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The eradication of *M. caprae* tuberculosis in wild boar (*Sus scrofa*) in the Bieszczady Mountains, southern Poland – an administrative perspective

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Abstract

Introduction: Animal tuberculosis (TB) is a zoonotic disease caused by acid-fast bacteria belonging to the *Mycobacterium tuberculosis* complex (MTBC). Both animals and humans are susceptible to infection by the MTBC. Interspecies transmission is also possible, including to livestock and humans. In the years 1997–2013, many tuberculosis cases were recorded in European bison in the Bieszczady Mountains; more alarmingly, TB was also recorded in wild boar in the years 2013–2020. **Material and Methods:** In the years 2013–2020, 104 wild boar from the Bieszczady Mountains were tested for TB through necropsy, mycobacterial culture, strain identification and spoligotyping. **Results:** The microbiological examination confirmed TB in 46 wild boar; these infections were identified as *M. caprae*, spoligotype SB2391. **Conclusion:** Free-living European bison are at risk of TB infection from wild boar carrying *M. caprae*. This situation also poses a risk to local cattle. There is a need for further activities aimed at monitoring the disease, preventing further transmission, and minimising the risk to public health.

Keywords: *Mycobacterium caprae*, spoligotype SB2391, tuberculosis, veterinary administration, wild boar.

Introduction

Animal tuberculosis (TB) is a zoonotic disease caused by acid-fast bacteria belonging to the *Mycobacterium tuberculosis* complex (MTBC) (23, 28). Of these, the main causes of tuberculosis in cattle, other livestock, companion animals, wild animals and even some species of carnivorous birds are *Mycobacterium bovis* and *Mycobacterium caprae* (1, 4, 13, 14, 28).

Earlier reports noted the presence of TB in wild boar in a few European countries (15, 16, 27). Its role in the transmission of TB is not always clear; however, it is believed to depend on various conditions, such as population density, the presence of tuberculosis in other species, feeding and climate (20). Most European cases of tuberculosis in boar have been reported in the Iberian Peninsula. This high incidence is probably related to the prevailing environmental and climatic conditions of the area; for example, during the dry summers typical of the region, animals tend to gather near water pockets and feeding grounds, which is conducive to infection (8). In France, most cases of boar tuberculosis are in areas where bovine tuberculosis has also been diagnosed; hence, the wild boar population is believed to be the key aetiological agent in this environment (21).

The first infection with TB (*M. caprae*) in the Bieszczady Mountains in Poland was confirmed in a fallen wild boar in 2013 (11). The carcass was found

near the town of Nasiczne in the Lutowiska district, in an area where an episode of the disease had been noted over the three-year period up to the carcass discovery. Animal tuberculosis was later confirmed in all individuals of the nearby Górny San bison herd, 26 of which were tested following death or culling (12). The herd itself previously lived in areas administered by the Stuposiany Forest District and the Bieszczadzki National Park. It was found that the *M. caprae* strains isolated from the fallen wild boar shared a molecular pattern with those of the infected bison, confirming the transmission of the disease between these two free-living animal species (11, 19).

Material and Methods

Animals. Submandibular and bronchial lymph nodes were collected from 104 wild boar (*Sus scrofa*) and tested for the presence of tuberculosis. Most boar carcasses (n = 99) were obtained from hunting, and the rest (n = 5) were natural deaths. The samples were collected in the years 2013–2020 in the Bieszczady Mountains. The largest numbers of tissue samples were collected in 2013 (n = 21), 2014 (n = 27) and 2017 (n = 29). In the remaining years, one to fifteen individuals were examined (Table 1).

Mycobacterial culture and strain identification. The laboratory diagnosis of tuberculosis was performed following the instructions of the Chief Veterinary Officer. The procedure comprised anatomopathological examination of the tissues sent, cultivation, microscopic examination, and genotypic identification of *Mycobacterium* isolates (7). The current relevant legislation is the Animal Health Law (6).

The lymph nodes were removed, cut into small pieces, and then processed for 3 min in an Interscience homogeniser (Schaffhausen, Switzerland). The tissues were then flooded with 5% oxalic acid solution and mixed thoroughly. The supernatant was pipetted into a separate 50 mL Falcon tube (Bionovo, Legnica, Poland) and incubated at 37°C for 20 min. The sample was then centrifuged for 10 min at 3500 × *g*. The supernatant was removed, and the pellet washed twice with sterile saline. The resulting sediment was examined microscopically and used for inoculation on solid substrates produced in house: Stonebrink (S) and Petragnani (P). Each sample was inoculated onto six plates (three with S media and three with P media) and incubated at 37°C for six weeks. All media for mycobacteria, including the reagents used in the study, were prepared by the Nutrient Department of the National Veterinary Research Institute in Puławy (10).

