ORIGINAL ARTICLE

Microbiological and molecular monitoring for bovine tuberculosis in the Polish population of European bison (*Bison bonasus*)

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Abstract

Introduction and objective. In recent years, bovine tuberculosis (BTB) has become one of the major health hazards facing the European bison (EB, Bison bonasus), a vulnerable species that requires active protection, including regular and effective health monitoring. Monitoring of zoonotic disease in wildlife is also an important part of public health protection. The aim of the study was to determine whether BTB still influences the EB population in Poland.
Materials and method. During 2017–2019, mandibular, retropharyngeal and mediastinal lymph nodes were collected from 90 EB during *post-mortem* examination, and then cultivated on Lowenstein-Jensen and Stonebrink media. Isolated strains were subjected to molecular analysis to determine the species, spoligotype and MIRU-VNTR pattern.
Results. Lesions were found in lymph nodes originating from eight EB (8.89%). Positive microbiological cultures for mycobacteria were obtained in samples from six (6.67%) EB. The isolated strains were identified as Mycobacterium caprae (material from four EB) and atypical mycobacteria (material from two EB). For *M. caprae* strains spoligotype M. bovis 4_CA 1600 was identified and the MIRU-VNTR pattern was identified as 345751355413232.
Conclusions. It is recommended that this potentially dangerous disease should be monitored in EB via a comprehensive strategy based on a combination of microbiological and molecular methods. Such monitoring will protect the health of both animals and humans.

Key words

monitoring, bovine tuberculosis, *Mycobacterium caprae,* European bison, *Mycobacterium avium* complex

INTRODUCTION

Almost a quarter of the global European bison (EB, Bison bonasus) population lives in Poland. The European bison *Pedigree Book* indicates that in 2018, the EB population in Poland comprised 1,820 individuals: 207 – closed farms, 1,613 – free-ranging herds (https://bpn.com.pl/index.php?option=com_content&task=view&id=1132&Itemid=306]). EB is a priority species for the European Union in terms of conservation measures (species code: 2674), and despite EB being included in the VU (vulnerable to extinction)

category in the International Union for Conservation (IUCN) *Red Book of Endangered Species*, its population is steadily growing (http://www.iucnredlist.org) [1]. The main goal of EB restitution now is to increase the number of free-ranging and enclosed-breeding herds [2, 3]. In Poland, the national EB protection project places a high priority on monitoring the health of the population [4]. Such monitoring is primarily based on *post-mortem* examination, during which clinical material is collected for further diagnosis [5].

Suspsceptibilty to infectious diseases seems to be one of the most crucial problems but there are also existing environmental threats [6]. In the 20th Century, a number of diseases affected the EB population in Poland, with the most significant ones being foot-and-mouth disease, posthitis and bovine tuberculosis (BTB) [7]. BTB, caused by *Mycobacterium bovis* and *Mycobacterium caprae,* is a chronic disease that has

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been found in many species of both livestock and wildlife [8, 9], including the genus *Bison* [10]. Not only is it important that endangered animals be monitored for BTB, but as the disease is known to have zoonotic potential, its presence in other species also needs to be tracked [11, 12]. Even though, currently, Poland has the officially tuberculosis free (OTF[1]) status, in recent years cases of *M. bovis* and *M. caprae* infection has been confirmed in wildlife [13, 14]. Sharing pastures by free-living animals with livestock can lead to the pathogens transmission and pose a real threat to public health. Wherefore, the monitoring of BTB in EB is important for two reasons – species protection and its zoonotic nature.

Currently, despite the appearance of many new methods, the current gold standard in tuberculosis diagnosis is based on a combination of culture and molecular methods. These methods are also the most appropriate for use in *post-mortem* monitoring. In such cases, the predilection tissue for mycobacteria isolation is that of the lymph nodes [15, 16].

OBJECTIVES

As the severity of the threat presented to European bison in Poland by BTB remains unclear, the aim of the present study was to determine the occurrence of mycobacteria within the population by examination of lymph nodes collected *post-mortem* from EB for BTB testing. The study also uses a combination of microbiological and molecular methods to classify any identified mycobacteria.

MATERIALS AND METHOD

Material collection. During 2017–2019, lymph nodes were collected from 90 European bison (EB) from various regions of Poland. The age of the EB ranged from three months to 25 years (mean age: 7.5 years). The ages of nine EB were unknown. Of the studied EB, 44.44% were from free-ranging herds (Knyszyńska Forest, Bieszczady Mountains, Borecka Forest, Białowieska Forest) and 55.56% from breeding centres. *Post-mortem* examination was performed on all EB, during which lymph nodes (mandibular, retropharyngeal and mediastinal) were collected for further examination. The nodes were delivered to the laboratory under refrigerated conditions and kept at -20 °C until cultures were performed.

