Epidemiology of paratuberculosis in two red deer (*Cervus elaphus*) populations of Trentino (Northern Italy)

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ABSTRACT

The objective of this study was to estimate the prevalence and describe the epidemiology of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in two distinct areas of Trentino. Both areas are characterized by high densities of red deer and domestic livestock (cattle and small ruminants) that share pasture. During the five-year period 1998-2002 the intestines of 246 red deer killed or found dead were examined in the western sector of the province. Between 1999 and 2002 the study was extended to the eastern part of the province, where the viscera of 156 red deer were collected. Isolates of MAP were isolated in 194 of the 402 carcasses examined by bacterial culture (HYEM). The results obtained show a significant difference in the prevalence of MAP in the two populations examined (66.2% in the western sector and 18.6% in the eastern sector). The typing of the strains identified as MAP by using IS900-based PCR technique was carried out by means of PCR-REA on the IS1311 sequence. The results of this analysis confirmed that the strains isolated in the deer were “cattle type”. The infection has been reported in other sympatric species of wild ungulates, present in both study areas, such as roe deer, chamois and ibex, as well as non-ruminant species, such as fox and hare. In order to verify the existence of a species-specific cycle of MAP, phylogenetic analysis by AFLP was carried out to clarify the role of each wild species in the epidemiology of paratuberculosis in the area.

Key words: *Mycobacterium avium* subsp. *paratuberculosis* (MAP), epidemiology, red deer, PCR, AFLP.

INTRODUCTION

Paratuberculosis is a chronic granulomatous enteritis caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) that affects both domestic and wild ruminants. Recent studies have revealed that MAP can also infect non-ruminant species (Beard et al., 1999; Greig et al., 1999). Cervids are particularly susceptible to MAP infection, and manifest clinical signs at a young age (Pacetti et al., 1994; De Lisle & Collins, 1995; Stehman, 1996).

In Italy the first reported case of paratuberculosis in wild ruminants was identified in two red deer originating from Stelvio National Park in the early 1990s (Pacetti et al., 1994). The disease also infects other sympatric species of wild ungulates found in the Alps such as ibex (*Capra ibex*), chamois (*Rupicapra rupicapra*), roe deer (*Capreolus capreolus*), and mouflon (*Ovis musimon*). The estimated prevalence of infection in these species varies by geographic area and the diagnostic technique used (Tolari et al., 1987; De Meneghi et al., 2000; Gennero et al., 1993; Ferroglio et al., 2000; Robino et al., 2000, Nebbia et al., 2003). However, the role played by wild ungulates and non-ruminants in the epidemiology of paratuberculosis is still poorly understood.

The objectives of this study were to estimate the prevalence and describe the epidemiology of MAP in wild red deer in two areas of Trentino (Northern Italy), and understand the implications for other species of wild...
animals, both ruminant and non-ruminant. The results of this study may prove useful to both wildlife and livestock managers.

**MATERIALS AND METHODS**

**Survey area**

The study was carried out in two areas of Trentino (Fig. 1) characterized by high densities of red deer and domestic livestock (cattle and small ruminants) that share pasture. One study area is in the western sector of the province including the Trentino sector of the Stelvio National Park and the surrounding areas (Peio, Rabbi and Vermiglio Reserves). The other study area is in the eastern sector of the province, including the Paneveggio-Pale di S.Martino Natural Park and its surrounding areas within the Travignolo watershed (Moen, Predazzo and Primiero Reserves) (Fig. 1 and Table 1). During the summer in the western sector of the province there were about 1,630 cattle and 390 small ruminants, whilst in the eastern sector there were 3,100 cattle and 4,730 small ruminants.

![Map of Trentino Region showing the two areas sampled in the survey](image)

