice versa.* In fact, since clinically infected deer can shed
149 5×10^6 MAP CFU/g (Schroen et al., 2003) and cows over than 10^8 MAP CFU/g of feces (Cocito et al., 1994),
150 animals can become infected grazing on pastures contaminated with infected feces (Carta et al., 2013; Daniels
151 et al., 2003a; Daniels et al., 2003b). In addition, C strains not only don't show host preference and have been
152 isolated from a very broad host range of ruminant and nonruminant wildlife animals, but they are also the
153 predominant type isolated from bovine specimens, not only in Italy (Ricchi et al., 2011), but also in all
154 countries of the world (Stevenson, 2015).
155 However, it is not possible to exclude that the isolation of only one type in our data could be due to the better
156 cultivability of this strain type; in fact, C strains are more easily isolated from clinical samples on solid and
157 liquid media than S strains. On the other hand, this finding could be due to the higher infection ability of C
158 strains and, particularly, to their higher capacity for infecting and surviving in macrophages compared with S
159 strains (Stevenson, 2015).
160 To the best of our knowledge, this is the first time that strains isolated from Italian red deer population have
161 been sub-genotyped and all isolates showed the same MIRU-VNR pattern profile (Table 2).
162 Furthermore, Mini-satellite loci analysis revealed the presence of the INMV1 genotype and up until now, the
163 INMV1 profile has been already described before in Italy only in cattle herds (Ricchi et al., 2011). On the
164 other hand, INMV1 has been previously detected in red deer in the Czech Republic, The Netherlands
165 (Stevenson et al., 2009), Germany (Fritsch et al., 2012) and in Austria (Gerritsmann et al., 2014) and,
166 according to the MAC-INMV database (http://mac-inmv.tours.inra.fr/), it is the second most diffused profile
167 in the world.
168 Moreover, short sequence analysis of two loci highlighted the presence of one dominant MAP subtype
169 (Table 2). In fact, all the isolates carried identical numbers of repeats in SSR1 and SSR8 loci and differed
170 only for a few bases at the SSR2 locus. A possible explanation for the difference found out in this locus
171 could be its instability, as previously suggested by (Ricchi et al., 2011) and investigated by (Kasnitz et al.,
172 2013). With reference to this, the application of SSR analysis could help to improve the knowledge of the

173 genetic distribution of MAP subtypes improving epidemiological data, as suggested by (Motiwala et al.,
174 2006).
175 Notably, the same MIRU-VNTR pattern and the same SSR profile were detected in Germany (Fritsch et al.,
176 2012), not only in a wild living deer population, but also in farmed cattle living in the same territory,
177 suggesting the possibility of interspecies transmission for this genotype. According to (Fritsch et al., 2012),
178 tracking MAP transmission by VNTR typing and SSR analysis among wild red deer and farmed cattle could
179 be the most cost-effective and efficient strategy for discriminating strains and highlighting any possible host
180 relation among genotypes which infected wildlife ruminant and those isolated from domestic species. In our
181 case, this analysis suggests how the field isolates here recovered, can have a common clonal origin.
182 However, a convergent evolution of VNTR loci has been recently described by (Ahlstrom et al., 2015), in
183 which the authors underlined how "caution should be used when using VNTR typing as a tool to assess the
184 diversity and relatedness of MAP isolates at both a national and herd-level". For this reason, we believe
185 whole genome sequencing data can probably be the best approach to investigate the clonal origin hypothesis
186 since, as previously reported also for *Mycobacterium bovis* (Biek et al., 2012), this last method provides the
187 highest resolution for such task.
188 In conclusion, more studies should be carried out to understand the epidemiological role of wildlife in the
189 transmission of paratuberculosis, including also nonruminant wildlife in the research, so that control
190 strategies could be adopted to avoid interspecific transmission. The use of more powerful methodologies,
191 such as whole genome sequencing approaches coupled with mathematical modelling (Biek et al., 2012),
192 could provide new insights in the epidemiology of paratuberculosis in wildlife animals.
193

194 **4. CONFLICT OF INTEREST STATEMENT**

195 The authors declare that there is no conflict of interest regarding the publication of this paper.
196

197 **5. ACKNOWLEDGEMENTS**

198 This work was supported partially by Public Health Service grant E87G12000160001- PRC2011012 from
199 the Italian Ministry of Health. We thank Dr. Savi Roberto for his help during the collection of the field
200 isolates and MAP subtyping and Mr. Bryan Manchi for his valuable revision of the English text.
201
202