Mycobacteria isolation. The collected lymph nodes were examined for the presence of anatomopathological lesions, with particular emphasis on tuberculous lesions. Following this, the material was subjected to standard methods for mycobacterial isolation according to Orlowska et al. (2017) [13]. Briefly, the material was homogenized in 5% oxalic acid and flushed twice in saline solution. The supernatant was then plated on Lowenstein and Stonebrink media, and incubated at 37ºC for 12 weeks, with the growth on the media being checked every seven days.

Genetic analysis. DNA was isolated using the Genolyse isolation kit (Hain Lifescience, Germany). Based on the obtained DNA, the strains were classified as MTBC (*Mycobacterium tuberculosis* complex) or as 27 NTM

1. OTF – officialy tuberculosis free.

(Non-tuberculosis mycobacteria) species using the GenoType®Mycobacterium CM test (Hain Lifescience). Within the MTBC group, the species were further differentiated using GenoType®MTBC assay (Hain Lifescience) which is based on the polymorphism of the gyrB gene. GenoType tests are based on DNA-STRIP method which can identify PCR products by allowing them to hybridize with specific oligonucleotide probes on a nitrocellulose strip. Both tests were carried out in accordance with the manufacturer's instructions. The isolated DNA was also subjected to spoligotyping using a commercial genotyping kit (Genutaur molecular products, Kampenhout, Belgium) (https://gentaur-spain.com/wp-content/uploads/2015/03/Spoligotyping-Manual.pdf). This method allows the polymorphism of the chromosomal DR (direct repeat) region occurring only in the genome of MTBC to be detected. The obtained patterns were compared to those of strains registered in the SpolDB4 database.

In addition, the DNA obtained for each strain was subjected to 15 amplification reactions using 15 pairs of primer sequences based on the MIRU-VNTR method. This method is based on the analysis of selected polymorphic microsatellite sequences within the mycobacterial genome. The PCR products were visualized by gel electrophoresis (2.5% agarose gel, large gel-65V, 5 hours). The following loci were analyzed: MIRU-4, MIRU-10, MIRU-16, MIRU-26, MIRU-31, MIRU-40, VNTR 424, VNTR 577, VNTR 2165, VNTR 2401, VNTR 3690, VNTR 4165, VNTR 2163b, VNTR 1955, VNTR 4052. The results are presented as a 15-digit code. The digits indicate the number of repetitions of subsequent MIRU-VNTR repeat sequences.

RESULTS

Assessment of lesions in lymph nodes. Lesions were observed in lymph nodes originating from eight EB (8.89%), among which four EB demonstrated caseous lesions and four presented purulent lesions.

Mycobacteria isolation. Material obtained from five EBs demonstrated growth on both types of media, manifested as rough, yellow colonies on the surface of the medium. Material from one EB was found to give rise to two types of colony.

Genetic analysis. Of the seven tested strains, four were classified as *M. caprae*, two as *Mycobacterium xenopii* and one as *Mycobacterium avium*. Further analysis of *M. caprae* found the spoligotype for these strains to be identified *M. caprae* – spoligotype M. bovis _4_ CA 1600 (octagonal pattern: 200003770003600) (SpolDB4 database). For the studied *M. caprae* strains, the MIRU-VNTR pattern was identified as 345751355413232.

DISCUSSION

BTB cases in EB should be should be carefully analyzed and monitored. In this study, 4.44% of tested samples were found to be BTB positive. These samples were obtained from EB kept in an enclosed breeding centre where BTB had been diagnosed previously. Although cases of BTB have been noted almost every year in this herd since 2013 [17], no new BTB outbreaks occurred in EB elsewhere in Poland in the

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period 2017–2019. However, as BTB cases have been recorded in EB in Poland in previous years, it is recommended that lymph nodes from each dead EB be collected *post mortem* and subjected to mycobacterial examination.

