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

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ABSTRACT

Paratuberculosis (or Johne’s disease) is an infectious disease which affects mainly ruminants and it is caused by Mycobacterium avium subsp. paratuberculosis (MAP). During a culling program (years 2011–2015) aimed at controlling the red deer (Cervus elaphus) population in Stelvio National Park (Italian Alps), where paratuberculosis was already described in this species, 382 tissue samples from the Lombardy Region and 102 fecal specimens from the Autonomous Province of Bolzano were analyzed by PCR. Of these, 77 samples (20.16%) from the Lombardy area and 19 specimens (18.63%) from the Bolzano area resulted PCR positive. The cultural test was carried out on PCR positive samples (n = 96), enabling the isolation of 19 MAP field strains which were genotyped using MIRU-VNTR typing and Short Sequence repeats (SSRs).

Our results suggest that all isolates share an identical VNTR profile corresponding to the INMV1 genotype. The only variation was on the locus SSR2, but the utility of this last locus has already been questioned because of its instability. Overall, these data suggest a common clonal origin and host adaptation during the diffusion of paratuberculosis in this population. Finally, this profile is the same as that which has already been described...
in the cattle population in Northern Italy, suggesting a possible inter-species disease transmission pattern from wildlife to domestic ruminants and vice versa

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

1. INTRODUCTION

Paratuberculosis (or Johne's disease) is an infectious disease which affects ruminants and is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Anonymous, 2014).

In Italian wildlife it has been detected in roe deer (Robino et al., 2008), alpine ibex (Ferroglio et al., 2000), wild boar (Zanetti et al., 2008) and red deer in many areas including that investigated in the present study (Ferroglio et al., 2000; Fraquelli et al., 2005; Nebbia et al., 2000; Robino et al., 2008).

Infection is usually acquired through fecal-oral and congenital routes (Mackintosh et al., 2004) (Thompson et al., 2007; van Kooten et al., 2006). Notably, the latter commonly occurs in red deer (van Kooten et al., 2006). In this regard, MAP was isolated from 90% of fetuses born from clinically affected hinds (van Kooten et al., 2006) and from 78% of fetuses born from subclinically infected red deer (Thompson et al., 2007).

There is also the possibility of inter-species transmission through the ingestion of grass contaminated by infected feces from domestic and wildlife ruminants and non-ruminants, especially wild rabbits (Carta et al., 2013; Daniels et al., 2003a; Daniels et al., 2003b). In this regard, a difference in the MAP load excreted by feces among the species has been described: cattle and sheep can excrete up to $10^8$ MAP CFU/g of feces (Cocito et al., 1994; Whittington et al., 2000), while wild rabbits shed up to $7.6 \times 10^5$ MAP CFU/g of feces (Daniels et al., 2003a).

In deer, signs of illness include weight loss, poor body condition and diarrhea with fecal staining around the perineum and hindquarters. Notably, during the differential diagnosis, also yersiniosis, abomasal parasitism, avian tuberculosis and chronic malignant catarrhal fever should be taken into account (Mackintosh, 1998).

During post-mortem examination, clinically affected deer frequently show enlargement of mesenteric lymph nodes, often with caseous lesions, prominent lymphatic drainage vessels from the jejunum to adjacent lymph nodes and omentum’s oedema; in addition, unlike cattle and sheep, ileum does not always show thickening. Microscopic lesions are characterized by the presence of acid-fast laden macrophages in affected lymph nodes, often with caseation and foci of calcification. In addition, the ileocecal valve often shows loss
of villous structure and contains mixed cellular infiltrate and numerous acid-fast organisms; notably, analogous lesions are detectable in ileum and jejunum (Mackintosh et al., 2004).

In order to improve the knowledge about the wildlife and its role in the transmission of the disease to cattle, our study was aimed at sub-typing MAP field strains isolated from the wild red deer population which inhabits Stelvio National Park (Italy), by a combination of MIRU-VNTR and Short Sequence Repeats sequencing (SSRs), as previously described (Ricchi et al., 2011).

2. MATERIALS AND METHODS

2.1 Sampling

The sampling plan was designed to include all the subjects culled during a program aimed at controlling the red deer population in Stelvio National Park from 2011 to 2015, in Lombardy Region (n= 382 tissues) and in the area of the Autonomous Province of Bolzano (n=102 feces and tissues) (Figure 1). The animal age was estimated based on the teeth eruption patterns: deer until two years old were classified as yearlings, while those with more than two years as adults.