203 **6. REFERENCES**

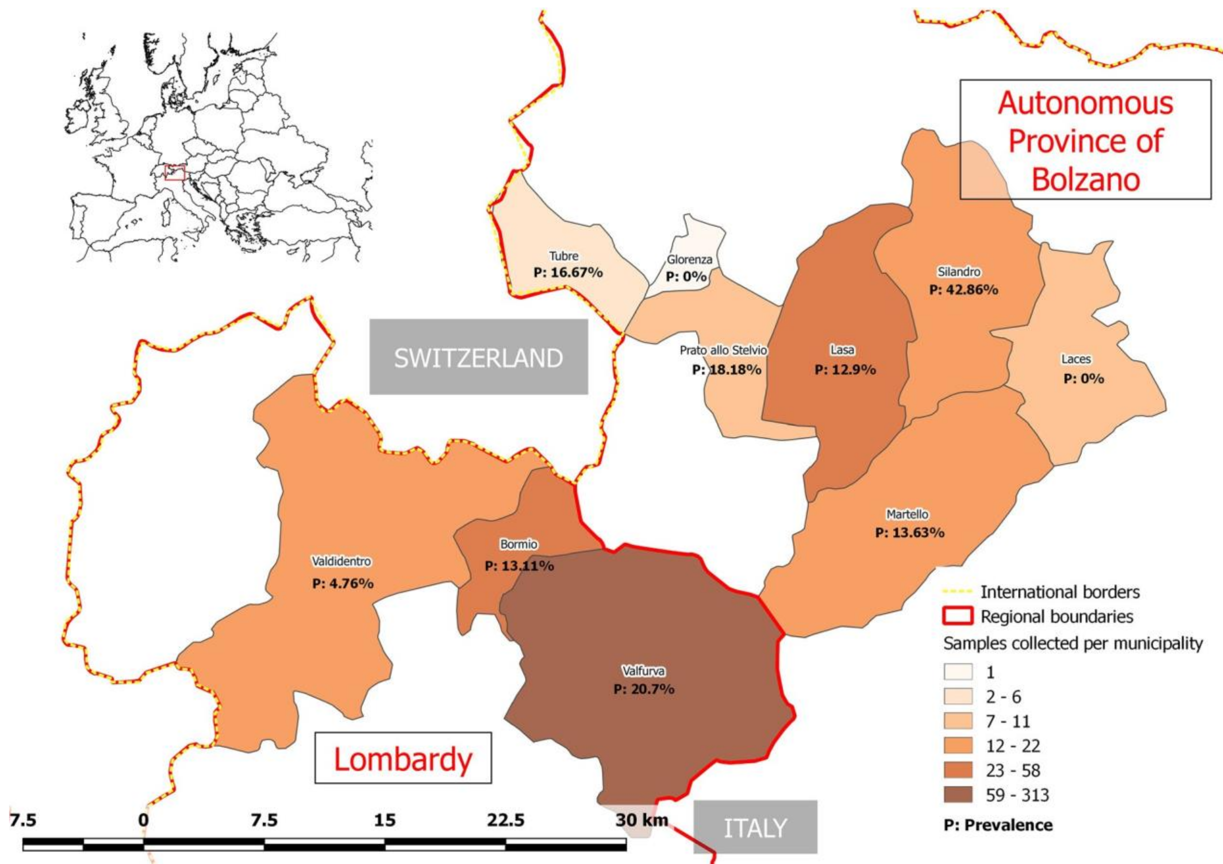
204 Ahlstrom, C., Barkema, H.W., Stevenson, K., Zadoks, R.N., Biek, R., Kao, R., Trewby, H., Haupstein, D.,
205 Kelton, D.F., Fecteau, G., Labrecque, O., Keefe, G.P., McKenna, S.L., De Buck, J., 2015.
206 Limitations of variable number of tandem repeat typing identified through whole genome
207 sequencing of Mycobacterium avium subsp. paratuberculosis on a national and herd level. BMC
208 Genomics 16, 161.
209 Amonsin, A., Li, L.L., Zhang, Q., Bannantine, J.P., Motiwala, A.S., Sreevatsan, S., Kapur, V., 2004.
210 Multilocus short sequence repeat sequencing approach for differentiating among Mycobacterium
211 avium subsp. paratuberculosis strains. J Clin Microbiol 42, 1694-1702.
212 Anonymous 2014. Chapter 2.11.1. Paratuberculosis (Johne's Disease) (OIE – World Organisation for animal
213 health. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals), 16.
214 Biek, R., O'Hare, A., Wright, D., Mallon, T., McCormick, C., Orton, R.J., McDowell, S., Trewby, H., Skuce,
215 R.A., Kao, R.R., 2012. Whole genome sequencing reveals local transmission patterns of
216 Mycobacterium bovis in sympatric cattle and badger populations. PLoS Pathog 8, e1003008.
217 Carta, T., Alvarez, J., Perez de la Lastra, J.M., Gortazar, C., 2013. Wildlife and paratuberculosis: a review.
218 Res Vet Sci 94, 191-197.
219 Cocito, C., Gilot, P., Coene, M., de Kesel, M., Poupart, P., Vannuffel, P., 1994. Paratuberculosis. Clin
220 Microbiol Rev 7, 328-345.
221 Collins, D.M., De Zoete, M., Cavaignac, S.M., 2002. Mycobacterium avium subsp. paratuberculosis strains
222 from cattle and sheep can be distinguished by a PCR test based on a novel DNA sequence
223 difference. J Clin Microbiol 40, 4760-4762.
224 Daniels, M.J., Henderson, D., Greig, A., Stevenson, K., Sharp, J.M., Hutchings, M.R., 2003a. The potential
225 role of wild rabbits Oryctolagus cuniculus in the epidemiology of paratuberculosis in domestic
226 ruminants. Epidemiol Infect 130, 553-559.
227 Daniels, M.J., Hutchings, M.R., Beard, P.M., Henderson, D., Greig, A., Stevenson, K., Sharp, J.M., 2003b.
228 Do non-ruminant wildlife pose a risk of paratuberculosis to domestic livestock and vice versa in
229 Scotland? J Wildl Dis 39, 10-15.
230 Ferroglio, E., Nebbia, P., Robino, P., Rossi, L., Rosati, S., 2000. Mycobacterium paratuberculosis infection
