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Molecular Characterization of *Mycobacterium* spp. Isolated from Cattle and Wildlife in Poland

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Abstract

Although Poland is officially tuberculosis (TB) free, meaning that less than 0.1% of her cattle herd is TB-positive, the problem of bovine TB in Poland may be re-emerging: its presence has recently been confirmed in domestic and companion animals, wildlife such as the European bison, and even humans. The aim of this chapter was to review all reports of bovine TB in Poland described to date, with particular emphasis on molecular studies, and determine further research directions. These studies include a range of molecular methods for diagnosis, including genotyping, spoligotyping and MIRU- VNTR; such methods successfully identifies a tuberculosis-positive European bison as the source of wild boar infection in the Bieszczady Mountains based on its spoligotype. This chapter argues that identified trains should be better archived, as such records would allow detailed epidemiological investigations and shed greater light on the activity of *Mycobacterium* spp. The current epidemiological situation in Poland highlights the need for further studies to determine epidemiological links and confirm possible routes of transmission based on whole genome sequencing; this need is accentuated by the zoonotic potential of such infections and the endangered species at risk.

Keywords: epidemiological investigation, European bison, molecular methods, *Mycobacterium caprae*, *Mycobacterium bovis*, Poland

1. Introduction

Bovine tuberculosis is a highly-contagious bacterial disease whose etiological agents are the acid-fast bovine mycobacteria species *Mycobacterium bovis* and *Mycobacterium caprae*. These two species can also cause tuberculosis in humans, although infection with *Mycobacterium tuberculosis* is more common. Although Poland has been an officially tuberculosis free (OTF) country since 2009, cases are still noted in cattle. In addition, *M. bovis* infection has been observed in emergency cases in alpacas and *M. caprae* has been found in endangered European bison (*Bison bonasus*). Tuberculosis infection has been observed in various other wildlife. The aim of this chapter is to present the epizootic situation of bovine tuberculosis in

Poland, including its molecular diagnostics, and to determine which molecular diagnostic methods would be useful in the future.

2. Tuberculosis in Poland as a zoonosis

Tuberculosis (TB) remains a leading cause of death worldwide. Its treatment requires supervision, efficient and reliable diagnostics, contact tracing and effective therapy. In 2019, 10 million people with tuberculosis were registered worldwide. The incidence of tuberculosis in Poland is slightly higher than the European average, being 13.9/100.000 in 2019 [1, 2].

Although most cases of human TB are caused by the bacterial species *M. tuberculosis*, this only represents part of a complex that includes various zoonotic forms. According to the latest nomenclature, some of the most prominent members of this complex known to cause disease in humans or/and animals are *M. tuberculosis* (human), *Mycobacterium africanum* (human), *Mycobacterium canetti* (human), *M. bovis* (cattle and other animals), *M. caprae* (goats, cattle and other animals), *Mycobacterium pinnipedii* (seal), *Mycobacterium microti* (voles and other small rodents) and *M. bovis* BCG (vaccine strain) [3, 4].

While transmission can take place directly, through the aerogenic route, bovine tuberculosis (bTB) is most commonly transmitted to humans through an indirect route, possibly through unpasteurized milk or dairy products and raw meat. Those at the highest risk of indirect exposure are people exposed to the source of infection at work, such as farmers and veterinarians, and those working with meat, such as slaughterhouse workers and hunters in contact with contaminated animals [5].

According to estimates by the World Health Organization (WHO), in 2016, 147,000 new cases and 12,500 deaths were associated with zoonotic tuberculosis worldwide. However, such figures are often underestimated due to financial constraints and the consequent lack of adequate routine control in countries where bovine tuberculosis is endemic. Zoonotic tuberculosis tends to be of low prevalence where its presence is correctly monitored in animals and appropriate safe food production procedures are followed [6, 7]. While over two thirds of human TB cases, i.e. those resulting from *M. tuberculosis* infection, primarily affect the lungs [8], zoonotic TB often affects extrapulmonary sites, including lymph nodes and other organs [9]. Since bovine mycobacteria causes clinical, radiological and pathological symptoms that are similar to *M. tuberculosis*, these strains can be distinguished only by bacterial culture, by biochemical and morphological analysis, and by genotyping.

