WILDLIFE

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

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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, diarrhoea 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.[1–5] In Belgian cattle, the disease is considered endemic with within-herd and herd prevalences of 4per 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on 191 wild red deer showed a seroprevalence of 7.8 per cent in Wallonia, the southern part of Belgium (Linden, unpublished results).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 a*vium* (*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 euthanased 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
Inprints 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 inprint 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-fuschin 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).[28] All cultures were periodically checked for mycobacterial growth or contamination. In this study, no macroscopic differences in colonies have been seen and a predetermined number of colonies were each time collected.

DNA extraction and PCR

Direct DNA extraction from tissue samples was performed using the QIAamp DNA Mini Kit.

Detection of *Map* was performed using qPCR (Adiavet ParaTB real-time kit, Adiagene) with a concentration step (Adiafilter).

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 QIAamp DNA Mini Kit.

Macroscopically, lesions induced by *Map* cannot be differentiated from other mycobacterial infections which can be of zoonotic concern (*MAC* or *Mycobacterium tuberculosis* complex (*MTC*), including *M bovis*).[9] In order to differentiate these infections, three different qPCR protocols using the Taqman technology were followed, targeting :
I.   *IS900*, *F57* and *ISMAP02* (specific DNA sequences for *Map*).
II. *IS901* and *IS1245* (specific DNA sequences for *MAC*, other than *Map*).
III. *DevR* (specific DNA sequences for *MTC*, including *M bovis*).

Map triplex qPCR

The specific genetic elements *IS900*, *F57* and *ISMAP02* of *Map* were targeted as previously described.[29]

The primers (f and r) and probes (p) used are for *IS900*, IS900-f (5′-TGCTGATCGCCTTGCTCA-3′), IS900-r (5′-GGGCCTGATCGGCGATGAT-3′) and IS900-p (5′-(FAM)-CCGGGCAGCGGCTGCTTTATATTC-[BHQ1]-3′), for *F57*, F57-f (5′-TTCATCGATACCCAAACTCAGAGA-3′), F57-r (5′-GTTCGCCGCTTGAATGGT-3′) and F57-p (5′-(Yakima Yellow)-TGCCAGCCGCCCACTCGTG-[BHQ1]-3′, and for *ISMAP02*, ISMAP02-f (5′-CGCCAGGAACGCAAACAT-3′), ISMAP02-r (5′-GTGCAGGGTCGCTCTGATG-3′) and ISMAP02-p (5′-(Dragonfly Orange)-ACTCCGCATCCACAACTCACGCTG-[BHQ-2]-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* (*Mah*) (*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′- GTGATCAAGCACCTTCGGAA-3′), IS901-r (5′-GCTGCGAGTAGCTTGATGAG-3′) and IS901-p (5′- (Yakima Yellow)- AACAACATCGACACGATCGCCGACAA-[BHQ1]-3′) and for *IS1245*, IS1245-f (5′-CCGGATCTGCAAAGACCTC-3′), IS1245-r (5′-CGACACCACCCGATGATTC-3′) and IS1245-p (5′-(FAM)- CCGTTGGGTTATCAGCGCTTTC-[BHQ1]-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'-CCGATGGCAACGGCATT-3'), devR-r (5'-GAGGATCAGACAGCGCAGATC-3') and dev-p (5'-(JOE)-AACTGTGCCGCGATCTGTTGTCCC-[BHQ-1]-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 perirenal fat. Lungs showed numerous yellowish and firm nodules (1–3 mm diameter). The lymphoid tissue was reactive with a marked bronchial adenomegaly. The bronchial lymph node was enlarged (6.2 cm×1.8 cm), firm, congestive and presented nodules similar to those observed in the lungs (figure 1). Furthermore, the respiratory tract was heavily infested by *Dictyocaulus viviparus*.
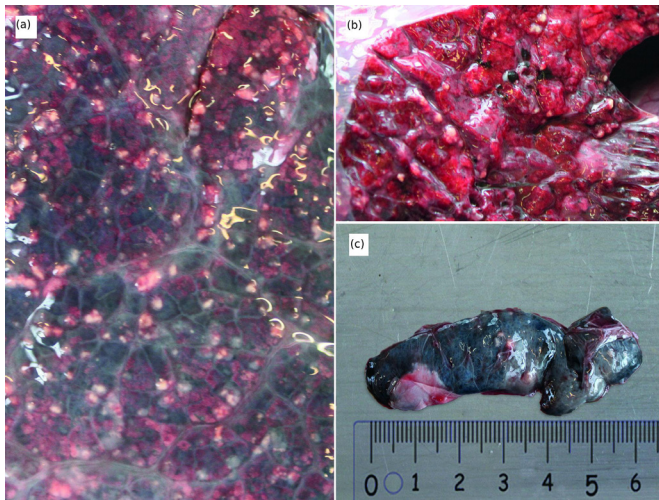
Figure 1 Postmortem examination of the respiratory tract: evidence of numerous yellowish nodules in lungs (a, b). At section, within the respiratory parenchyma, nodules are well defined (1–3 mm diameter), yellowish and firm (b). Enlarged and congestive bronchial lymph node (6.2 cm×1.8 cm), showing similar lesions similar to those observed in the lungs (c).

