WILDLIFE

Mixed *Mycobacterium avium* subspecies *avium* and *M. avium* subspecies *paratuberculosis* infection in a wild red deer (*Cervus elaphus*) in Belgium

Rosario Volpe,¹ Thomas Fett,¹ Dominique Cassart,¹ Jacques Godfroid,²,³ Annick Linden¹

SUMMARY

This report describes a mixed *Mycobacterium avium* subspecies *avium* and *M. avium* subspecies *paratuberculosis* infection in a free-ranging red deer (*Cervus elaphus*). The gross presentation was consistent with clinical paratuberculosis as previously reported in red deer and for other ruminants, with poor body condition, diarrhea and mesenteric lymphadenitis. However, this animal presented unusual lung lesions, with necrosis and calcification similar to those reported for *Mycobacterium bovis* infection in wild and domestic ruminants. A mixed *M. avium* subspecies *avium* and *M. avium* subspecies *paratuberculosis* was shown by quantitative PCR (qPCR) performed on digestive tract samples. In addition, *M. avium* subspecies *avium* was also detected in the respiratory tract (lungs and bronchial lymph nodes) by qPCR. These results were confirmed by classical bacteriology. Lesions induced by those mycobacteria cannot be differentiated from *M. bovis* lesions. This point is particularly important with regards to increasing interactions between livestock and wild animals.

BACKGROUND

Paratuberculosis (*Mycobacterium avium* subspecies *paratuberculosis* (*Map*)) is a chronic granulomatous enteritis of ruminants responsible for significant economic losses for the cattle and the deer industries.¹⁻³ In Belgian cattle, the disease is considered endemic with within-herd and herd prevalences of 4 per cent and 21.6 per cent, respectively.⁶ The sheep and goat sector is very small in Belgium, mostly consisting of hobbyist farmers and data are poorly documented.⁷⁻⁸

In wild ruminants, the presence of *Map* is largely documented in several European countries.⁹⁻¹² Lesions are mainly observed in the distal part of the small intestine and associated mesenteric lymph nodes.¹⁰ In Belgium, the first description of Johne’s disease in farmed red deer (*Cervus elaphus*) was documented in 2000.¹³ A survey performed on 191 wild red deer showed a seroprevalence of 7.8 per cent in Wallonia, the southern part of Belgium (Linden, unpublished results). A study on 190 roe deer (*Capreolus capreolus*) revealed a seroprevalence of 4.1 per cent in Flanders the Northern part of Belgium.¹⁴ In 2005, a study highlighted the use of molecular tools to differentiate mycobacterial infections in red deer in Wallonia.¹⁵

Natural extraintestinal lesions due to *Map* have been reported in domestic ruminants.¹⁶⁻¹⁷ Several studies have described extraintestinal lesions in spontaneous and experimental paratuberculosis in some wild ruminant species and farmed deer.¹⁸⁻²⁰ In wild cervids, natural disseminated infections are rarely reported.²¹ The Surveillance Network of Wildlife Disease operating in Wallonia performs general and targeted surveillance to report wildlife infections to local and national authorities and proposes adaptive solutions to improve the sanitary management of local wildlife.²²

In this study, the authors report a case of severe extraintestinal lesions in a wild red deer male fawn, euthanased for sanitary reasons in May 2012. A mixed *Map* and *M. avium* subspecies *avium* (*Maa*) infection was shown by quantitative PCR (qPCR) and cultures performed on hepatic lymph node, mesenteric lymph node, ileo-caecal junction and liver samples, while a single *Maa* infection was detected in the lungs and bronchial lymph nodes. Taken together, these observations suggest that mixed mycobacterial infections and systemic dissemination of mycobacteria may occur in cervids.²³⁻²⁴

CASE PRESENTATION

Materials and methods

Animal and samples

In May 2012, the Surveillance Network of Wildlife received one juvenile male red deer for postmortem examination. The deer (Forest District of Spa, Liege, Belgium) was euthanised for sanitary reasons (emaciation and diarrhoea). Infection sources and interspecies transmissions of *Map* strains between cattle and red deer have been described.²⁵⁻²⁷ However, such studies have not yet been performed in Belgium.