M. bovis used to be differentiated from other complex members based on its resistance to pyrazinamide (PZA); however, following the discovery of PZA-susceptible strains of *M. bovis*, the species was split into two subspecies: the PZA-resistant *M. bovis* subsp. *bovis*, and the PZA-sensitive *M. bovis* subsp. *caprae* [10, 11]. PZA is one of the four essential drugs used in the current standard first-line anti-TB treatment regimen. However, as most healthcare providers initiate treatment without performing any drug susceptibility testing, patients with zoonotic TB caused by *M. bovis* may demonstrate poorer treatment outcomes and may develop further resistance to other anti-TB drugs; for example, additional resistance to rifampicin and isoniazid has been detected in some *M. bovis* isolates [12].

Like other bacterial species, the resistance of *Mycobacterium tuberculosis* complex members to antimycobacterial drugs arises from the selection of naturally-resistant mutants that are constantly present in every bacterial population. Wild strains of mycobacteria belonging to the *M. tuberculosis* complex that have never been

exposed to drugs are naturally sensitive to tuberculostats, with one exception; the PZA-resistant *M. bovis*.

In addition to mutations, mycobacteria can develop phenotypic resistance through a change in cell wall permeability, which can impair penetration of the drug into the cell, or by employing efflux pumps, which allow the active removal of the drug from the cell. Metabolic pathways that bypass “drug-sensitive” sites in the cell may also be altered. Regardless of its mechanisms, mycobacterial drug resistance always occurs as the result of a selection process, i.e. a change in the ratio of drug-sensitive to drug-resistant cells [13, 14].

With the growth of research of the *Mycobacterium* genome and its drug resistance pathways, the main mechanisms and genes by which mutations cause drug resistance have been recognized. Currently, commercial tests based on PCR reactions are used to detect the most common mutations determining the resistance of mycobacteria to antibiotics that are crucial in treatment. One such example is the line probe assay (LiPA); these employ targeted amplification of specific regions of the MTB genome using biotinylated primers followed by reverse hybridization of the amplicons to oligo probes immobilized on nitrocellulose strips. Hybridization is then detected by a colorimetric reaction. Currently, the most widely used tests are those developed by Hain Lifescience (Nehren, Germany): Genotype MTBDRplus and Genotype MTBDRsl, detecting resistance to the most important antituberculosis drugs of the I- and II- lines [13].

From the epidemiological and therapeutic point of view, the identification of MDR (Multi Drug Resistant), XDR (eXtremely Drug Resistant) and TDR (Totally Drug Resistant) MTBC strains is of key importance [15, 16].

Drug-resistant tuberculosis is more difficult to treat than drug-resistant tuberculosis. Patients do not recover from the standard six-month treatment regimen, but undergo long-term therapy requiring the use of less effective, more toxic and more expensive drugs (Table 1) [17].

In Poland and most other developed countries, the threat of bTB in humans decreased significantly in the middle of the 20th Century following the introduction of tuberculosis management strategies [18]. Thanks to the combined implementation of appropriate eradication and surveillance programs, in 2009, Poland was awarded the status of a tuberculosis-free country.

However, in 2020, the first Polish case of bTB in humans was recorded in a retrospective study by Kozińska and Augustynowicz-Kopeć [19], which described the case of a 46-year-old male detected in 2012 with bacteriologically-confirmed pulmonary infection with *M. caprae*. Changes typical for pulmonary TB were

| Drug | Type of resistance |
|--|--------------------|
| Isoniazid + rifampicin | MDR |
| Isoniazid + rifampicin + streptomycin | |
| Isoniazid + rifampicin + ethambutol | |
| Isoniazid + rifampicin + streptomycin + ethambutol | |
| MDR + fluoroquinolone + one of the injectable drugs (amikacin or kanamycin or capreomycin) | XDR |
| INH + RMP + SM + EMB + fluoroquinolone + aminoglycoside + polypeptide + thioamide + cycloserine + para-aminosalicylic acid | TDR |