European bison appear to be particularly sensitive to BTB infection, as indicated by the large number of cases observed in the past [18]. In the period 1996–2013, 45 cases of BTB were confirmed in EB (*Bison bonasus caucasicus*) in the Bieszczady Mountains in south-east Poland [18]. The first case concerned a three-year-old cow in which generalized tuberculosis was found during *post-mortem* examination [19]. Following this, in the period 2005–2008, BTB was confirmed in two EB from the Bieszczady area [20]. In 2009, an entire free-ranging herd in the Bieszczady Mountains was culled following the discovery of BTB: tuberculosis lesions were found during *post-mortem* examination in all herd members, and BTB was confirmed microbiologically in 23 out of 24 individuals [21].

It should be noted that since 2012, BTB cases have also been reported in wild boars in the Bieszczady Mountains. Interestingly, the strain identified in the boars demonstrated an identical spoligotype to a herd in which BTB had previously been diagnosed in 13 out of 18 individuals [14]; although the decision was made to cull the entire herd, several EB separated from the herd and could not be found [22]. The long history of BTB in the Bieszczady Mountains justifies greater monitoring for BTB, particularly in this region. In addition, in 2016, two cases of BTB were also confirmed in free-ranging EB in a herd in the Borecka Forest [23] in the Warmia-Mazuria region of north-east Poland, but no cases where confirmed in this region later [24].

Attempts have been made to isolate mycobacteria *ante-mortem* from animals in captivity, such as in zoos and breeding centres, using materials such as bronchopulmonary lavage from lions [25] or EB [26]. However, due to the difficulties associated with collecting material, indirect tests are typically used for *ante-mortem* diagnostics in free-ranging animals, such as the tuberculin test [27, 28], gamma-interferon test [29, 30, 31, 32] or serological tests [33, 34, 35]. These tests, however, are less sensitive than *post-mortem* microbiological monitoring, and they often lack standardization for different species, including EB. For this reason, *ante-mortem* monitoring for BTB is not sufficient for monitoring the epizootic situation in the EB population, and material obtained *post-mortem* remains extremely valuable.

One of the goals of sustainable development set by the World Health Organization is to combat the global tuberculosis epidemic. It should be emphasized that data on the number of tuberculosis cases in humans are considered to be underestimated and the real scale of the problem remains unknown [36]. BTB is a dangerous zoonosis, and monitoring is needed to protect public health. Although in recent years the number of BTB infections has decreased significantly in developed countries, mainly due to wide-scale milk pasteurization and eradication programmes, cases are still observed, even in Poland [37]. Infected EB in zoos or bison breeding centres can pose a potential health risk to tourists, keepers or veterinarians.

BTB testing is also important in the EB population because wildlife often share pastures with livestock, and this has been found to contribute to the spread of mycobacteria [38, 39, 40]. Such sharing clearly presents a risk for EB which may come into contact with potentially infected cattle; conversely, infected EB can also pose a zoonotic risk for humans by coming into contact with livestock. For Poland to maintain the OTF status, the prevalence of BTB in free-ranging animals, especially the EB and wild boars of the Bieszczady Mountains, needs to be effectively monitored. The possible loss of the OTF status would be associated with economic losses and trade restrictions, therefore it is of a great importance to monitor BTB, especially in highly sensitive species.

M. caprae have been isolated previously in European wildlife such as red deer (*Cervus elaphus*) [41, 42, 43, 44], red fox (*Vulpes vulpes*) [45] and wild boar [46]. It has also been described in wild boar (*Sus scrofa*), roe deer (*Capreolus capreolus*) and wolves (*Canis lupus*) in Poland [47]. The MIRU-VNTR pattern obtained in the present study has not been described previously. It may be of great importance in the case of outbreaks of tuberculosis in European bison, or in other wildlife or livestock animals, especially those from the same region. Such information can help establish transmission routes and allow epidemiological investigations [48].

It is notable that atypical mycobacteria were isolated from the EB in the present study. Even though *M. avium* infection in animals can be asymptomatic, their occurrence should be monitored in protected species as infection is often associated with loss of condition, and the occurrence of diarrhea and weight loss [49]. In addition, the isolated mycobacteria (*M. avium* and *M. xenopi*) tend to be etiological factors of serious diseases in humans, particularly those with immune deficiency [50, 51, 52].

CONCLUSIONS

Microbiological monitoring in 2017–2019 indicated that BTB does not currently pose a significant threat to the EB population. However, among the samples taken from bison in a breeding centrer where BTB had previously been detected, 4.4% were found to be positive for bovine mycobacteria, and 2.2% for atypical mycobacteria. No new BTB outbreaks were detected in the period 2017–2018. Despite these negative results, it is recommended that further microbiological monitoring for BTB be performed in this species.

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