Identification of Mycobacterium avium subsp. paratuberculosis in wild cervids (Cervus elaphus hippelaphus and Capreolus capreolus) from North-Western Italy

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SHORT COMMUNICATION

Identification of *Mycobacterium avium* subsp. *paratuberculosis* in wild cervids (*Cervus elaphus hippelaphus* and *Capreolus capreolus*) from Northwestern Italy

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Abstract Seventy-seven red deer (*Cervus elaphus hippelaphus*), 40 roe deer (*Capreolus capreolus*) from the Northwestern (NW) Alps (Turin Province, NW Italy) and 29 roe deer from the NW Apennines (Alessandria province, NW Italy) were examined for the presence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) by culture, IS900 nested polymerase chain reaction (PCR) and IS1311 PCR restriction endonuclease analysis for strain characterisation. MAP identification (nested PCR and/or culture) allowed us to detect 32.9% MAP-infected red deer and 22.5% infected roe deer in the NW Alps and 41.4% MAP infected roe deer in the NW Apennines. On the basis of the polymorphism present in the IS1311 sequence, all MAP isolates were characterised as cattle strains. Our results show that MAP circulates widely among populations of wild cervids in NW Italy.

Keywords Paratuberculosis · Red deer (*Cervus elaphus hippelaphus*) · Roe deer (*Capreolus capreolus*) · Italy

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Introduction

Interest in wildlife diseases has greatly increased in the last decade (Kuiken et al. 2005). The possible transmission of pathogens among wild species, domestic animals and, potentially, humans is of great concern when a disease is present in a wild host (Gortázar et al. 2007). The risk of spillover from wildlife to livestock is to be considered when wild animals can act as a reservoir for disease agents whereby eradication plans are applied in livestock. The transmission risk of pathogens is high when wild and domestic species share the same range and pasture areas. Paratuberculosis is chronic enteritis of both domestic and wild ruminants, caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). MAP has also been isolated from a wide range of wild non-ruminant species (e.g. foxes, stoats, weasels, crows, hares, rabbits; Beard et al. 2001; Greig et al. 2003; Deutz et al. 2005; Corn et al. 2005; Judge et al. 2006), but their role in maintaining the infection is not adequately known for all species. Indeed, the possibility of vertical and horizontal transmission of MAP as determined in rabbits—asymptomatic MAP carriers—could contribute to the maintenance of infection among these animals and the environment (Judge et al. 2006).

Generally, adult red deer are infected, but only young animals (8–15 months old) may show a sudden onset of clinical signs, with rapid worsening of symptoms, weight loss and frequent diarrhoea. Adults rarely develop clinical signs, although they may be sero-positive and/or show pathological lesions only on postmortem examination (Mackintosh et al. 2004).

Restriction fragment length polymorphism analysis has identified two groups of MAP, the sheep strain and cattle strain. The latter is known to infect cattle, sheep, goats and other species, including humans, while the former is strictly

related to sheep and goat infections (Collins et al. 1990). The possibility to quickly differentiate sheep and cattle strains is particularly useful for paratuberculosis control plans whenever both cattle and small ruminants graze on pastures.

As the most recent records of MAP in free-ranging cervids from the Italian Alps date back to the late 1990s (Nebbia et al. 2000; Fraquelli et al. 2005), we have deemed it interesting to present the results of a survey on red and roe deer, carried out in selected study areas of Northwestern Italy.

Materials and methods

The present study was carried out in two study areas, with different ecological and climatic characteristics: the Northwestern Alps (NW Alps) and the Northwestern Apennines (NW Apennines).

In the NW Alps, we monitored a hunting district in the Upper Susa Valley (USV), located in an Alpine valley with elevation ranging from about 500 to 3,500 m of altitude characterised by Alpine meadows, mixed broad-leaved trees and coniferous forests. From the beginning of June to the end of September, roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus hippelaphus*) and chamois (*Rupicapra rupicapra*) partially share their home range and pasture with livestock, mainly cattle and sheep.