Table 1.
Definitions of drug resistance on MTBC.

identified on chest X-ray, and a tuberculin test result of 18 mm was obtained. In addition, direct staining of sputum revealed the presence of acid-fast mycobacteria (AFB ++), and *Mycobacterium* colonies were identified after four weeks of culture on Löwenstein-Jensen (LJ) medium. Initial identification in the hospital laboratory confirmed that the isolated strain belonged to the *M. tuberculosis* complex. Phenotypic and molecular methods revealed drug susceptibility, and the strain was thus classified to the species *M. caprae*. Further genotyping identified the unique spoligotype 200003757377600; although this strain was not registered in the international spoligotype databases SpolDB4 and SITVIT WEB, it was found to match SB1690 in Mbovis. Org, this being a Spanish isolate from 2009 (**Table 2**) [20]. The source of infection remained unknown: the patient's history revealed that he had not had recent contact with any person with tuberculosis, nor had he been close to farm animals which had not been tested for tuberculosis. Until now, this has been the only documented case of zoonotic TB in Poland.

As tuberculosis is an infectious disease with a complex epidemiology and pathogenesis, it is essential to employ molecular typing (genotyping) methods when testing for *M. tuberculosis*: such tools are fundamental for guiding effective epidemiological research, defining the dynamics of transmission, and enabling global surveillance of the disease. In addition, genotyping provides an insight into the biodiversity and evolution of the pathogen.

Various genotyping methods are used in human and bovine TB research, such as IS6110-RFLP (*Insertion Sequence 6110-Restriction Fragment Length Polymorphism*), spoligotyping, MIRU-VNTR (*Mycobacterial Interspersed Repetitive Units-Variable Number Tandem Repeats*), and WGS (*Whole Genome Sequencing*) [21].

The spoligotyping method takes advantage of a polymorphism within the chromosomal region DR (*Direct Repeat*) found in mycobacteria belonging to the *M. tuberculosis* complex. This region, first described by Hermans in the *M. bovis* BCG P3 strain, is formed by a variable number of direct repeat (DR) sequences, 36 bp long, with short (35–41 bp) unique spacer sequences between them [22]. The spacer sequences are detected by synthetic oligonucleotide probes complementary to the 43 known sequenced spacer sequences identified in *M. tuberculosis* H37Rv and *M. bovis* BCG strains.

Being a PCR-based method, spoligotyping requires very little DNA and thus, can be used to detect and identify *M. tuberculosis* complex bacteria directly in clinical specimens, bypassing the culture step.

Another advantage of spoligotyping is the ease with which typing results can be recorded, i.e. in binary and octagonal formats, cataloged, and compared in central


| Results of microbiological and molecular testing | |
|--|---|
| Clinical material | Sputum |
| Bacterioscopy | ++ |
| Culture | Growth after four weeks on LJ medium |
| Phenotype | Sensitive to SM, INH, RMP, EMB, PZA |
| Strain identification | <i>Mycobacterium caprae</i> |
| Spoligotyping | Hybridization pattern  |

Table 2.
Characteristics of Mycobacterium caprae – The first human isolate in Poland.

databases [23] (SpolDB4, SITVIT WEB, Mbovis. Org databases). It is therefore commonly employed as a screening method in molecular epidemiological investigations. It can be used to identify species within the *M. tuberculosis* complex, provide information regarding the lineage of various strains, determine their placement in major genetic families and indicate the directions of the global spread of molecular families of mycobacteria [24].

However, the detection of tuberculosis transmission foci in closed populations of humans and animals, as well as their interspecific transmission, requires the use of methods with a higher genome differentiation potential. Therefore, spoligotyping studies are commonly complemented by the use of MIRU-VNTR analysis and WGS [25].

The largest group of VNTR sequences in the *Mycobacterium* genome are the 46–100-nucleotide MIRU fragments. Of these 15 to 24 known *loci* with the highest variability were selected for the genetic typing of mycobacteria.