The liver was enlarged (hepatomegaly) but normal at section (without nodular lesions). The hepatic lymph nodes was 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 particularly prominent and thick (figure 2).

The digestive content was liquid, on all the length of the digestive tract. Some portions of the jejunum were bluish whereas the digestive mucosa was partially thickened specially the ileocaecal junction and the proximal colon. No haemorrhagies and no parasitic nematodes were observed.

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.[16 35] The same pattern of macrophagic infiltration (focal–paucibacillary form) was observed in other tissues, suggesting a systemic disease.[36]

Figure 2 Postmortem examination of the digestive tract: severe enlargement of mesenteric lymph nodes (a, see black *). When cut, they exhibit numerous firm and thick nodules (chalky appearance) (b). Hepatomegaly is shown by the rounded edge to the liver with an enlarged hepatic lymph nodes (c).
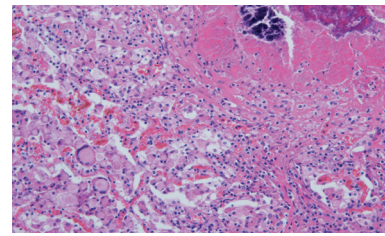
Figure 3 Histopathology of the lungs shows Langhan's giant cells infiltration and necrosis with central calcification surrounded by few haemorrhagies (upper right corner) (haematoxylin and eosin stain, x 100 magnification).

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.

qPCRs was performed on DNA extracted from organs. All digestive tissues were positive for *Map* while all other tissues (including respiratory tissues) were positive for *Maa*. On digestive organs, a *map/maa* mixed infection was confirmed.

After 3 months incubation (July 26, 2016), the *Maa*-specific qPCR (from colonies isolated on HEYM without mycobactin) targeting *IS901* and *IS1245* sequences yielded positive results for (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 Coletsos 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*, *Mah* and *Mycobacterium intracellulare*. Factors likely to affect the susceptibility of deer to mycobacterial infection include age, environment, population density, exposure and genetics.[37]

Bacterial isolation is considered the 'gold standard' and the most sensitive method for the diagnosis of mycobacterial diseases.[28 37]

In cervids, paratuberculosis is described as a disease affecting mainly the digestive system. Disseminated infections are less frequent. In domesticated and wild cervids, avian mycobacterial infections (excluding *Map*) are frequent. They can lead to macroscopic lesions (mesenteric lymph nodes hypertrophy and/or abcedation, digestive mucosa thickening) in animals that show no clinical signs.[37 38] Those opportunistic mycobacteria may induce clinical symptoms (such as emaciation and diarrhoea) in immunocompromised individuals and are mainly observed in farmed deer. This suggests that stress linked to the farming of wild species was a key factor for the clinical expression of the infection.[20 37 38] However, such cases have also been documented in wild animals.[23]

Although mixed infections in deer are well documented,[15 23 39–41] cases of mixed infections within the same individual are rarely reported.[24 42 43] In this study, only the digestive tract was positive for *Map*, while several organs (liver, ileo-caecal 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.[15 40] 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.

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