In the USV hunting district, a focus of paratuberculosis had previously been identified, and clinical cases had been found in red deer (Nebbia et al. 2000). During the present study, samples were collected from both roe and red deer in the area within the previous focus (FA) and from other areas (OFAs) of the USV. The OFA is located more than 10 km away from the FA, and no cases of paratuberculosis have been reported from there.

The other study area is a hunting reserve, Monteacuto Game reserve (MGR), located in the NW Apennines on the Liguria–Piedmont border, characterised by hills covered with deciduous woods, meadows and fallow land (altitude 300–540 m). Small-scale mixed farming is the main agriculture and livestock production system in the area. Small flocks of goat and sheep are raised within and near the borders of MGR. Roe deer is the only free-ranging wild ruminant present throughout MGR and bordering areas. Roe deer density has been estimated (spring game counts) at about 15 heads/100 ha (Regione Piemonte 2007).

During 1999–2004, 77 red deer (49 in FA and 28 in OFA, USV) and 69 roe deer (21 in FA, 19 in OFA and 29 in MGR) harvested and/or killed by road accident underwent a postmortem examination, with collection of mesenteric lymph nodes. Ziehl–Neelsen (ZN) staining was performed on lymph nodes to detect the presence of acid-fast bacteria. Conventional culture (in-house) on Herrold's egg yolk medium supplemented with mycobactin J and amphotericin B was carried out (AA. VV. 2006, OIE, Manual of Diagnostic Tests). All tubes were incubated at 37°C and examined over 8 to 24 weeks.

Deoxyribonucleic acid (DNA) was extracted from colonies and lymph nodes following a modified protocol for mycobacteria (Robino et al. 2003) and MAP was identified using IS900 nested polymerase chain reaction (PCR) previously described by Englund et al. (1999). Restriction endonuclease analysis (REA) of the insertion sequence IS1311 was carried out on all MAP strains isolated, to discriminate sheep and cattle strains, according to the technique described in Marsh et al. (1999). Agreement between results of ZN, isolation and nested PCR was evaluated by K coefficient, using the software WIN Episcope 2.0 (Thrusfield et al. 2001). The EPI INFO (Dean et al. 1995) version 6 statistical package was used to compare prevalence data among species and origin of animals with a χ^2 test. Differences in prevalence were considered as significant when $p \leq 0.05$.

Table 1 Paratuberculosis in red and roe deer in the two study areas (NW Italy): results of the tests performed

Species	Study area	Locality	Animals tested	Number positive ZN	Number positive bacterial culture	Number positive IS900-PCR	Number total MAP-positive[c]
red deer	NW Alps (USV)	FA[a]	49	20	11 (22.4%)	27 (55.1%)	27 (55.1%)
		OFA[b]	28	7	3 (10.7%)	3 (10.7%)	3 (10.7%)
roe deer	NW Alps (USV)	FA	21	8	3 (14.3%)	7 (33.3%)	7 (33.3%)
		OFA	19	5	0 (0%)	2 (10.5%)	2 (10.5%)
	NW Apennines	MGR	29	13	5 (17.2%)	11 (37.9%)	12 (41.4%)
total			146	53	22 (15.1%)	50 (34.2%)	51 (34.9%)

[a] Previous focus of paratuberculosis in NW Alps
[b] Other areas where no cases of paratuberculosis have never been reported.
[c] PCR and/or culture positive