In the MIRU-VNTR method, individual sequences are amplified, and the size of the resulting products depends on the number of repeats of the core unit. For each locus, the number of repeats of the MIRU or VNTR motif is calculated, which allows the results to be cataloged using a 15- or 24-digit MIRU-VNTR code. The MIRU-VNTR method is characterized by high sensitivity and repeatability. It allows the analyzed strains to be differentiated to a large extent, is relatively easy and is distinguished by a short analysis time [26].

Although spoligotyping, MIRU-VNTR and RFLP have a very high diagnostic value, they are not suitable for accurately determining the dynamics of TB transmission. The spread of tuberculosis may also occur through short contacts, or in a high-risk population where epidemiological links between patients are difficult to establish. In addition, as they screen less than 1% of the genome, standard genotyping techniques therefore have limited discriminatory power and cannot optimally detect potential transmission chains.

These limitations can be circumvented by the use of whole genome sequencing (WGS). WGS provides comprehensive genetic data as well as information on drug resistance, virulence factors, and genome evolution. However, such sequencing analysis requires high expenditure, the possession of specialized equipment and complex bioinformatic analysis of the results [27].

An accurate confirmation of the molecular relationship of the studied strains, supplemented with epidemiological data, can form the basis for identifying the transmission of infection between closely-related patients, such as family members, as well as among homeless people and immigrant populations, between wild animals and livestock, and between humans and animals. Unfortunately, not all diagnostic laboratories have the appropriate equipment to perform specialist testing based on the analysis of the mycobacterial genome. As a result, current data on the transmission of tuberculosis as zoonosis may well be underestimated.

Preventing the development of zoonotic TB in humans requires reducing the risk of exposure and transmission at the human-animal interface. However, while the principal routes of transmission are known, more information is needed about their underlying sociocultural and economic bases, and how to promote safer alternatives.

3. Epizootic situation of bovine tuberculosis in cattle and other animal species in Poland, and the molecular characteristics of isolated strains

Bovine tuberculosis is an infectious disease that mainly affects cattle. In 2020, seven outbreaks in cattle were recorded in Poland; in the rest of Europe, only France

(n = 105) and Germany (n = 10) reported higher numbers of outbreaks, while seven outbreaks were noted in Italy and Belgium [28]. Bovine bacilli can cause tuberculosis in other farm species (**Figure 1**). They show high virulence in natural conditions in goats, pigs, sheep and cats [29]; however, the disease is less common in horses and dogs [30, 31]. Cattle are not very susceptible to human bacilli, but infections with *M. tuberculosis* are known in this species: one case of bovine tuberculosis due to *M. tuberculosis* has been reported in Poland so far [32]. Wild animals living in the close vicinity of farms can also be a mycobacterial reservoir. The largest reservoir of bovine bacilli in Great Britain is the badger population [33]. However, in Spain, wild boar populations represent the largest reservoir of tuberculosis [34]. The transmission of tuberculosis bacilli occurs in shared pastures, less often as a result of fighting or biting.

In Poland, the largest reservoir of bovine bacilli is believed to be sick cattle. The spread of infection between herds is usually due to the movement of asymptomatic vector animals. Introducing infected animals into a tuberculosis-free herd may cause infection of other animals and disease development in immunocompromised animals. However, following the eradication program carried out in Poland in 1959–1975, its prevalence has significantly fallen, especially in the eastern part of the country. Further progress in the control of the disease in cattle herds has been made possible by the application of strict rules and their consistent enforcement. As in other European countries, Poland operates a special bovine tuberculosis control program, described in detail in the Regulation of the Minister of Agriculture and Rural Development and in the amended Instruction of the General Veterinary Inspector. These documents require the testing of 1/5 of the total cattle population in each county based on bovine and avian purified protein derivative (PPD) tuberculin using both single and comparative tuberculin tests. All positively reactive animals are eliminated, and all samples from these animals are tested in the National Reference Laboratory of Bovine Tuberculosis, located in the