2.2 Post-mortem examination

The post-mortem inspection was carried out on all subjects: signs of illness including weight loss, poor body condition and diarrhea and any visible lesion in mesenteric lymph nodes and gastrointestinal tract were recorded. With reference to this, macroscopic lesions were graded according to the classification described by Fraquelli (Fraquelli et al., 2005).

2.3 qPCR and cultural assay

Lombardy area

DNA extraction and quantitative PCR targeting IS900 (Ricchi et al., 2014) was carried out by examining tissue samples (pool consisting of ileal mucosa, ileocecal valve and mesenteric lymph nodes). Positive samples were then cultured in VersaTREK® system and on Herrold's egg yolk medium (HEYM) supplemented with mycobactin J (ID.VET, Montpellier, France) as reported in the OIE manual (Anonymous, 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 (≤ 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
In the Lombardy area, out of 382 investigated deer, 77 were PCR positive, giving an apparent prevalence of 20.16% (CI 95% 16.44 - 24.47), while in the Bolzano area, out of 102 fecal samples, 19 were PCR positive, with an apparent prevalence of 18.63% (CI 95% 12.26 - 27.27); no statistical difference was observed between the two areas. The slightly lower prevalence rate recorded in Bolzano area could be attributed to the different diagnostic matrices. In fact, sub-clinically infected deer can only intermittently shed MAP and the consequently low load in feces could lead to a false negative result despite the animal is truly affected (Maroudam et al., 2015).

The results suggested that yearlings showed a higher risk of infection compared to adults, although the comparison of the two age categories showed no statistic difference in the disease’s prevalence between yearlings (5/16, 31.25%) and adults (14/86, 16.28%) in Bolzano area ($\chi^2= 1.99; p$-value = 0.157) and yearlings (41/199, 20.60%) and adults (36/183, 19.67%) in Lombardy area ($\chi^2=0.051; p$-value = 0.82). A possible explanation for the greater number of positive subjects in the yearlings population rather than in older animals, could be the quick progression from infection to clinical signs (Carta et al., 2013), and the subsequent increase in the rate of mortality; in this regard, as suggested by (Fraquelli et al., 2005), in a deer’s population exposed to MAP, the lower disease’s prevalence of the adults subjects could be attributed to the death of the younger animals.

Pathological examination revealed most of the infected subjects did not show macroscopic lesions, while lesions associated with MAP infection were recorded in few subjects (Table 1). The importance and extensions of lesions, according to the scheme proposed by (Fraquelli et al., 2005) varied from limited affection of lymphatic system to chronic enteritis with thickening of the wall and wrinkling of the mucosa associated with severe enlargement of mesenteric lymph nodes (Table 1) (Figure 2, 3 and 4).

By DMC PCR, all MAP isolates resulted as type C, which is considered more virulent for red deer than type S. In fact, in domestic deer, it has been demonstrated by cultural examination of tissue samples that oral inoculation with type C strain determines a higher infection rate (100%) than type S strain (69%) (Mackintosh et al., 2007; O’Brien et al., 2006). Moreover, type C strains induce a higher immunological response (O’Brien et al., 2006), a lower weight gain, more serious histopathological lesions and higher seropositive rates (ELISA) (Mackintosh et al., 2007) than type S. Comparing this finding to those of similar studies previously performed in Northern Italy (Fraquelli et al., 2005; Nebbia et al., 2000; Robino et al., 2008), it can be stated that in our
country red deer population is infected with C type strains. Furthermore, from an epidemiological point of view, the detection of MAP in wildlife red deer population points out to a possible inter-species disease transmission from wildlife to domestic ruminants and *vice versa*. In fact, since clinically infected deer can shed $5 \times 10^6$ MAP CFU/g (Schroen et al., 2003) and cows over than $10^8$ MAP CFU/g of feces (Cocito et al., 1994), animals can become infected grazing on pastures contaminated with infected feces (Carta et al., 2013; Daniels et al., 2003a; Daniels et al., 2003b). In addition, C strains not only don’t show host preference and have been isolated from a very broad host range of ruminant and nonruminant wildlife animals, but they are also the predominant type isolated from bovine specimens, not only in Italy (Ricchi et al., 2011), but also in all countries of the world (Stevenson, 2015).

However, it is not possible to exclude that the isolation of only one type in our data could be due to the better cultivability of this strain type; in fact, C strains are more easily isolated from clinical samples on solid and liquid media than S strains. On the other hand, this finding could be due to the higher infection ability of C strains and, particularly, to their higher capacity for infecting and surviving in macrophages compared with S strains (Stevenson, 2015).