Results

On postmortem examination, none of the sampled animals showed lesions indicating paratuberculosis. The results of the tests are presented in Table 1. Results of ZN-stained lymph nodes smears were poorly correlated with identification by cultural (k=0.41, 95% confidence interval [CI]=0.23–0.58) and molecular methods (k=0.47, 95% CI=0.32–0.62) because of low sensitivity and specificity of the ZN staining method. Isolates were detected in 14 red deer (18.1% in the NW Alps) and in 8 roe deer (N=3 in the NW Alps, 7.5%; N=5 in the NW Apennines, 17.2%). Moreover, MAP DNA was demonstrated by a bio-molecular method from 30 red deer (39.0% in the NW Alps) and in 21 roe deer (N=9 in the NW Alps, 22.5%; N=12 in the NW Apennines, 41.4%). Culture test showed a lower sensitivity than nested PCR, and the agreement was low (k=0.41, 95% CI=0.24–0.59). After enzymatic digestion (IS1311 PCR-REA), restriction pattern of digests was assessed: All samples have been typed as cattle strains, by the presence of a band, absent in the sheep strain digestion pattern, indicating such a mutation.

These results from Western Italy are in accordance with the data obtained by Fraquelli et al. (2005) in red deer from Northeastern Italy.

Prevalence of PCR-positive roe deer in the FA area of the USV was higher (33.3%) than that observed in OFA of the same USV (10.5%). However, this difference was not significant (χ^2 1.81; p=0.18). Furthermore, the differences observed in roe deer between the two study areas (USV and MGR), 22.5% in the NW Alps and 41.4% in NW Apennines, were not significant (χ^2 2.8; p=0.09). Comparing the roe and the red deer from the USV study area, no statistical difference was observed (χ^2 3.1; p=0.07). Conversely, MAP PCR prevalence in red deer from the “infected” area FA in USV (55.1%) was higher than that in the neighbouring areas (OFA 10.7%; χ^2 14.8; p=0.0001).

Discussion

Although gross lesions pointing to paratuberculosis were not observed in the study animals, MAP identification (nested PCR and/or culture) allowed us to detect 22.5% MAP-infected roe deer in the NW Alps and 41.4% in the NW Apennines. Samples were positive in most animals when tested in nested PCR, which is more sensitive than the cultural method. Moreover, higher sensitivity observed could be due to the use of tissue samples instead of faecal extracts, which are known to contain PCR inhibitors (Englund et al. 1999). The opposite—sample negative at DNA amplification test but positive at bacteriological culture—was observed only in one case and could be explained by the fact that only a small sample of tissue was used for PCR analysis, whereas a larger section of tissue was adopted for the isolation method. These findings are in accordance with Ceelen et al. (2007).

The results demonstrate that MAP is present in both study areas, where it circulates in both roe and red deer populations, in the absence of clinical/pathological signs. Our data also suggest that in the wild cervids population of the USV hunting district, MAP infection is not restricted to a small focus (FA) previously described (Nebbia et al. 2000), but it is now widespread throughout the whole valley, even in areas where clinical cases have not previously been reported. Roe deer are also exposed to MAP and become infected, but roe deer seem to have a low degree of susceptibility to paratuberculosis and do not usually develop clinical signs, which is contrary to what has been reported in red deer (Gilmour and Nyange 1989; Goddard 1994). The presence of infected animals in all study areas shows that MAP is widespread in wild cervids from NW Italy. In these areas, MAP infection was detected by serological and molecular tests (agar gel immunodiffusion, enzyme-linked immunosorbent assay, PCR) even in domestic ruminants, both, respectively, in cattle grazing on a summer pasture area in the USV hunting district (Nebbia et al. 2000) and in small ruminants living not far from the borders of MGR (Robino et al. 2003). Bearing in mind that even the strains isolated from domestic animals were cattle strains, sharing pasture areas may allow the transmission of the same strains between one population and another. It is still not clear how MAP shedding takes place. It has been suggested (Corn et al. 2005) that MAP transmission may occur from wildlife to wildlife, wildlife to livestock and/or livestock to wildlife. Wild ruminants, as possible reservoirs for MAP, play an important role in paratuberculosis control/eradication programmes in domestic ruminants, as they may possibly affect the success of such programmes. Moreover, it would be interesting to investigate the extent of MAP infection, in NW Italy, in the non-ruminant wild species (i.e. foxes, hares) sharing the same pasture areas because they may serve as reservoirs and could infect or re-infect domestic ruminant species.

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