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A correlation of Mycobacterium bovis SB0134 infection between cattle and a wild boar (Sus Scrofa) in Campania region

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ABSTRACT

A case of *Mycobacterium bovis* infection is described in a death adult female wild boar in the province of Avellino, Campania Region (Southern Italy). The carcass was sent to the Istituto Zooprofilattico Sperimentale del Mezzogiorno (IZSM) of Portici, Naples, Italy, where postmortem examination was performed. At necropsy, a disseminated granulomatous infection was observed, with involvement of various lymph node districts, spleen and lungs. Therefore, all lymph nodes were collected, together with spleen and lung lesions, in order to carry out bacteriological and molecular analyses that confirmed an uncommon disseminated *Mycobacterium bovis* infection. Subsequently, an analysis of the spoligotype, performed by the National Reference Center of *Mycobacterium bovis* in Brescia (Northern Italy), resulted in the spoligotype SB0134, previously identified in bovine outbreaks in the same area where the wild boar was found.

1. Introduction

Mycobacterium (M.) bovis is the causative agent of tuberculosis (TB), a zoonotic disease that primarily affects species of zootechnical interest, where cattle are mainly invested as the target species, involving public health implications, trade limitations and severe economic losses due to eradication plans (Richomme et al., 2019; Schiller et al., 2011). However, the attention on wildlife is increasing for their possible role of reservoir in bovine tuberculosis (bTB) epidemiology (Corner, 2006; Mentaberre et al., 2014), mostly for their attitude of super-shedder, causing environmental contamination and the maintenance of tuberculosis in endemic areas (Gortázar, Fernández-Calle, Collazos-Martínez, Mínguez-González & Acevedo, 2017; Richomme et al., 2019). In Europe, some difficulties have been found in achievement bTB eradication and it has been hypothesized the possible role of wildlife as source of infection (Gormley & Corner, 2018; Varela-Castro, Alvarez, Sevilla & Barral, 2020). Indeed, new geographical areas are increasingly considered endemic for the isolation of *M. bovis* in wild animals (Crispell et al., 2020), mostly wild boars (*Sus scrofa*) and deer (*Cervus elaphus*) (Réveillaud et al., 2018; Richomme et al., 2019). Wild boars are susceptible to the same *M. bovis* genotypes that infect cattle (Réveillaud et al., 2018). Moreover, some authors have found that exposure to *Mycobacterium tuberculosis* complex (MTBC) in wild boars was related to shorter distances between them and TB outbreaks in cattle, highlighting the inter-species transmission (1). On the other hand, Varela-Castro and colleagues found an inverse correlation between the prevalence of TB in

Abbreviations: IZSM, Istituto Zooprofilattico Sperimentale del Mezzogiorno; TB, Tuberculosis; bTB, bovine tuberculosis; MTbC, Mycobacterium tuberculosis Complex; S.I.G.L.A., Informative System for Analysis Laboratories Management; BDN, National Informative System Database; ORSA, Epidemiologic and Biostatistic Regional Observatory.

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wild boars and infected livestock locations, and associated these findings to higher intra-species spread of the pathogen (Varela-Castro et al., 2020).

In wild boars, tuberculosis is mostly observed as 1 mm miliary caseo-calcareous tubercles lesions in the head lymph nodes (1), while only the 11% of them show generalized forms (Mentaberre et al., 2014).The distribution and characteristics of the lesions, in relation to their possible role in the transmission of the disease, as well as epidemiological factors, are essential for determining the meaning of infected wild animal species as a reservoir for bovine tuberculosis (García--Jiménez et al., 2013; Mentaberre et al., 2014).

The aim of this study was to better understand the role of wildlife related to the pathogen persistence in the environment and the essential involvement of these animals in an eradication plan also through geomorphological based evaluations.

2. Methods

2.1. Anatomopathological and Microbiological examinations

A complete necropsy was performed by post graduated veterinarians of the Unit of Forensic Veterinary Medicine on the dead wild boar, according to standard protocols (de Lisle, Bengis, Schmitt & O'Brien, 2002). Samples from lymph nodes, lungs and spleen lesions were collected and processed for histopathological analysis, stained with hematoxylin and eosin (H&E), microbiological and molecular examinations. Notably, *M. bovis* was determined according to the procedures described by the World Organization for Animal Health in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE Manual of Diagnostic Tests & Vaccines for Terrestrial Animals. Manual of diagnostic tests & vaccines for terrestrial animals, 2020).