**Fig. 1.** Map of Trentino Region showing the two areas sampled in the survey

**Table 1.** Characteristics of the study areas and of the red deer populations surveyed

<table>
<thead>
<tr>
<th>Altitude (min - max) (m)</th>
<th>Western sector of Trentino</th>
<th>Eastern sector of Trentino</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total area (hectare)</td>
<td>1500-3770</td>
<td>1200-2300</td>
</tr>
<tr>
<td>Red deer density (head/100 ha)</td>
<td>9850</td>
<td>7085</td>
</tr>
<tr>
<td>1998</td>
<td>11.5</td>
<td>3.7</td>
</tr>
<tr>
<td>1999</td>
<td>13.2</td>
<td>6.6</td>
</tr>
<tr>
<td>2000</td>
<td>14.1</td>
<td>6.6</td>
</tr>
<tr>
<td>2001</td>
<td>9.3</td>
<td>6.9</td>
</tr>
<tr>
<td>2002</td>
<td>12.6</td>
<td>7.3</td>
</tr>
</tbody>
</table>
Collection of samples
During the five-year period 1998-2002 in the western sector of the province, the internal organs of 246 red deer, killed during hunting seasons, or found dead, were examined and samples collected for culture. The study was extended to the eastern part of the province, in 1999, where, up to 2002, the viscera of 156 red deer were collected (Table 2). The age, sex, weight and biometric measures were recorded for each animal. The small and large intestine was examined at necropsy for visible lesions of paratuberculosis. Samples for bacteriologic culture were taken from the ileum, ileo-cecal-colic valve and mesenteric lymph nodes. The anatomo-pathological lesions found during autopsy were classified as type A, B, C on the basis of severity, location and distribution (Perez et al., 1996). During necropsy both the kidneys and the perirenal fat were removed in order to evaluate the body condition by Kidney Fat Index (KFI). Further analyses were carried out to exclude the possibility that lesions were caused by other bacteria or viruses. To evaluate the possible involvement of other wildlife species thought to be susceptible to paratuberculosis infection, analyses were carried out on 228 roe deer (Capreolus capreolus), 88 chamois (Rupicapra rupicapra) and 6 ibex (Capra ibex) obtained from the study areas. Non-ruminant species, such as foxes (Vulpes vulpes) and hares (Lepus europaeus) were included in these analyses from 2000 onwards.

Table 2. Red deer survey data

<table>
<thead>
<tr>
<th>Red Deer</th>
<th>Trentino: Western sector</th>
<th>Trentino: Eastern sector</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hunted</td>
<td>151</td>
<td>149</td>
<td>300</td>
</tr>
<tr>
<td>Found dead</td>
<td>95</td>
<td>7</td>
<td>102</td>
</tr>
<tr>
<td>Total carcasses examined</td>
<td>246</td>
<td>156</td>
<td>402</td>
</tr>
</tbody>
</table>

Bacteriological culture
For every carcass, culture was completed to detect MAP in a pool of tissues consisting of the proximal part of the ileum, the ileo-cecum-colic valve and the mesenteric lymph nodes. Tissue homogenates were prepared by following the methods outlined below. Five to ten grams of tissue were decontaminated by adding Butterfield buffer and leaving overnight at room temperature. Each sample was then subjected to trypsin digestion (0.4 % in PBS) (15 ml), homogenised in a stomacher blender, and left to stand for 15 minutes at 37°C. The supernatants were centrifuged at 1800-2000 rpm for 10 minutes and each pellet was resuspended in 7 ml of sterile distilled water. 5 ml of each supernatant sample was finally resuspended in 10 ml of sterile distilled water and 1.65 ml of decontaminant Benzalkonium chloride (BAC 0.3%), and incubated overnight at room temperature. 200 µl of inoculum was added to each of three slants of Herrold’s egg yolk medium. The first slant contained mycobactin J (2 mg/l) and amphotericin B (50 mg/l) (HEYM); the second slant contained HEYM with sodium pyruvate (4 g/l); and the third slant contained HEYM with cloramphenicol (30 mg/l) (Belletti e Zavanella, 1987). All the tubes were incubated at 37°C and examined once every 4 weeks for up to 16 weeks. The strains isolated were classified according to the definition criteria proposed by Stevenson (1996).

Molecular typing of MAP isolates
The strains isolated by culture were screened by PCR for the presence of the MAP species-specific IS900 insertion sequence. The following primer sequence was used: forward (p90) GAAGGCTTTGCGGGCCGTCGGTTAGG and reverse (p91) GGCCTTTGAGTCGATCAGCAGTGA. These PCR primers amplify a 413-bp target sequence. In order to identify the bovine or ovine type of strain isolated, the PCR-restriction endonuclease analysis (REA) based on IS1311 sequence was used. Strains identified as MAP by PCR-IS900 that were isolated from cattle, small ruminants and red deer were typed by PCR using primers: forward (M56) GCGTGAGGCTCTGTGGTGAA, reverse (DD2) GTCGGGTTGGGCGAAGAT. The PCR-amplified product, resulting in a 909-bp probe, was digested by the restriction endonuclease Hinfl. The PCR products were subjected to agarose gel electrophoresis. Two negative controls and one positive control (DNA from Mycobacterium phlei ATCC 11758, Mycobacterium flavescens ATCC 14474, and Mycobacterium avium subsp. paratuberculosis ATCC 19698), were included in each amplification run.
Kidney Fat Index calculation