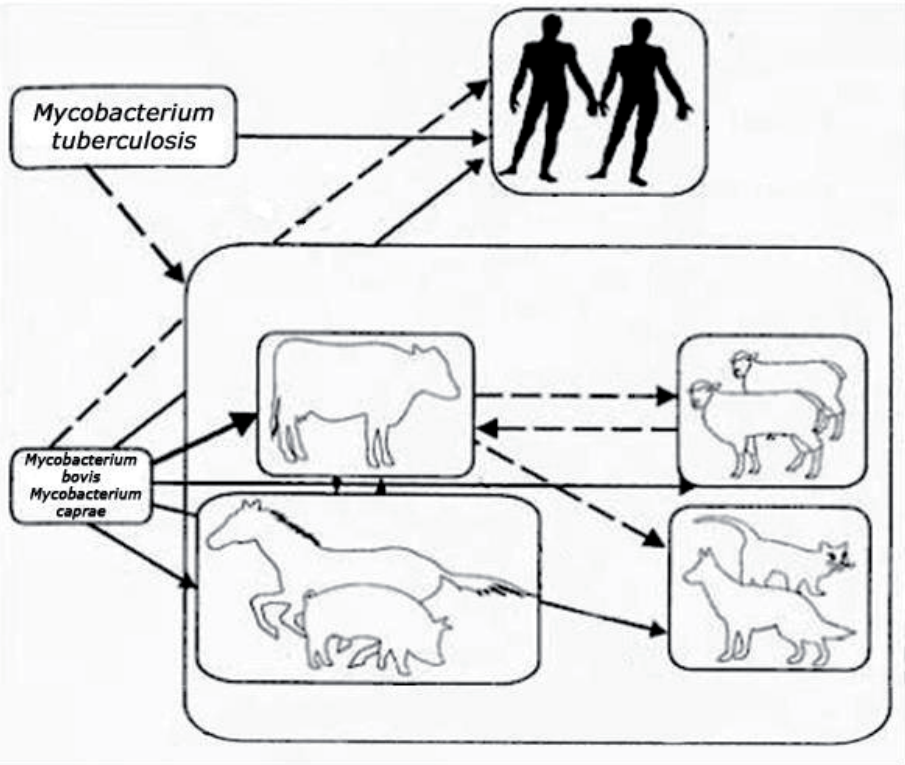


Figure 1. Diagram illustrating transmission of potential tuberculosis cases caused by mycobacteria from the MTBC complex.

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All tissue samples are collected *post mortem*, prepared and cultured on Stonebrinck and Petragnani media, as stipulated by the State Veterinary Inspectorate's Instructions for Laboratory Diagnosis of Bovine Tuberculosis [35]. The culture is also supplemented by a biological analysis performed using two guinea pigs, and the strain extraction procedure is complemented by GenoType MTBC (HAIN Lifescience, Germany) typing kits.

The numbers of outbreaks and sick animals in cattle breeding were found to fall during the course of the program, and Poland was recognized as free from bovine tuberculosis in 2009. However, a total of 372 outbreaks were recorded in cattle herds during the following 10-year period, i.e. in 2009–2019. Almost 1/3 of these outbreaks were found in the Masovian Voivodeship, the central region of Poland, especially in its northern part [36]. A significant number of outbreaks were also recorded in the Greater Poland (Wielkopolskie) ($n = 68$), Łódzkie ($n = 28$) and Lesser Poland (Małopolskie) voivodeships ($n = 24$). The smallest number of disease outbreaks concerned the Lubusz (Lubuskie) ($n = 2$) and Opole voivodeships ($n = 1$). Molecular studies to date indicate that 70% of cattle suffering from bovine tuberculosis were infected with *M. bovis* and 30% with *M. caprae*. In 2010, the first case of tuberculosis in a calf caused by *M. tuberculosis* was confirmed [33]. It should be noted, however, that the status of Poland as being officially free of bovine tuberculosis was never threatened; on the contrary, compared to other European countries, Poland has very favorable data on disease control, particularly considering that almost six million cattle are farmed there.