Tissue samples from lungs and liver were homogenized and decontaminated. The bacteriological culture was first performed by using the BD BACTEC Mycobacteria Growth Indicator Tube (MGIT) 960 System (Bec-ton, Dickinson and Company) according to manufacturer's instructions and incubated up to 42 days. Therefore, 0.2 ml of liquid media were transferred to solid Stonebrink TB medium (Becton, Dickinson and Company) and incubated at 37 $^{\circ}C \pm 1$ °C. The development of characteristic colonies was verified in first reading after 2–5 days and then weekly for a maximum of 8 weeks starting from the date of inoculation into the liquid medium. Colonies suspected to be *Mycobacterium spp*. were subjected to Ziehl-Neelsen staining.

2.2. Molecular characterization

Suspected colonies were submitted to molecular analysis for species identification. For this purpose, 250 μL of a bacterial colony suspension were boiled at 99 °C for 15 min, then centrifuged at 10,620 xg for 10 min, successively the collected supernatant was used for molecular analyses. Amplification was carried out according to the protocol of Chimara and colleagues (13) in a final volume of 25 μL with the QuantiFast Pathogen kit (QIAGEN) and the following set of primers and probe: forward primer EXT-1 5′-CCCGGACAGGCCGAGTTT-3′ 0.5 μM, reverse primer, INT-1 5′-CCCCATCGACCTACTACG-3′ 0.5 μM, probe IS6110 5′-FAM-AACTCAAGGAGCAGTCAGGCH-BHQ1–3′ 0.2 μM. Thermal cycling conditions included an initial denaturation step at 95 °C for 5 min, followed by 45 cycles at 95 °C for 15 s and 60 °C for 30 s, and were performed on a CFX 96 touch thermal cycler (BIO-RAD).

Species identification was performed by the High Resolution Melting (HRM) according to Issa et al. protocol (14). The reaction was performed in a total volume of 20 μl. 1 μl of sample DNA was added to a reaction mixture containing 10 μl 2X SSoFast EvaGreen Supermix (BIORAD), 1 μl of each primer (10 μM) and 7 μl of DNAse – RNAse free water. The PCR thermocycling conditions were as follows: initial denaturation at 98 °C for 3 min, 50 cycles with denaturation at 98 °C for 5 s and annealing/extension at 60 °C for 10 s followed by a second cycling step at 95 °C for 1 min and 65 °C for 1 min followed by a HRM ramping from 65 °C to 95 °C. Fluorescence data were acquired at 0.2 °C increments every 10 s to generate specific melting curves. For each experiment, the three reference strains *M. microti* ATCC 19,422, *M. bovis* ATCC 19,210 and *M. tuberculosis* ATCC 27,294 were included as melting curve standards and positive controls. To exclude contaminations in the reaction mixture, ultrapure water was added as a negative control in each experiment. Next, the specimens were submitted to Mycobacterial interspersed repetitive units (MIRU) variable number tandem repeat (VNTR) by the National Reference Laboratories for Tuberculosis (Istituto Zooprofilattico Sperimentale of Lombardia and Emilia Romagna-IZSLER- Brescia, Italy) that graciously provided the results.

2.3. Database

A georeferenced analysis was conducted in the area surrounding the wild boar tested positive carcass, highlighting bovine herds, pastures and hunting reserves. Positive SB0134 cattle data were investigated over eight year period (2011 – 2019) obtained from the database of the IZSM, Informative System for Analysis Laboratories Management (S.I.G.L.A.). The produced map highlights the area where the farms found to be positive for the spoligotype under examination, are located. In addition, all intensive, transhumant and extensive livestock farms and those with unknown breeding management, as the in-formation is not present in the national informative system database (BDN), were traced. Data were carried out by the Epidemiologic and Biostatistic Regional Observatory (ORSA) by using ArcMap 10.7 software with epsg projection 32,633 - wgs 84 / utm zone 33n spatial reference system.

3. Results

In March 2019, an adult female wild boar weighing approximately 200 kg was found dead in the municipality of Montoro (Campania, Southern Italy; 40°49′N, 14°45′E), and transferred to the Istituto Zooprofilattico del Mezzogiorno (IZSM) of Portici, Naples, Italy, in order to investigate the cause of death. Physical examination showed a severe state of malnutrition. Proceeding with the necropsy, prescapular, mandibular, bronchial, mediastinal, hepatic and inguinal lymph nodes were found enlarged and cheese-like in appearance (Fig. 1).Lungs showed pleural thickening with fibrin deposition and multifocal granulomatous areas (Fig. 2); at last, the spleen surface showed 1–2 cm in diameter nodules, firm in appearance and pale yellow in color, which also presented a solid white caseo-calcareous material (Fig. 3).Lungs showed pleural thickening with fibrin deposition and multifocal granulomatous areas (Fig. 2); at last, the spleen surface showed 1–2 cm in diameter nodules, firm in appearance and pale yellow in color, which also presented a solid white caseo-calcareous material (Fig. 3).

