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Short communication

Evaluation of susceptibility to pyrazinamide and streptomycin, isoniazid, rifampin and ethambutol of *Mycobacterium caprae* strains isolated from European bison (*Bison bonasus caucasicus*) in the Bieszczady Mountains (Southern Poland)

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Abstract

The material for drug resistance testing was 28 strains of *Mycobacterium caprae* isolated from tissue collected post mortem from a free-living Bieszczady Mountain European bison (*Bison bonasus caucasicus*) herd. All drug susceptibility tests were carried out on an automated Bactec mycobacterial growth indicator tube (MGIT) 960 system, using Bactec MGIT 960 streptomycin, isoniazid, rifampin and ethambutol (S.I.R.E.) and Bactec MGIT 960 PZA kits. The analyzed *M. caprae* strains demonstrated susceptibility to PZA and the complement of four basic anti-mycobacterial drugs: S.I.R.E. Considering that we are dealing with multidrug-resistant and extremely drug-resistant tuberculosis more and more often, and that no new drugs have been discovered or developed for over 60 years, the study of drug resistance in free-living animal strains of MTBC is of great importance for the deepening and broadening of our knowledge of TB.

Keywords: isoniazid, ethambutol, pyrazinamide, rifampin, streptomycin, *Mycobacterium caprae*, SB2391, tuberculosis, European bison, Poland

Introduction

The European bison is the largest land mammal in Europe and at the same time national pride in Poland. Currently, the population of European bison in Bieszczady Mountains (Poland) ranges 802 animals. This is the second largest group of wisent in Poland. The major health problem in this population has been tuberculosis (TB) so far. First case was reported in 1996, then single cases were diagnosed over the next 15 years. Ultimately, the General Director for Environmental Protection decided to cull of the whole "Górny San" herd. Elimination of infected bison was the only way to prevent the disease from spreading among wildlife in the Bieszczady Mountains.

The history of modern TB treatment begins with the discovery of streptomycin (SM) in 1943. The remaining anti-tuberculosis drugs: rifampicin (RMP), isoniazid (INH), ethambutol (EMB) and pyrazinamide (PZA) were later approved for the treatment of *Mycobacterium tuberculosis* complex (MTBC) infections. Seventy years after PZA effectiveness against tuberculosis was discovered, its remains one of the main anti-tuberculosis drugs used in the treatment regimen recommended by the WHO in humans. This drug plays a key role in the first phase of antibiotic therapy, because it acts powerfully inside macrophages, where tubercle bacilli survive (Napiórkowska et al. 2011).

The aim of the study is presentation of phenomenon of drug resistance to anti-mycobacterial drugs among strains of *Mycobacterium caprae* isolated from a herd of free-living European bison (*Bison bonasus caucasicus*) from the "Górny San" ("Upper San (river)") herd in the Bieszczady Mountains. Additionally, the complete phenotypic and genotypic analysis of the etiological agent will contribute to better knowledge of endemic tuberculosis in this region.

Materials and Methods

Animals and samples

Twenty-eight MTBC strains isolated in 2012-2013 from 28 European bison (*Bison bonasus caucasicus*) from the "Górny San" ("Upper San (river)") herd in the Bieszczady Mountains were analyzed. All strains were isolated according to standard procedures in accordance with the guidelines for laboratories for the diagnosis of bovine animal tuberculosis contained in the Animal Health Law. The following clinical materials were analysed: fragments of the lungs, pleura, liver, spleen, visceral peritoneum and lymph nodes. The cultivation was carried out on solid Stonebrink and Petragnani medium for a period of 4-6 weeks at 37°C. Molecular characterization was performed using the GenoType MTBC kit (Hain Lifescience, Nehren, Germany) and the spoligotyping method (Isogen Bioscience, the Netherlands) as previously described by Krajewska et al. (2015).

No ethical committee permission was required as the samples were collected post-mortem. All individuals were eliminated by certified hunters according to Polish General Directorate for Environmental Protection decisions.

The pyrazinamide resistance phenotype

The pyrazinamide resistance phenotype of the strains was determined in the Bactec 460-Tb isotope system (Beckton Dickinson, Franklin Lakes, NJ, USA). Resistance to this drug was determined on Middlebrook 7H12 liquid medium containing 14C-labeled palmitic acid with a pH in the range of 5.9-6.0 and a PZA limit of 100 μg/mL (Napiórkowska et al. 2011).

The S.I.R.E. resistance phenotype

Phenotypic tests for susceptibility to anti-TB drugs were performed with the method recommended by the World Health Organization (WHO). All drug susceptibility tests were carried out on an automated Bactec mycobacterial growth indicator tube (MGIT) 960 system according to the manufacturer's protocol, using Bactec MGIT 960 SM, INH, RMP and EM (SIRE) and Bactec MGIT 960 PZA kits (Becton Dickinson, Franklin Lakes, NJ, USA). The concentrations of drugs were as follows: 1.0 μg/mL for SM, 0.1 μg/mL for INH, 1.0 μg/mL for RMP, 5.0 μg/mL for EM and 100 μg/mL for PZA. Quality control was performed during drug susceptibility testing (DST) using the H37RV M. tuberculosis reference strain. Analysis of fluorescence in test tubes containing drugs compared with fluorescence in control test tubes was automated in the machine to define sensitivity.

Genotyping methods

Genotyping of the strains was conducted in 2 stages – spoligotyping and mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU--VNTR) typing. Spoligotyping was performed (Ocimum Biosolutions, Hyderabad, India) using commercially available membranes according to a standard protocol described previously by Krajewska et al. (Krajewska et al. 2015). Spoligotype shared type were assigned according to the SITVIT2 database (Couvin et al. 2019).