Among the *M. bovis* strains isolated from cattle in Poland, the most common individual spoligotype was SB0856, being present in 44% of the tested strains. In addition, SB0127 and SB0119 were also frequently observed [37]. Among *M. caprae* strains isolated from cattle, the most common spoligotypes were SB0418, SB2390 and SB2393 [38].

However, the incidence of bTB is not limited to cattle. In the period of 2009–2010, cases of bTB were recorded in three zoos in Poland. Of the 12 strains isolated from 12 captive animals, *viz.* six antelopes, three giraffes, two tapirs and one alpaca, those from ten animals were identified as *M. bovis* and two were identified as *M. caprae* [37, 39, 40]. Transmission was only confirmed in the antelope herd and between tapirs. Unfortunately, it has been suggested that zoos may withhold epidemiological data, making it very difficult to conduct epidemiological investigations and trace the source of infection among rare and valuable animals threatened with extinction. During a tuberculosis outbreak in the Śląski Ogród Zoologiczny (Silesia Zoo), the decision was made to treat a giraffe with active tuberculosis, with unfortunately negative results [41]; this raises the question of whether the research team should have undertaken the treatment of an animal with active tuberculosis, particularly when considering the potential consequences for public health.

In 2014, a bovine tuberculosis outbreak was also identified among American bison (*Bison bison*) farmed in Poland [42]. In total, three cases of *M. caprae* strains were isolated, all of which were characterized by the spoligo pattern SB1912. Most cases of TB among free-living animals and exotic animals kept in zoos in Poland are caused by *M. caprae* strains, with spoligotypes SB1912, SB2391 and SB2392 predominating [38].

In contrast, sporadic cases of transmission to other species of livestock and domestic animals have been reported. For example, one case was found in pigs (*Sus scrofa* f. *domestica*) kept in the vicinity of a herd in which advanced disease was diagnosed [43]. In 2018, bovine tuberculosis was reported among alpacas of British origin in Poland [44]. In both cases, the strain was identified as *M. bovis* spoligotype

SB0666, according to the international spoligotype database (www.Mbovis.org); this type was first isolated in Great Britain in 2003. bTB has also been confirmed in both free-living wild animals and those in breeding centers [45].

Animal strains of MTBC have been analyzed for drug resistance to five basic anti-tuberculosis drugs: streptomycin (SM), isoniazid (INH), rifampicin (RMP) and ethambutol (EMB), known as SIRE, and PZA. Fortunately, the findings indicate that Polish strains of bTB obtained from animals do not show environmental resistance [38, 40, 46].

A gap exists in Polish veterinary legislation regarding bovine tuberculosis: so far, it makes no explicit mention of *M. caprae* causing tuberculosis in animals. In the Act of 11 March, 2004 on the Protection of Animal Health and Control of Infectious Diseases of Animals, Annex 2, bovine tuberculosis is listed as a notifiable disease without a disease-causing pathogen. While the disease is mentioned in the Regulation of the Minister of Agriculture and Rural Development of 23 November 2004 on eradication of bovine tuberculosis, it does not indicate an etiological agent. Despite the Amendment of the Instruction of the Chief Veterinary Officer No. GIWpr-02010/2016 of 8 February 2016, the only pathogenic species listed as causing bovine tuberculosis is *M. bovis*.

Poland was declared OTF in 2009 [47], and the fact that the country has remained this way for the subsequent 10 years indicates that the procedures used to control the disease are effective. Only minor incidents have been reported, and they usually occur as a result of incidental errors in anti-epizootic management and the carelessness of animal owners. More importantly, such errors do not seem to have a decisive impact on the overall bovine tuberculosis situation. Poland currently has a consistent policy of eradicating *M. bovis*/*M. caprae* infections in cattle herds, and the country still meets the formal requirements for a TB-free status.

4. Bovine tuberculosis in European bison in Poland and the use of molecular methods

Even though bTB-positive cattle are considered to constitute the primary reservoir of the bovine mycobacterium in Poland, tuberculosis has also been found in wildlife such as badgers (*Meles meles*), wild boar (*Sus scrofa*), wolves (*Canis lupus*) and European bison (*Bison bonasus*) [45, 48, 49].