231 in two free-ranging Alpine ibex. Rev Sci Tech 19, 859-862.
232 Fraquelli, C., Carpi, G., Bregoli, M., Ostanello, F., Pasolli, C., Pozzato, N., Rosati, S. 2005. Epidemiology of
233 paratuberculosis in two red deer (C*ervus elaphus)* populations of Trentino (Northern Italy). In:
234 Proceedings of the 8th International Colloquium on Paratuberculosis, Copenhagen, Denmark, 605-
235 612.
236 Fritsch, I., Luyven, G., Kohler, H., Lutz, W., Mobius, P., 2012. Suspicion of Mycobacterium avium subsp.
237 paratuberculosis transmission between cattle and wild-living red deer (Cervus elaphus) by
238 multitarget genotyping. Appl Environ Microbiol 78, 1132-1139.
239 Galiero, A., Turchi, B., Pedonese, F., Nuvoloni, R., Cantile, C., Colombani, G., Forzan, M., Cerri, D.,
240 Bandecchi, P., Fratini, F., 2017. Serological, culture and molecular survey of Mycobacterium avium
241 paratuberculosis in a goat flock in Tuscany. Folia Microbiol (Praha).
242 Gerritsmann, H., Stalder, G.L., Spergser, J., Hoelzl, F., Deutz, A., Kuebber-Heiss, A., Walzer, C., Smith, S.,
243 2014. Multiple strain infections and high genotypic diversity among Mycobacterium avium subsp.
244 paratuberculosis field isolates from diseased wild and domestic ruminant species in the eastern
245 Alpine region of Austria. Infect Genet Evol 21, 244-251.
246 Kasnitz, N., Kohler, H., Weigoldt, M., Gerlach, G.F., Mobius, P., 2013. Stability of genotyping target
247 sequences of Mycobacterium avium subsp. paratuberculosis upon cultivation on different media, in
248 vitro- and in vivo passage, and natural infection. Vet Microbiol 167, 573-583.
249 Mackintosh, C.G., 1998. Deer health and disease. Acta Vet Hung 46, 381-394.
250 Mackintosh, C.G., de Lisle, G.W., Collins, D.M., Griffin, J.F., 2004. Mycobacterial diseases of deer. N Z
251 Vet J 52, 163-174.
252 Mackintosh, C.G., Labes, R.E., Clark, R.G., de Lisle, G.W., Griffin, J.F., 2007. Experimental infections in
253 young red deer (Cervus elaphus) with a bovine and an ovine strain of Mycobacterium avium subsp
254 paratuberculosis. N Z Vet J 55, 23-29.
255 Maroudam, V., Mohana Subramanian, B., Praveen Kumar, P., Dhinakar Raj, G., 2015. Paratuberculosis:
256 Diagnostic Methods and their Constraints | Open Access Journals. Journal of Veterinary Science &
257 Technology 6, 1.