Tissue samples were obtained from lungs, spleen and lymph node

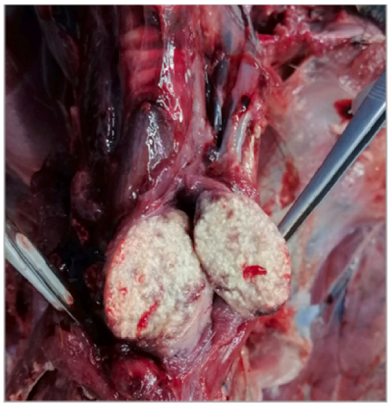

Fig. 1. Cutting section of the bronchial lymph node.

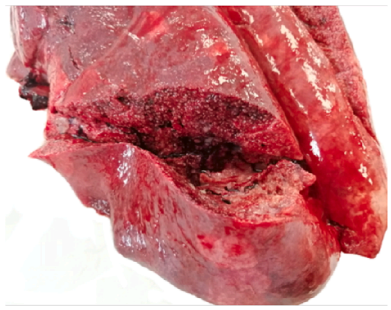

Fig. 2. Multifocal granulomatous lesions in lungs.

Fig. 3. Caseo-calcific nodules in spleen.

lesions and submitted to microbiological and molecular examinations. Suspected Mycobacterial colonies were first processed to Real-time PCR protocol for the detection of *Mycobacterium tuberculosis Complex*, then to High Resolution Melting (HRM) and M. bovis was identified. Finally, the specimens were submitted to both spoligotyping and 12 genomic loci MIRU-VNTR typing that showed the isolates could be classified as Spoligotype bovis 0134 (SB0134).

At georeferenced analysis eight SB0134 positive cattle, belonging to six different herds in the last 8 years, were found between 4 and 15 Km from the wild boar carcass (Fig. 4).Above eight cattle, five of them were Podolica breed (*Bos promigenius*), one mixed breed and two Italian Pezzata Rossa (*Bos taurus*). Moreover, it was observed that this same area presents bovine grazing and hunting reserves.

4. Discussion

Mycobacterium bovis is recognized as the major agent of animal tuberculosis, infecting many species of wild, domestic mammals and humans (Luciano & Roess, 2020). Different epidemiologic situations may occur, from dead-end spillover hosts to maintenance hosts and reservoirs (Richomme et al., 2019). Same spoligotype identification in both domestic and wildlife species favors the hypothesis of inter-species transmission of the infection. The transmission from wild animals to livestock or vice versa may take place when the animals share the same grazing [16)] Indeed, Reis and colleagues, by evaluating molecular data, reinforced and supported the inter-transmission of bTB from livestock to wildlife and wildlife-wildlife intra-transmission. Thus, wildlife may negatively contribute to the efficiency of TB eradication programs in cattle (Chimara, Ferrazoli & Leão, 2004; Issa et al., 2014; Reis et al., 2020).

For many years, bTB eradication in Campania region has been a real challenge. It was estimated that in 2018, *M. bovis* infected farm prevalence and incidence were 0.62% and 0.47%, respectively, and the prevalence for the area under study (Avellino province) was 0.53% (Relazione annuale del Piano regionale Integrato- Anno, 2018). Little is known about the regional epidemiology of wild boars and wildlife in general. Iovane and colleagues in a serosurvey on wild boars observed that the 10.6% of the tested animals presented antibodies against *M. bovis* (19). In Italy, a different range of this pathogen infection in wild boars has been observed, from 2.4% in Central-Northern Italy to 8.4% in Southern Italy, and genotype SB0120, SB0841 and SB1565 were mostly identified (Casalinuovo, Ciambrone, Grillone & De Gori, 2017; Regione Abruzzo, 2021), but no data are available on SB0134 in this species. Our