In recent years, of all species diagnosed with bTB in Poland, the European bison is the most common [50]. A total of 45 cases of tuberculosis were confirmed in European bison in the Bieszczady Mountains during the years 1996–2013 [51]. An autopsy identified generalized tuberculosis in a three-year-old female from a free-living herd in the Brzegi Dolne Forest District. Around the same time, in the years 1997–2001, 13 out of 18 culled European bison from the same *Brzegi Dolne* herd were microbiologically confirmed to have tuberculosis and the decision was made to liquidate the entire herd [49]; however, not all animals were culled, and several bison from the herd have still not been found [51].

Other scattered cases have been found in the region. Tuberculosis was confirmed in two European bison in the Bieszczady Mountains in 2005–2008 [52]. In addition, a positive result in the *Górny San* herd from Bieszczady in 2009 resulted in the entire herd of 24 European bison being culled. Tuberculosis-like lesions were found in all individuals, and tuberculosis was microbiologically confirmed in 23 [51]. It is possible that the source of infection for the European bison from the *Brzegi Dolne* herd was locally grazed cattle, while the source of infection in the *Górny San* herd may have been individuals that separated from the *Brzegi Dolne* herd. Unfortunately, as no strains from the *Brzegi Dolne* herd were archived, it

was not possible to compare the mycobacteria strains between the two herds; this underlines the importance of using molecular methods when studying epidemiology among wildlife. Interestingly, a strain isolated from the *Górny San* was found to have the same spoligotype as one isolated from wild boar from the same area, i.e. the Bieszczady Mountains [49].

Cases of bTB have been recorded in captive European bison in Poland: in Warsaw Zoo, Wolisko and the Smardzewice Bison Breeding Centre. Spoligotyping and MIRU-VNTR analysis of the European bison from Smardzewice identified the presence of as *M. caprae*–spoligotype *M. bovis* _4_ CA 1600 (octagonal pattern: 200003770003600) (SpolDB4 database) [53]. The source of infection remains unknown due to a lack of archived *Mycobacteria* strains, but there are suspicions that it may have been acquired from an individual from *Silesia Zoo*.

A number of studies have been undertaken recently to address the problems associated with the *ante mortem* diagnosis of tuberculosis in wildlife [54–56]. Such studies have also been conducted in European bison [57]. Although a range of serology methods have been tried [58], the material for direct detection is collected from tracheobronchial lavage, and from swabs and biopsy from retropharyngeal lymph nodes. A more recent approach is to combine microbiological testing with molecular tests, allowing accurate results to be obtained in a much shorter time. In one case, MTBC genetic material was confirmed in laryngeal swab and tracheobronchial lavage using the BD ProbeTec *Mycobacterium tuberculosis* Complex (DTB) Direct Detection Reagent Pack (Becton Dickinson, US) which allows direct detection of mycobacterial genetic material in a clinical specimen [57]. The test acts by amplifying and identifying the target DNA simultaneously. However, the method is characterized by *inter alia* intermittent mycobacterial shedding, which can lead to false positive results.

With the current situation of bTB in European bison in Poland in mind, it would clearly be advisable to include molecular methods in routine diagnostics, thus facilitating more accurate epidemiological investigations and more effective disease control.

5. Tuberculosis in wildlife in Poland, other than European bison, including molecular diagnostic methods

Currently, no wildlife tuberculosis monitoring program exists in Poland, except when visible lesions suggestive of TB are found in the animal. Despite this, it seems that tuberculosis cases are rarely found in wildlife in Poland and are limited to the area of the Bieszczady Mountains in Southeast Poland: a region bordered by Slovakia and Ukraine, with the highest peak being Tarnica (1346 m a.s.l.). This area is characterized by high forest coverage, low human population and low livestock abundance [59], unpublished data of the County Veterinary Inspectorate, Ustrzyki Dolne, Sanok]. Between 1996 and 2020, most TB cases in this area were found in European bison and in wild boar [49, 51, 53, 60–63], and no cases have been reported in domestic animals or livestock since 2005. Outside this region, only two single cases of TB have been described in wildlife in Poland: the first in a roe deer (*Capreolus capreolus*) near Gdańsk and the second in a European bison in Borecka Forest [64, 65].