The obtained sediment was also used for microscopic examination. The samples were stained using a Gram Staining Kit (Merck Life Science, Darmstadt, Germany) according to the manufacturer's protocol.

The strains grown on S media were identified using a MGIT TBc kit (Becton Dickinson, Franklin Lakes, NJ, USA), which is an immunochromatographic mycobacterial growth indicator tube (MGIT) test for detecting the MPT64 protein fraction secreted by MTBC cells during cultivation.

The isolates were identified to the species level using the GenoType MTBC test (Hain Lifescience, Nehren, Germany) according to the manufacturer's protocol (24). The strain was identified by spoligotyping using a genotyping kit (Gentaur Molecular Products, Kampenhout, Belgium) following the manufacturer's recommendations. This method detects polymorphisms in the chromosomal direct repeat region which are present only in the MTBC genome.

Results

In 22 of the 46 TB positive wild boar (47.8%), tuberculous nodules ranging in size from 2 to 4 mm were found to be present in the submandibular lymph nodes. In the mediastinal lymph nodes, the changes were larger, reaching diameters of approximately 10 mm (Fig. 1), but less common, being present in only four individuals (8.7 %). In all wild boar with tuberculous lesions in the mediastinal lymph nodes, nodules were also visible in the submandibular lymph nodes.

Table 1. The number of wild boar samples tested in 2013–2020 and the number of confirmed TB cases

Year	2013	2014	2015	2016	2017	2018	2019	2020	Total
Number of wild boar tested	21*	27**	2	8	29	1	15	1	104
Number of confirmed positives (*M. caprae*)	9*	14**	2	5	9	1	5	1	46

* 2013 – including one wild boar from the Leski county (bordering the Bieszczady county)
** 2014 – including one wild boar from the Przemyśl county (bordering the Bieszczady county)
Another wild boar was obtained from the Bieszczady county

Fig. 1. A segment of a mediastinal node with nodules of approximately 10 mm in diameter

Table 2. Description of SB2391 spoligotype

Pattern	Octal code	Hex code	Spoligotype	Animal (number of strains)
□■□□□□□□□□□□□□□□■■■■■■■■■■■ □■■■■■■■■■□□□□□□	200003777377400	20-00-1F-7E-FF-40	SB2391*	wild boar (46)

* — Assigned by the *Mycobacterium bovis* spoligotype database

The mycobacteria showing growth on the S medium were initially classified as *M. bovis* and *M. caprae*. Their growth was visible as slightly moist and brittle white colonies. Based on the MGIT TBc identification test results, 46 strains were qualified to the MTBC complex. GenoType MTBC molecular testing confirmed the presence of *M. caprae* in tuberculosis strain cultures isolated from 46 boar (Table 1). Genetic relationship analysis confirmed that all strains classified as *M. caprae* had a common SB2391 spoligotype (Table 2); this was first introduced to the *Mycobacterium bovis* spoligotype database (25) by Krajewska in 2016 and has so far been confirmed only in Poland.

Discussion

In wild boar, control of TB is not mandated by national or European Union regulations. Pursuant to the provisions of the Act of March 11, 2004 (9) on the protection of animal health and combating infectious animal diseases, TB caused by *M. bovis* is classified as a zoonotic disease caused by a zoonotic agent and must be monitored as such; however, Polish law lacks specific rules and criteria for conducting such monitoring in free-living animals. Nevertheless, under Regulation (EU) 2016/429 (6) in force from April 21, 2021, bovine tuberculosis outbreaks caused by infection with the *Mycobacterium tuberculosis* complex (*M. bovis*, *M. caprae* and *M. tuberculosis*) are category B diseases, and hence subject to compulsory control in all EU Member States if they occur in the specified species (*i.e. Bison* spp. – European bison, *Bos* spp. – cattle, or *Bubalus* spp. – buffalo).

Control measures apply to outbreaks of TB as a category B disease in cattle, buffalo, and bison, both as livestock and free-living animals. However, among other species of even-toed ungulates, including wild boar, the disease is classified as category D or E and no such control is needed; category D makes it obligatory only to meet specific requirements and standards in the

trade of animals, especially in international trade, and category E imposes only the surveillance and notification of identified outbreaks of such diseases.