258 Motiwala, A.S., Li, L., Kapur, V., Sreevatsan, S., 2006. Current understanding of the genetic diversity of
259 Mycobacterium avium subsp. paratuberculosis. Microbes Infect 8, 1406-1418.
260 Nebbia, P., Robino, P., Ferroglio, E., Rossi, L., Meneguz, G., Rosati, S., 2000. Paratuberculosis in red deer
261 (Cervus elaphus hippelaphus) in the western Alps. Vet Res Commun 24, 435-443.
262 O'Brien, R., Mackintosh, C.G., Bakker, D., Kopecna, M., Pavlik, I., Griffin, J.F., 2006. Immunological and
263 molecular characterization of susceptibility in relationship to bacterial strain differences in
264 Mycobacterium avium subsp. paratuberculosis infection in the red deer (Cervus elaphus). Infect
265 Immun 74, 3530-3537.
266 Overduin, P., Schouls, L., Roholl, P., van der Zanden, A., Mahmmod, N., Herrewegh, A., van Soolingen, D.,
267 2004. Use of multilocus variable-number tandem-repeat analysis for typing Mycobacterium avium
268 subsp. paratuberculosis. J Clin Microbiol 42, 5022-5028.
269 Pozzato, N., Gwozdz, J., Gastaldelli, M., Capello, K., Dal Ben, C., Stefani, E., 2011a. Evaluation of a rapid
270 and inexpensive liquid culture system for the detection of Mycobacterium avium subsp.
271 paratuberculosis in bovine faeces. J Microbiol Methods 84, 413-417.
272 Pozzato, N., Stefani, E., Capello, k., Muliari, R., Vincenzoni, G., 2011b. Mycobacterium avium
273 subsp.paratuberculosis as a template in the evaluation of automated kits for DNA extraction from
274 bovine organs. World J. Microbiol. Biotechnol. 27, 31-37.
275 Ricchi, M., Barbieri, G., Taddei, R., Belletti, G.L., Carra, E., Cammi, G., Garbarino, C.A., Arrigoni, N.,
276 2011. Effectiveness of combination of Mini-and Microsatellite loci to sub-type Mycobacterium
277 avium subsp paratuberculosis Italian type C isolates. Bmc Veterinary Research 7.
278 Ricchi, M., De Cicco, C., Kralik, P., Babak, V., Boniotti, M.B., Savi, R., Cerutti, G., Cammi, G., Garbarino,
279 C., Arrigoni, N., 2014. Evaluation of viable Mycobacterium avium subsp paratuberculosis in milk
280 using peptide-mediated separation and Propidium Monoazide qPCR. Fems Microbiology Letters
281 356, 127-133.
282 Robino, P., Nebbia, P., Tramuta, C., Martinet, M., Ferroglio, E., De Meneghi, D., 2008. Identification of
283 Mycobacterium avium subsp. paratuberculosis in wild cervids (Cervus elaphus hippelaphusand
284 Capreolus capreolus) from Northwestern Italy. Eur. J. Wildl. Res. 54, 357-360.
285 Savi, R., Ricchi, M., Cammi, G., Garbarino, C., Leo, S., Pongolini, S., Arrigoni, N., 2015. Survey on the
286 presence of Mycobacterium avium subsp. paratuberculosis in ground beef from an industrial meat
287 plant. Vet Microbiol 177, 403-408.
288 Schroen, C., Bradley, T., McDonald, W., Condron, R. 2003. Diagnostic tests for Johne's Disease in deer.
289 (Rural Industries Research and Development Corporation report. Australian Government, Victoria,
290 Australia).
291 Stevenson, K., 2015. Genetic diversity of Mycobacterium avium subspecies paratuberculosis and the
292 influence of strain type on infection and pathogenesis: a review. Vet Res 46, 64.
293 Stevenson, K., Alvarez, J., Bakker, D., Biet, F., de Juan, L., Denham, S., Dimareli, Z., Dohmann, K.,
294 Gerlach, G.F., Heron, I., Kopecna, M., May, L., Pavlik, I., Sharp, J.M., Thibault, V.C., Willemsen,
295 P., Zadoks, R.N., Greig, A., 2009. Occurrence of Mycobacterium avium subspecies paratuberculosis
296 across host species and European countries with evidence for transmission between wildlife and
297 domestic ruminants. BMC Microbiol 9, 212.
298 Thibault, V.C., Grayon, M., Boschiroli, M.L., Hubbans, C., Overduin, P., Stevenson, K., Gutierrez, M.C.,
299 Supply, P., Biet, F., 2007. New variable-number tandem-repeat markers for typing Mycobacterium
300 avium subsp. paratuberculosis and M. avium strains: comparison with IS900 and IS1245 restriction
301 fragment length polymorphism typing. J Clin Microbiol 45, 2404-2410.
302 Thompson, B.R., Clark, R.G., Mackintosh, C.G., 2007. Intra-uterine transmission of Mycobacterium avium
303 subsp paratuberculosis in subclinically affected red deer (Cervus elaphus). N Z Vet J 55, 308-313.
304 van Kooten, H.C., Mackintosh, C.G., Koets, A.P., 2006. Intra-uterine transmission of paratuberculosis
305 (Johne's disease) in farmed red deer. N Z Vet J 54, 16-20.
306 Whittington, R.J., Marsh, I., McAllister, S., Turner, M.J., Marshall, D.J., Fraser, C.A. 1999. Evaluation of
307 Modified BACTEC 12B Radiometric Medium and Solid Media for Culture of Mycobacterium
308 avium subsp. paratuberculosis from Sheep, In: J Clin Microbiol. 1077-1083.
309 Whittington, R.J., Reddacliff, L.A., Marsh, I., McAllister, S., Saunders, V., 2000. Temporal patterns and
310 quantification of excretion of Mycobacterium avium subsp paratuberculosis in sheep with Johne's
311 disease. Aust Vet J 78, 34-37.
312 Zanetti, S., Bua, A., Molicotti, P., Delogu, G., Mura, A., Ortu, S., Sechi, L.A., 2008. Identification of
313 mycobacterial infections in wild boars in Northern Sardinia, Italy. Acta Vet Hung 56, 145-152.