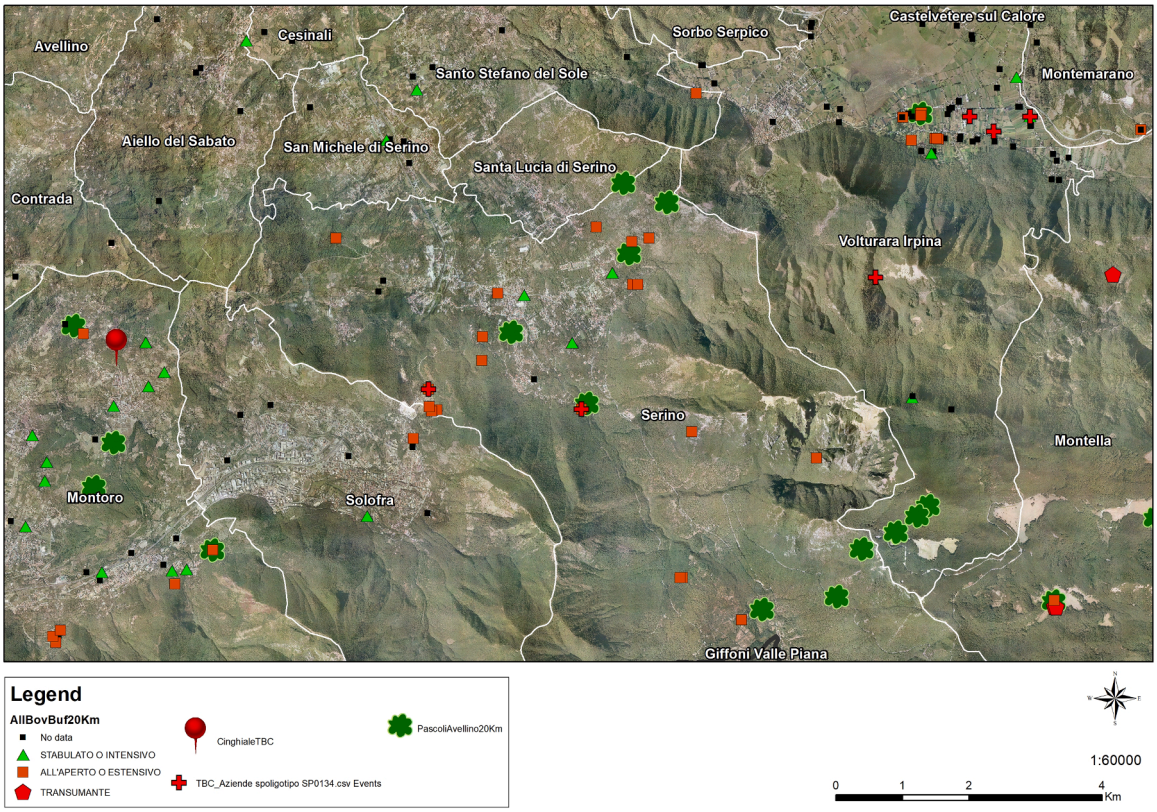

Fig. 4. Georeferenced map showing herd, pasture and hunting overlapping areas with the tested positive wild boar.

spoligotype analysis shows that SB0134 is circulating throughout the Campania region (Pacciarini & Zanoni, 2015). The overlapping wild boar hunting and cattle grazing areas allows to assume that feeding of wild boars in the pastures of infected cattle, thus, indirect contact could represent a reasonable transmission route (Naranjo, Gortazar, Vicente & de la Fuente, 2008). However, it is necessary to consider that the establishment of persistence infection mechanisms in wild populations it is a complex phenomenon resulting from the combination of various factors such as the density of wild species, social interactions between individuals, the biology and eating habits of animals, the persistence of bacteria vital in the environment, interaction with potentially infected pets and the influence of man, through hunting practices, also making available trophic resources for animals and habitat modification, such as construction of fences and obstacles, or the abandonment of agricultural lands. This epidemiological complexity, which involves several animal species, is represented by the infection maintenance mechanisms in some areas of Spain among wild boar and deer populations, with possible infection transfer in cattle (Gortázar et al., 2012). Conditions believed to locally affect the transmission and persistence of bTB can be found in the high density of wild animals, the concentration of animals around feeding and watering areas and a Mediterranean climate, with hot and dry summers, which promotes greater aggregation of animals around watering points (Palmer, 2013). Furthermore, by nature, wild boars are able to move up to 80 km per week searching for food (Scherer et al., 2020), indeed, a range from 0 to 20 Km (average 7.38 km) has been observed between positive subjects and bovine tuberculosis outbreaks (Iovane et al., 2020). Considering that wild animals and extensive livestock farms share the same spaces, which can be used for roaming grazing, it is possible to hypothesize an indirect contact also through manure, combined with the presence of positive grazing animals can favor environmental contamination and a possible inter and intra-species contagion.

5. Conclusions

From 2011 to 2019 various cattle with SB0134 were found and later, in 2019, a wild boar with the same spoligotype. This could suggest that the transmission took place from cattle to the wild boar, however it remains of firm importance the correlation found. The importance of what is highlighted in this work lies in the fact that the eradication plan implemented in Italy should be extended and integrated with a surveillance plan dedicated to wildlife of TB interest, especially in areas such Campania Region where bovine tuberculosis is endemic and difficult to eradicate.

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Ethic statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. The Istituto Zooprofilattico Sperimentale is the official laboratory designed by the Italian Ministry of Health. According to National regulation and internal policy, ethical approval was deemed unnecessary.

Data availability

The data that support the findings of this study are included in the manuscript.

Author contribution

All the authors equally contributed to the study. ES, LC and CdM writing—original draft preparation. GG, NDA, MGL, VMT and GF, conception and supervision. AC, PC and RP methods. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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