Most *Sus scrofa* samples were collected in 2013 and 2014, which is related to the time point of the culling of the 'Górny San' herd and training of hunters to collect tissue samples from wild boar prey. In addition, in these years the highest number of confirmed cases of animal tuberculosis in wild boar was recorded. The locations of the TB-positive tissue samples obtained from wild boar in 2013–2020 are given in Fig 2. Clearly, with this scale of risk and the possible environmental pressure associated with the disease, there is a strong possibility that further transmission may occur between infected wild boar and bison, resulting in the emergence of TB in free-living bison herds. Furthermore, this risk of transmission increases with the number of free-living bison in the Bieszczady Mountains, which rose from 256 in March 2013 to 739 in March 2021 (26).

The problem of TB in the wild boar population in the Bieszczady Mountains is growing, and it is possible that the disease is endemic in the Lutowiska district, as implied by the number of confirmed infections among hunted animals. Animal tuberculosis in the Bieszczady Mountains affects not only bison and wild boar, but also other species of free-living animals. In this area, *M. caprae* has been isolated from the lymph nodes of several wolves and a roe deer (18, 19, 20), the last isolation being from a wolf in April 2022 (unpublished data).

The distribution and prevalence of the disease are also driven by the dynamic growth of the bison population in these areas, which is highly susceptible to infection with bovine tuberculosis (3, 12, 22, 29).