314

315 Fig. 1. Comparison of samples collected and disease prevalence per municipalities. The map shows the
316 location of the Lombardy Region and the Autonomous Province of Bolzano (Northern Italy) and the
317 municipalities into which they are divided. Coloured areas indicate the municipalities in which animals were
318 sampled during the study period. The colours are proportional to the number of samples collected. Disease
319 prevalence for each municipality (in bold) is included

320



321

322

323 Table 1 Prevalence of macroscopic lesions observed in Yearlings and Adults positive to PCR assay carried out on tissue specimens (Lombardy Region, n = 77)
324 and fecal samples (Autonomous Province of Bolzano, n = 19). The lesions are classified according to the degree of severity. The number and the percentage of
325 deer with lesions within each age class are reported.

326

327

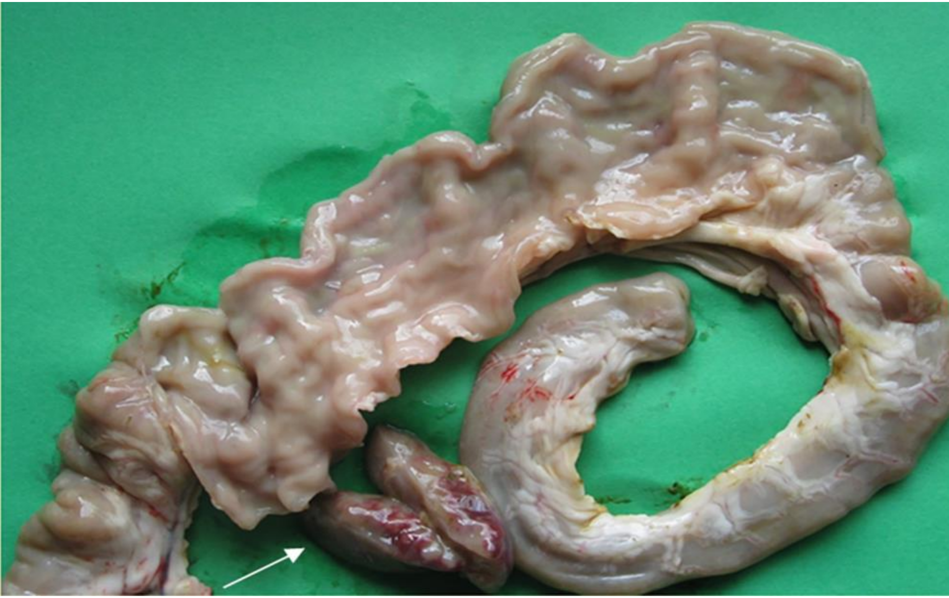
Region of sampling	Age class	Lesion status*					
		NL	A	B	C1	C2	C3
			Minimal	Mild	Moderate		Severe
Lombardy	Yearling (n = 41)	35 (85.36%)	1 (2.44%)	1 (2.44%)	2 (4.88%)	0 (0%)	2 (4.88%)
	Adults (n = 36)	28 (77.78%)	5 (13.89%)	3 (8.34%)	0 (0%)	0 (0%)	0 (0%)
Autonomous Province of Bolzano	Yearlings (n = 5)	1 (20%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	4 (80%)
	Adults (n = 14)	12 (85.71%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (14.28%)