Postmortem examination was performed at the Faculty of Veterinary Medicine (FVM) of the University of Liege according to a systematic protocol based on (1) gross lesions, (2) histopathology and (3) targeted microbiological analysis.

Microscopic examination

Imprints slides were made of bronchial lymph nodes, hepatic lymph nodes, mesenteric lymph nodes, ileo-caecal junction and stained by the Ziehl-Neelsen (ZN) method to detect acid-alcohol resistant bacilli.
Each imprint was examined under an oil immersion objective (x 100, Olympus BX41).

Fixed tissues to the formalin 10 per cent were previously dehydrated in baths of alcohol of increasing concentration, then placed in an isoparaffin-based clearing agent (UltraClear, J.T. BAKER) before being incorporated into some paraffin wax (Tissue-Tek VIP (Vacuum Infiltration Processor)). Afterwards, two sections of 4 µm of thickness (microvolume Reichert-Jung (LEICA)) were made on each sample. Sections were then stained either with haematoxylin and eosin (HE) or with carbo-fuscin ZN to detect acid-alcohol resistant bacilli.

Histopathological sections were examined under a light microscope (Olympus BX41) using x 4, x 10, x 20, x 40 and x 100 magnifications.

Bacterial cultures
Specific mycobacterial cultures on (I) Herrold’s Egg Yolk Medium (HEYM) with and without mycobactin J media (Becton Dickinson, USA) and (II) on Coletsos (Biorad) media were made, for M avium complex (MAC, including Map and Maa) and Mycobacterium bovis isolation, respectively. All manipulations were performed in a class II vertical laminar flow hood.

Tissue decontamination steps for the cultures of MAC and M bovis were realised according to the manufacturer’s instructions. The authors followed the World Organisation for Animal Health (OIE) recommendations for the diagnostic test on solid media and fast-growing colonies were first eliminated. After five weeks of incubation, colonies were subcultured to verify mycobactin dependency (on HEYM solid media with and without mycobactin J). All cultures were periodically checked for mycobacterial growth or contamination. In this study, no macroscopic differences in colonies have been seen and a predeter-

DNA extraction and PCR
Direct DNA extraction from tissue samples was performed using the QIAdvant DNA Mini Kit.

Detection of Map was performed using qPCR (Adiavet ParaTB real-time kit, Adiagene) with a concentration step (Adiajax). After incubation (three or six months, depending the mycobacteria subspecies), DNA was extracted by taking a sterile loop-full from the colony. The loop was then inserted into microtubes containing nuclease-free water (Molecular BioProduct, USA) and vortexed before incubation for 10 min at 99°C in heating block. After centrifugation, the supernatant was stored at −20°C until used. DNA extraction was performed using the QIAmp DNA Mini Kit.

Macroscopic differences in colonies have been seen and a predeter-

Map triplex qPCR
The specific genetic elements IS900, F57 and ISMAP02 of Map were targeted as previously described.29

The primers (f and r) used for are for IS900, IS900-f (5′-TGGTGTACGGTGGCGCTGA-3′), IS900-r (5′-GGGCGAGCGTGGGCTATATCTTTTATTTATTTTTT-3′), and IS900-p (5′-(FAM)-CCGGGGCGGCGCTGGGCTATATCTTTTATTTTTT-3′), for F57, F57-f (5′-TTTATATACCGCTACCAACCAACCATG-3′), F57-r (5′-GTTGCGCCGGTTGTTGAGGTTT-3′), and F57-p (5′-(Yakima Yellow)-TCTGGCGCCGGGACCTCTG-3′), and for ISMAP02, ISMAP02-f (5′-CGCCAGGAGGCGGCCACA-3′), ISMAP02-r (5′-GTTGCGCCGGTTGTTGAGGTTT-3′), and ISMAP02-p (5′-(Dragonfly Orange)-ACTCCGCGATCCAACAACCTG-3′) and were synthesised by Eurogentec (Belgium). The PCR mixture (20 µl) includes 1X qPCRBIO Probe Mix Hi-ROX, the primers (0.375 µM), the probes (0.25 µM) and 2 µl of genomic DNA.