In the second stage of genotyping used the PCR-based MIRU-VNTR method, which distinguishes between strains by assessing the number and length

Fig. 1. Necrotic lesions in the mesenteric lymph node in the European bison.

of tandem repeats at each locus of each isolate. Fifteen independent MIRU-VNTR loci were amplified, i.e.: 580 (MIRU04 or ETRD), 960 (MIRU10), 1644 (MIRU16), 2996 (MIRU26), 3192 (MIRU31 or ETRE), 802 (MIRU40), 424 (VNTR42), 577 (VNTR43 or ETRC), 2165 (ETRA), 2401 (VNTR47), 3690 (VNTR52), 4156 (VNTR53 or QUB-4156c), 2163b (QUB-11b), 1955 and 4052 (QUB-26), as described elsewhere (Supply et al. 2006).

The genetic cluster was defined as all TB isolates sharing the same spoligotype, as well as the 15 MIRU -VNTR profile loci. However, since the genetic turnover of the bacteria was also taken into consideration, additionally a wider definition of clustering was applied on basis of previous studies (Alonso-Rodriguez et al. 2009). In this definition one difference in one of the MIRU/VNTRs explored in MIRU-VNTR typing were tolerated.

Results and Discussion

Twenty eight European bison from which the strains were isolated had extensive multi-organ TB (Fig. 1). Twenty eight analyzed *Mycobacterium caprae* strains demonstrated susceptibility to PZA and the complement of the four SIRE basic anti-mycobacterial drugs. All investigated isolates were *Mycobacterium caprae* of SB2391 spoligotype pattern (Octal code 2000037777377400) (Krajewska et al. 2015). The MIRU-VNTR results are grouped in one cluster and presented in Table 1. Among the 28 strains analyzed by MIRU-VNTR, 3 DNA profiles were identified: 16 strains with identical MIRU-VNTR patterns 453552362412223, 9 strains - 453552362413223, 3 strains – 453552342411223 (Table 1). Differences in DNA patterns were located only in locus 4156, which allowed all strains to be classified into a common genetic cluster. The strains genotyping results of this study confirmed the TB transmission between 28 European bison from the "Górny San" ("Upper San (river)") herd in the Bieszczady Mountains.

The endemic presence of bovine TB in free-living animals in the Bieszczady Mountains has severe consequences for dairy cattle and wildlife conservation and poses a potential risk to public health, especially for humans in close contact with these animals (hunters, foresters, veterinarians).

Despite the a very low incidence of human TB disease due to *M. caprae*, its zoonotic potential poses a serious threat to public health (Cöllü et al. 2022, Martínez-Lirola et al. 2023). In 2020, Kozińska et al. published the first case of *M. caprae* tuberculosis in a human in Poland. The source of the infection was not established and it appears to be an incidental case of this type of TB (Kozińska et al. 2020). According to the European Union One Health 2022 Zoonoses Report, *Mycobacterium caprae* TB was diagnosed in 5 patients in the European Union in 2022. According to the available literature, the source of infection for humans is sick animals or products of animal origin (Cadmus et al. 2019, Verdugo Escárcega et al. 2020).

The results of full susceptibility of *M. caprae* obtained in the current study are not surprising due to the fact that resistant and multidrug-resistant TB strains most often appear as a result of incorrect therapy that is used in humans. The reason for the more frequent emergence of drug resistance is also the endemic occurrence of TB and the associated more frequent exposure of bacterial strains to drugs, especially first-line drugs. In the case of animals, which were investigated in this study, no treatment was undertaken, therefore the risk

Table 1. Twenty eight repetitive units-variable number of tandem repeats (MIRU-VNTR) patterns identified among the analyzed genetic cluster.

No.	Animal species	Year of death	Identyfication		
			Mycobacterium species	Spoligotype	MIRU-VNTR
1.	European bison (*Bison bonasus caucasicus*)	2010	*Mycobacterium caprae*	SB2391 spoligotype pattern (Octal code 2000037777377400)	453552362412223
2.		2011			453552362412223
3.		2011			453552362412223
4.		2012			453552362412223
5.		2012			453552362412223
6.		2012			453552362412223
7.		2012			453552362412223
8.		2012			453552362412223
9.		2012			453552362412223
10.		2012			453552362412223
11.		2012			453552362412223
12.		2012			453552362412223
13.		2012			453552362412223
14.		2012			453552362412223
15.		2012			453552362412223
16.		2012			453552362412223
17.		2012			453552362413223
18.		2012			453552362413223
19.		2013			453552362413223
20.		2013			453552362413223
21.		2013			453552362413223
22.		2013			453552362413223
23.		2013			453552362413223
24.		2013			453552362413223
25.		2013			453552362413223
26.		2013			453552342411223
27.		2013			453552342411223
28.		2013			453552342411223

of developing drug resistance among the strains causing tuberculosis in European bison was negligible. However, it has been shown that some species of bacteria (*Enterococcus faecium* and *E. faecalis*) isolated from free-living animals (different species of bats), and therefore also not subjected to targeted therapy, showed a fairly common high-level resistance to RMP (Nowakiewicz et al. 2020), one of the drugs used in treatment of TB. Resistance to this drug is most often associated with the occurrence of mutations in the rpoB gene, encoding the beta subunit of bacterial RNA polymerase, also in bacteria belonging to *Mycobacterium* genus.

Considering the increasing incidence of resistance to both first- and second-line drug among bovine mycobacteria (Vázquez-Chacón et al. 2021) and the lack of an effective vaccine for animals, the diagnosis of TB in cattle and other animal species as well as assessment of drug resistance is of great importance for veterinary public health protection.

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