328

329 * NL = no macroscopic lesions; A = lesions limited to lymphatic system with hyperplasia of Peyer patches, enlargement of mesenteric lymph nodes
330 and/or lymphangectasia; B = catarrhal or catarrhal-haemorrhagic ileitis; C1 = catarrhal or catarrhal-haemorrhagic enterocolitis and typhlitis; C2 = chronic
331 enterocolitis and typhlitis with thickening of the intestinal wall and corrugation of the mucosa; C3 = lesions from both class C1 and C2 associated with severe
332 enlargement of mesenteric lymph nodes.

333

334 Fig. 2. Red deer. Isolated segment of small intestine showing marked thickening of the intestine wall and
335 corrugation of the mucosa. Also note enlargement of lymph node (arrow).



336
337

Table 2 Genotypes of MAP obtained by MIRU-VNTR and Short Sequence repeats (SSRs) from tissue samples collected from yearlings and adults positive to PCR assay carried out on tissue specimens (Lombardy Region) and fecal samples (Autonomous Province of Bolzano). Ten mini-satellite loci were analyzed: VNTR1067 and VNTR3527, VNTR25, VNTR47, VNTR292, VNTRX3, VNTR3, VNTR7, VNTR10 and VNTR32. Moreover, three micro-satellite loci, SSR1, SSR2 and SSR8 were also investigated

Region of	ID strain[a]	ID animal	sex	age	No. of copies MLVA-VNTR								No. of copies SSR			INMV profile[b]
					VNTR 292	MLVA 1658[c]	VNTR 25	VNTR 47	VNTR 3	VNTR 7	VNTR 10	VNTR 32	SSR 1	SSR 2	SSR 8	
Lombardy	1	A	f	6 mos.	4	2	3	3	2	2	2	8	7	11	4	1
	2	B	f	1 yr.	4	2	3	3	2	2	2	8	7	10	4	1
	3	C	m	4 yrs.	4	2	3	3	2	2	2	8	7	11	NA	1
	4	D	m	6 mos.	4	2	3	3	2	2	2	8	7	10	4	1
	5	E	f	7 yrs.	4	2	3	3	2	2	2	8	7	10	4	1
	6	F	f	1 yr.	4	2	3	3	2	2	2	8	7	10	4	1
	7	G	f	1 yr.	4	2	3	3	2	2	2	8	7	10	NA	1
	8	H	f	1 yr.	4	2	3	3	2	2	2	8	NA	NA	NA	1
	9	I	m	1 yr.	4	2	3	3	2	2	2	8	7	12	4	1
	10	L	m	1 yr.	4	2	3	3	2	2	2	8	7	10	4	1
	11	M	m	2 yrs.	4	2	3	3	2	2	2	8	NA	9	NA	1
Autonomous Province of Bolzano	12	N	f	5–6 yrs.	4	2	3	3	2	2	2	8	7	11	4	1
	13	O	f	3–4 yrs.	4	2	3	3	2	2	2	8	7	10	4	1
	14	P	m	1 yr.	4	2	3	3	2	2	2	8	7	12	4	1
	15	Q	m	1 yr.	4	2	3	3	2	2	2	8	7	12	4	1
	16	R	m	1 yr.	4	2	3	3	2	2	2	8	7	13	4	1
	17	S	f	3–4 yrs.	4	2	3	3	2	2	2	8	7	10	4	1
	18	T	f	1 yr.	4	2	3	3	2	2	2	8	7	12	4	1
	19	U	f	5–8 yrs.	4	2	3	3	2	2	2	8	7	12	4	1

m = male; f = female; NA = not available.

a From each animal it was possible to isolate only one strain.

b According to MAC-INMV Database (http://mac-inmv.tours.inra.fr/).

c Alias X3.