Maa and M avium subspecies hominisuis duplex qPCR
The specific genetic elements IS901 and IS1245 of Maa (IS901+ and IS1245+) and M avium subspecies hominisuis (Mab) (IS901− and IS1245+) were targeted as previously described.30

The primers (f and r) and the probes (p) used are for IS901, IS901-f (5′-GTGATCAAGACCTTGGCA-3′), IS901-r (5′-GCTGCGAGCTTGGCATGAC-3′) and IS901-p (5′-(Yakima Yellow)-AACACACTGCGACACACCTGGCA-3′) and for IS1245, IS1245-f (5′-CCGGATCTTGCAAGACCCT-3′), IS1245-r (5′-CGACACACCCGTTGATTCC-3′) and IS1245-p (5′-(FAM)-CCGTTGGTTTATCAGGGCTTTCC-3′) and are synthesised by Eurogentec (Belgium). The PCR mixture PCR (20 µl) includes 1X qPCRBIO Probe Mix Hi-ROX, the primers (0.375 µM), the probes (0.25 µM) and 2 µl of genomic DNA.

MTC qPCR
The specific MTC devR gene was targeted as previously described.31

The primers (f and r) and probes (p) used are devR-f (5′-CCGATGGACGACCCTGATT-3′), devR-r (5′-GAGGATCA GACAGCGCAGATC-3′) and dev-p (5′-(JOE)-AACTGTGCG CGGATCTTGCTTGGCCC-3′) and are synthesised by Eurogentec. The PCR mixture (20 µl) includes 1X qPCRBIO Probe Mix Hi-ROX, the primers (0.375 µM), the probes (0.25 µM) and 2 µl of DNA genomic extract.

The amplification conditions for all qPCRs consist of a first denaturation of 3 min at 95°C that is followed by 40 cycles consisting of 5 s at 95°C and 20 s at 60°C successively (StepOne Plus qPCR System, Applied Biosystems).

PCRs on colonies were performed after three months (July 26, 2016), six months (October 12, 2016) and 9 months (January 11, 2017) incubation.

INVESTIGATIONS
Results and discussion
Poor body condition was associated with liquid faeces soiling the anal margin and hind limbs. At the opening of the carcass, cachexy was confirmed by the absence of pericoronal and peri-

Further, the respiratory tract was heavily infested by Dictyocaulus viviparus.
The liver was enlarged (hepatomegaly) but normal at section (without nodular lesions). The hepatic lymph nodes were enlarged, firm and presented yellowish pigmented nodular foci found at the cutting surface (figure 2). Of note, hepatic lesions induced by Map are infrequently reported in cervids.32

The mesenteric lymph nodes were enlarged (10 times normal size), pale and firm. Once cut, they presented numerous yellowish and firm nodules (1–3 mm diameter) with central calcification. The mesenteric lymphatic vessels of the small intestine were enlarged heptogally lymph nodes (c). (b). Hepatomegaly is shown by the rounded edge to the liver with an enlarged hepatic lymph nodes (c).

Lungs lesions showed a chronic pleuritis and interstitial pneumonia with focal infiltration of macrophagic cells. Some Langhan’s giant cells were also observed with necrosis and calcification (figure 3). Histopathological lesions on bronchial lymph nodes could not be described because of severe histolysis.