To the best of our knowledge, this is the first time that strains isolated from Italian red deer population have been sub-genotyped and all isolates showed the same MIRU-VNR pattern profile (Table 2). Furthermore, Mini-satellite loci analysis revealed the presence of the INMV1 genotype and up until now, the INMV1 profile has been already described before in Italy only in cattle herds (Ricchi et al., 2011). On the other hand, INMV1 has been previously detected in red deer in the Czech Republic, The Netherlands (Stevenson et al., 2009), Germany (Fritsch et al., 2012) and in Austria (Gerritsmann et al., 2014) and, according to the MAC-INMV database (http://mac-inmv.tours.inra.fr/), it is the second most diffused profile in the world.

Moreover, short sequence analysis of two loci highlighted the presence of one dominant MAP subtype (Table 2). In fact, all the isolates carried identical numbers of repeats in SSR1 and SSR8 loci and differed only for a few bases at the SSR2 locus. A possible explanation for the difference found out in this locus could be its instability, as previously suggested by (Ricchi et al., 2011) and investigated by (Kasnitz et al., 2013). With reference to this, the application of SSR analysis could help to improve the knowledge of the
genetic distribution of MAP subtypes improving epidemiological data, as suggested by (Motiwala et al., 2006).

Notably, the same MIRU-VNTR pattern and the same SSR profile were detected in Germany (Fritsch et al., 2012), not only in a wild living deer population, but also in farmed cattle living in the same territory, suggesting the possibility of interspecies transmission for this genotype. According to (Fritsch et al., 2012), tracking MAP transmission by VNTR typing and SSR analysis among wild red deer and farmed cattle could be the most cost-effective and efficient strategy for discriminating strains and highlighting any possible host relation among genotypes which infected wildlife ruminant and those isolated from domestic species. In our case, this analysis suggests how the field isolates here recovered, can have a common clonal origin.

However, a convergent evolution of VNTR loci has been recently described by (Ahlstrom et al., 2015), in which the authors underlined how “caution should be used when using VNTR typing as a tool to assess the diversity and relatedness of MAP isolates at both a national and herd-level”. For this reason, we believe whole genome sequencing data can probably be the best approach to investigate the clonal origin hypothesis since, as previously reported also for Mycobacterium bovis (Biek et al., 2012), this last method provides the highest resolution for such task.

In conclusion, more studies should be carried out to understand the epidemiological role of wildlife in the transmission of paratuberculosis, including also nonruminant wildlife in the research, so that control strategies could be adopted to avoid interspecific transmission. The use of more powerful methodologies, such as whole genome sequencing approaches coupled with mathematical modelling (Biek et al., 2012), could provide new insights in the epidemiology of paratuberculosis in wildlife animals.

4. CONFLICT OF INTEREST STATEMENT

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

5. ACKNOWLEDGEMENTS

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6. REFERENCES


Fig. 1. Comparison of samples collected and disease prevalence per municipalities. The map shows the location of the Lombardy Region and the Autonomous Province of Bolzano (Northern Italy) and the municipalities into which they are divided. Coloured areas indicate the municipalities in which animals were sampled during the study period. The colours are proportional to the number of samples collected. Disease prevalence for each municipality (in bold) is included.
Table 1 Prevalence of macroscopic lesions observed in Yearlings and Adults positive to PCR assay carried out on tissue specimens (Lombardy Region, n = 77) 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 deer with lesions within each age class are reported.

<table>
<thead>
<tr>
<th>Region of sampling</th>
<th>Age class</th>
<th>Lesion status*</th>
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<td>Adults</td>
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* NL = no macroscopic lesions; A = lesions limited to lymphatic system with hyperplasia of Peyer patches, enlargement of mesenteric lymph nodes and/or lymphangectasia; B = catarrhal or catarrhal-haemorrhagic ileitis; C1 = catarrhal or catarrhal-haemorrhagic enterocolitis and typhlitis; C2 = chronic 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 enlargement of mesenteric lymph nodes.
Fig. 2. Red deer. Isolated segment of small intestine showing marked thickening of the intestine wall and corrugation of the mucosa. Also note enlargement of lymph node (arrow).
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.

<table>
<thead>
<tr>
<th>Region of</th>
<th>ID strain</th>
<th>ID animal</th>
<th>sex</th>
<th>age</th>
<th>No. of copies MLVA-VNTR</th>
<th>No. of copies SSR</th>
<th>INMV profile</th>
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<td>VNTR 292</td>
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<td>VNTR 25</td>
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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.