The Kidney Fat Index (KFI) proposed by Riney (1955) is a quick and easy method for assessing body fat and is commonly used as an indicator of body condition in ungulates. To determine the KFI, both kidneys were extracted from each carcass along with all the peri-renal fat. The kidneys were then dissected from the fat and the two weighed independently. The KFI was calculated using the following equation: KFI = (kidney fat weight/kidney weight) * 100.

Kidney weight is included in the index to allow a comparison between animals of different sizes, assuming that the kidney weight is proportional to body weight. (Mitchell et al., 1976; Finger et al., 1981).

Statistical analysis

Chi-square tests based on contingency tables were carried out to test for an association between prevalence and (I) sex, (II) age class, (III) type of animal (hunted or found dead) and (IV) KFI/weight, with analyses stratified by geographical areas. Continuous variables were dichotomised on either side of the median value. The median of KFI/weight was calculated for each age class by sex. The level of risk was estimated using an odds ratio according to the Mantel Haenszel method. Confidence intervals on annual prevalence by geographical areas were estimated assuming a hypergeometric distribution in the data.

RESULTS

MAP was isolated in 194 (48.2%) of the 402 deer examined. The prevalence of infection varied in the western sector, from a maximum of 80.0% (95% CI: 68.20 - 88.63) in 1999 to a minimum of 55.0% (95% CI: 45.26 - 64.37) in 2001. In the eastern sector, the maximum prevalence was 36.4% (95% CI: 16.92 - 60.77) in 1999, and the minimum was 5.9% (95% CI: 2.21 – 13.24) in 2001. Prevalences calculated using the data from the hunted animals only did not differ significantly.

In the western sector of the province the average prevalence of infection was 66.2%, significantly higher than the one in the Travignolo watershed, which was 18.6% ($\chi^2 = 75.88; P = 0.000$). There was no significant difference ($P > 0.05$) in the prevalence of the infection in relation to the median of weight, median of KFI, and cause of death (hunted or found dead), stratified by geographical areas.

In both study areas, the sex of the animal was not associated with infection prevalence (western Trentino $\chi^2 = 0.537 ; P = 0.309$; eastern Trentino $\chi^2 =0.372; P = 0.318$). For different age groups, the distribution of the infection was similar to what was found by other authors (Pacetti et al., 1994; Manning et al., 1998; Godfroid et al., 2002). The greatest percentage of positive results from culture examinations in both sectors were found in the youngest animals. There was a significant difference between the values of prevalence found in calves and yearlings in the western area ($\chi^2 = 10.25; P = 0.017$) (Fig. 2). Such values tend to decrease in adults examined. The age-related prevalence pattern can be explained if we consider mortality caused by the infection. The disease decreases the life expectancy at birth of each individual, according to the prevalence of infection observed over the entire population: a lower disease prevalence in adults occurs when infected animals in the population die at a young age. Furthermore, it would seem that adults may act as a vector of the infection. In the eastern area, the youngest age group had the highest prevalence of infection however, it was not significantly different from other age classes.

The odds ratio for the western area was 9.06 (95% CI: 5.59 – 14.68).
The infection was also found in other species sympatric with wild ungulates from both study areas such as roe deer, chamois and ibex (Table 3) as was seen in previous research in Italy and abroad (Tolari et al., 1987; et al., 2000; Ferroglio et al., 2000; Pavlik et al., 2000; Robino et al., 2000; Machackova et al., 2002; Nebbia et al., 2003). Natural infection of paratuberculosis was not limited to ruminants: similar to observations in Scotland (Beard et al., 1999; Greig et al., 2002) MAP was also isolated from fox (Vulpes vulpes) and hare (Lepus europaeus) (Table 3). Furthermore, according to the authors mentioned above, in foxes, the anatomo-pathological lesions concerned only the lymphatic tissues, whilst no lesions related to paratuberculosis were observed in hares.