A low rank focal macrophagic infiltration without intracellular mycobacteria, despite the specific ZN staining, was observed in the digestive tract (lesions not shown). These lesions correspond to the paucibacillary form of Johne’s disease.33 34 and are correlated to the immune response of the host to the bacterium.35 36 The same pattern of macrophagic infiltration (focal-paucibacillary form) was observed in other tissues, suggesting a systemic disease.36

Cultures for Salmonella species or Yersinia species performed on lymph nodes and faeces yielded negative results on Rappaport-Vassiliadis media at 42°C and Yersinia CIN media at 30°C for 24 and 48 hours, respectively. No Pasteurella species was isolated from the respiratory tract.

qPCR results were positive for Map after three months incubation. After 3 months incubation (July 26, 2016), the Maa-specific qPCR targeting IS900, IS1245 and ISMAP02 sequences yielded positive results (1) the liver, (2) the lung, (3) the ileo-caecal junction, (4) the bronchial lymph nodes, (5) the hepatic lymph node and (6) the mesenteric lymph nodes. After six months incubation (12 October 2016), the Maa results were similar to those obtained after three months incubation. qPCR targeting IS900, F57 and ISMAP02 sequences were realised on DNA extracted from colonies isolated on HEYM with mycobactin (on all the aforementioned organs) and Map was only confirmed for the hepatic lymph nodes-derived colonies. Surprisingly, qPCR targeting Map on the other digestive organs remained negative to the contrary of Maa PCR results (positive for all tested organs, even respiratory).

Because Map was not detected after six months incubation, it was decided to extend the incubation time on HEYM with mycobactin to nine months to rule out a possible overgrowth of Maa. After nine months incubation (January 11, 2017), results were similar to those obtained after six months incubation: the hepatic lymph node was the only one organ to be positive for both Map and Maa qPCRs. Other tissues were positive for Maa and negative for Map.

All samples were negative for M bovis (on qPCR and Coletso media culture—after three months incubation).

**OUTCOME AND FOLLOW-UP**

In 2013, Del-Pozo and others reported a mixed Map-Maa infection in reindeer.24 There are some similarities in both studies: gross lesions are comparable and M bovis infection was excluded. However, the authors were not able to isolate Map from the respiratory tract as opposed to Del-Pozo and others who were able to isolate both Maa and Map from the respiratory tract.

The authors report here an unusual case of mixed infection with Maa isolated in both the respiratory and digestive tracts and with Map only isolated from the digestive tract and the hepatic lymph node. This study highlights that single or co-infections with mycobacteria are clinically and pathologically indistinguishable and must therefore be differentiated by bacteriology and/or molecular tools.15 23–26
DISCUSSION

Most frequent mycobacterial infections in deer are bovine tuberculosis (bTB), paratuberculosis and avian tuberculosis due to Maa, Mab and Mycobacterium intracellulare. Factors likely to affect the susceptibility of deer to mycobacterial infection include age, environment, population density, exposure and genetics.²⁹³⁰

Bacterial isolation is considered the ‘gold standard’ and the most sensitive method for the diagnosis of mycobacterial diseases.²⁹³²³³ However, such cases have also been documented in farmed deer. This suggests that stress linked to the farming of immunocompromised individuals and are mainly observed in rural infections (excluding Maa, Mab cases of mixed infections within the same individual).¹⁵²³³⁹–⁴¹ cases of mixed infections within the same individual are rarely reported.²⁴²²⁴²⁴²⁵ In this study, only the digestive tract was positive for Map, while several organs (liver, ileocaecal junction, lungs, bronchial, hepatic and mesenteric lymph nodes) were positive for Maa.

DNA detection in a sample can be masked by the differential growth of the predominant mycobacteria in this sample.¹³⁰ However, after nine months incubation, no indication of Map infection outside the digestive tract and the hepatic lymph node could be documented, confirming the culture results. Altogether, the results suggest that this animal showed a mixed Maa/Map infection with a systemic dissemination of Maa in the host (including the respiratory tract), while Map infection was limited to the digestive tract.

Learning points

► Cervids are susceptible to mycobacterial infections.
► Mycobacterial lesions due to Maa and/or Map cannot be differentiated from M. bovis lesions and different mycobacterial species, alone or in association, may induce lesions in different organs in the same host.
► This point is particularly important with regards to the increasing interactions between domestic and wild animals.

REFERENCES


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ORCID iD Jacques Godfroid http://orcid.org/0000-0002-0782-7858