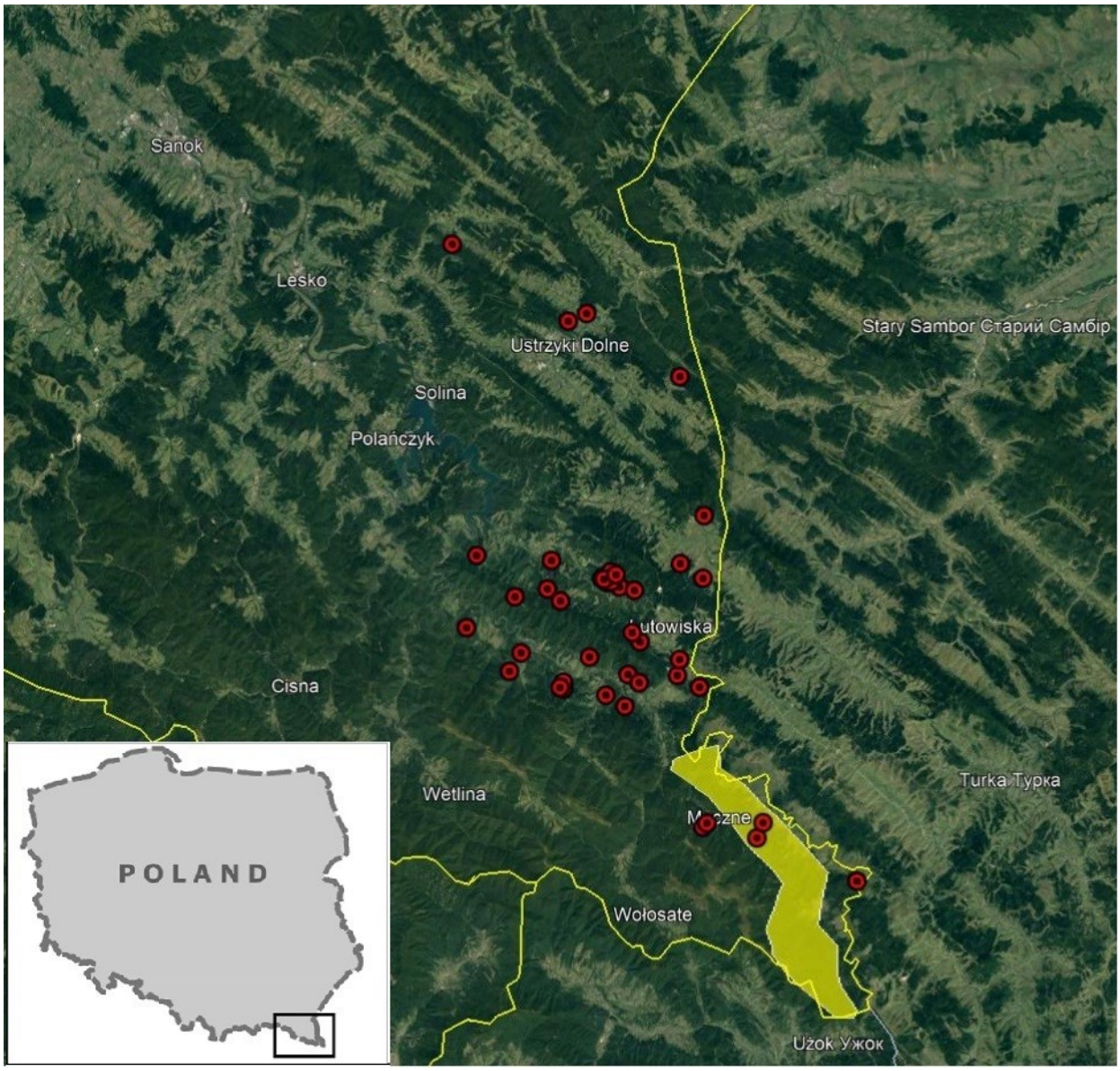

Fig. 2. Locations of TB-positive samples obtained from wild boar (red points) in 2013–2020. Yellow area – the range of bison from the Górny San herd

There is a pressing need to continue current administrative activities, including live monitoring of the health of wild boar and other free-living animals in the natural environment. In addition, passive monitoring is needed for tuberculosis in predators, especially wolves, which function as indicator species of tuberculosis infections in free-living animals in the Bieszczady Mountains (17). Additionally, considering that the problem of TB in wild boar in this region is related to the occurrence of infections in European bison, it may be worth introducing active monitoring of the bison population. This could consist of limited annual random shooting and examination of at least 10% of bison from each separate subpopulation, *i.e.* herds at risk of tuberculosis, or of the selective elimination and examination of weakened, emaciated, old and sick animals. Such activities in relation to bison are allowed by EU Regulation 2016/429. Implementation of such monitoring in Poland would require the activities to be a mandatory and centrally planned programme for monitoring and eradicating tuberculosis in bison which was enacted into national law.

It should be noted that, in accordance with Commission Delegated Regulation 2020/689 (5), a confirmation of MTBC infection in animal populations other than farmed cattle does not affect the TB-free status of a Member State or MTBC-free zone, provided that effective measures are implemented and periodic assessment is undertaken to prevent transmission of MTBC infection to farmed cattle. The endemic nature of tuberculosis in free-living animals in the Bieszczady Mountains poses a real risk of infection to local cattle and may hence have a direct impact on the official TB-free status of Poland (2). Planned action must be taken to monitor the disease, prevent further transmission of infection and minimise threats to public health.

Following the discovery of tuberculosis in wild boar in the Bieszczady region, a potential threat to both human and animal health, the Provincial Veterinary Inspectorate in Krosno carried out a risk analysis and subsequent administrative measures; these efforts were supported by subordinate veterinary services, State Forests and the Polish Hunting Association. The following *ad hoc* and short-term measures were implemented: (i) stricter veterinary supervision at venison collection points in Bieszczady and neighbouring counties, (ii) notification of the Provincial Sanitary Inspector in Rzeszów, the District Board of the Polish Hunting Association in Krosno, the Regional Directorate of State Forests in Krosno, the Chief Veterinary Officer of the Podkarpackie Voivodeship, and all district veterinarians, particularly those of the Podkarpackie voivodeship, of the risk of tuberculosis in wild boar, and issuance of advice regarding supervision over their respective game processing sites.

The local media were also informed, in order that information about the threat could be disseminated to the local population and tourists. Medium-term and long-term activities were undertaken, which were the preparation and distribution of information leaflets and general materials about the prevalence of tuberculosis in free-living animals. Training was also provided for hunters and employees of the State Forests and the Bieszczady National Park: in 2013, 14 such training sessions were held for a total of 411 participants. Hunters were issued recommendations for dealing with hunted wild boar in areas at immediate risk of tuberculosis outbreaks as well as adjacent areas (Bieszczady, Lesko, Sanok and Przemyśl counties).

In the Bieszczady county, the wild boar population was monitored passively, all samples from dead animals were tested for tuberculosis, and wild boar with suspicious organ changes were monitored actively. With the consent of the Chief Veterinary Officer, the requirement that a fifth of the herds in the Bieszczady county should undergo annual surveillance for tuberculosis was changed to a blanket requirement applying to all the county's cattle herds until 2019. Other species of free-living animals, especially bison and predators of protected species, *e.g.* wolves, were monitored passively for tuberculosis; this was supplemented by active testing in cases when wolves were eliminated. Ongoing research, analysis and expert support was provided by the Faculty of Veterinary Medicine, Warsaw University of Life Sciences, and the National Veterinary Research Institute in Puławy, as well as the Institute of Tuberculosis and Lung Diseases in Warsaw.

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