Table 3. Prevalence of MAP in different wild species examined from 1999 to 2002

<table>
<thead>
<tr>
<th>Species</th>
<th>Western area</th>
<th>Eastern area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Percent positive</td>
</tr>
<tr>
<td>Roe deer</td>
<td>106</td>
<td>24.4%</td>
</tr>
<tr>
<td>Chamois</td>
<td>27</td>
<td>33.3%</td>
</tr>
<tr>
<td>Ibex</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Fox</td>
<td>39</td>
<td>7.7%</td>
</tr>
<tr>
<td>Hare</td>
<td>11</td>
<td>18.2%</td>
</tr>
</tbody>
</table>

In order to verify whether a domestic-wild animal infection cycle exists, or whether MAP behaves as a species-specific pathogen, a phylogenetic analysis of isolated MAP by means of AFLP is underway. Strain typing, carried out by means of PCR-REA on the IS1311 sequence, confirmed that isolates were of the “bovine type”.

The primary lesions found in deer that were culture positive to the culture were located in the intestine. The lesions affected, to varying degrees, the distal tract of the jejunum, the ileum, the ileo-cecum-colic valve, the cecum and the local lymphatic system. A broad range of lesions were observed, varying by location, severity and distribution. In some cases the distal small intestine and the lymphatic system appeared normal or lightly congested. In other cases typical bovine lesions were apparent such as thickening and corrugation of the mucosal layer of the small intestine, edema, and enlargement of mesenteric lymph nodes, Peyer patches and ileo-cecum-colic valve. The considerable variety observed made it possible to classify the lesions according to location, severity and distribution, useful to the study of the disease in the
different categories, distinguished by age, sex and type of recovery (killed or found dead). On the basis of these data and taking as a reference the classification of the immunohistochemical lesions suggested by Perez et al. (1996), an attempt was made to classify the observed lesions into three main classes and further subclasses (Fig. 3).

Class A: Effects limited to lymphatic system with thickening Peyer patches, enlargement of mesenteric lymph nodes and/or thickening of lymphatics in mesentery and serosa.

Class B: Catarrhal or catarrhal-haemorrhagic enteritis confined to the ileum.

Class C:
C1: catarrhal or catarrhal-haemorrhagic enteritis associated with severe lesions to the ileum-cecum-colic
C2: chronic enteritis with thickening of the wall and wrinkling of the mucosa
C3: lesions from both class C1 and C2 associated with severe enlargement of mesenteric lymph nodes

Fig. 3. Prevalence of lesions observed in positive animals in the western and eastern areas

No significant differences were found in the distribution of lesions between hunted and found dead subjects, an indication that paratuberculosis was not affecting population dynamics. A similar result was obtained when evaluating the distribution of lesions by age groups in each study population.

CONCLUSION

The results showed a significant difference in the prevalence of MAP between the two deer populations examined. The statistical analysis highlighted that area of origin is a significant risk factor: for study animals from the Travignolo watershed area, characterized by a much lower density of deer than in the western area, the risk of infection was lower.

The isolation of MAP in many wild species, ruminant and non-ruminant, as documented in the current study has implications for animal health and for the control of the disease. Besides transmitting the infection to conspecifics, infected animals may act as a reservoir of infection for other species, particularly domestic ruminants, with which they share the pastures. Conversely, infected domestic livestock may also be the source of infection for wildlife.

Strain typing, carried out by means of PCR-REA on the IS1311 sequence, confirmed that the isolates were the bovine strain. This may suggest a risk of transmission of the infection between domestic and wild animals during the summer season, when mountain pastures are shared by all species. (Moreira et al., 1999; Pavlik et al., 2000; Robbi et al., 2000; Machackova et al., 2002; Nebbia et al., 2003).

In the two populations of deer analysed, it appears that paratuberculosis at even a quite high prevalence has not had a significant effect on population dynamics. Significant differences were not found between
hunted animals and those found dead in both the areas (western area: \( \chi^2 = 2.41; P = 0.121 \); eastern area: \( \chi^2 = 0.36; P = 0.546 \)); furthermore, in animals found dead, only mild lesions were seen at necropsy.

It is necessary to continue molecular biology research to phylogenetically characterize MAP in order to evaluate the relationship among the different strains isolated. A better understanding of the role of the wildlife, non-ruminants included, in the maintenance and transmission of paratuberculosis will allow a better understanding of the risk factors to wildlife and disease control strategies in domestic ruminants. Considering the possibility of interspecific transmission, through contaminated pastures, the current detection and cull policy will be inefficient for the control of the disease in areas where livestock interact with wildlife.

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