In the Bieszczady Mountains, the first TB case in wildlife was described in 1996 in a European bison from the Brzegi Dolne Forest District [52]. Between 1997 and 2013, TB was recorded in a total of 40 European bison in the region, resulting in the culling of two bison herds (*Bison bonasus caucasicus*) (see section 4) [37, 39, 49, 52, 60, 66–68]. Since then, no new TB cases have been detected within this species

in the Bieszczady region [63]. Even so, over the past 20 years, TB has been found in other species of wild animals in the Bieszczady Mountains, mostly in wild boars.

The first case of TB in a wild boar was reported in 2012 in a four-year-old female from Nasiczne in the Bieszczady, which was found dead due to *Metastrongylus* spp. invasion. Postmortem examination showed small caseous, yellowish tubercles in submandibular lymph nodes, from which *M. caprae* was isolated (at that time *M. bovis* ssp. *caprae*). The strain was found to have the same spoligo pattern as those strains isolated in 2011 from European bison from the Bieszczady area [45], this being 200003777377400, or SB2391 as assigned by www.Mbovis.org [69].

Since then, a number of cases of TB have been found in the Bieszczady wild boar population each year. Between 2012 and 2017, *M. caprae* was isolated from the lymph nodes of 21 out of 55 investigated wild boar [63]. These strains were subjected to molecular analysis based on spoligotyping according to Kamerbeek et al. [70], and MIRU-VNTR typing, as given in the public protocol [71]. A total of 15 loci were investigated: MIRU 4, MIRU 10, MIRU 16, MIRU 26, MIRU 31, MIRU 40, VNTR 424, VNTR 577, VNTR 2165, VNTR 2401, VNTR 3690, VNTR 4156, VNTR 2163b, VNTR 1955 and VNTR 4052. All 21 isolated strains shared an identical spoligotype 200003777377400 – SB2391. From this group, 19 strains shared a single MIRU-VNTR pattern (464652364413423), while the other two had patterns that differed with regard to a single locus (464552364413423 and 463652364413423) [63].

To describe the occurrence of TB in wildlife other than European bison and wild boar, both within the Bieszczady Mountain region and elsewhere, lymph node samples were collected for analysis from red foxes, wolves, badgers, red deer, roe deer and brown bear between 2011 and 2017. *M. caprae* was isolated from the lymph nodes of one roe deer and three wolves. Those animals had no visible TB-like lesions [48, 63].

All molecular research of *M. caprae* strains isolated from wildlife in the Bieszczady Mountains has been performed based on hsp65 sequence analysis, the GenoType®MTBC (Hain Lifescience, Germany) test, spoligotyping and MIRU-VNTR analysis. Further studies to determine the epidemiological link and the possible route of transmission of the source of infection are needed based on whole genome sequencing.

6. Conclusions

In conclusion, bovine tuberculosis remains a real threat in Poland, as indicated by the increasing number of cases observed in wildlife and the recent report of the first confirmed case of *M. caprae* infection in human. *M. caprae* is the main etiological agent of bovine tuberculosis in wildlife, and *M. bovis* in cattle.

We recommend that in Poland, bovine tuberculosis should not only be monitored in cattle but also in wildlife. This is especially true in the European bison population, which seems to be highly sensitive to infection. This is highly important for protecting public health, maintaining the OTF status of Poland and of course, protecting the European bison themselves. In which case, particular attention should be paid to the free-living animal population in the Bieszczady Mountains.

There is also a particular need to monitor alpacas, as TB-positive animals pose a particular risk to children and disabled people due to increased contact during animal therapy.

We recommend the more intensive use of molecular tests in monitoring and the proper archiving of the identified DNA. Such molecular methods play an essential role in epidemiological investigations, as these can accurately identify the source

of infection and effectively control the disease. Their findings also allow steps to be taken to reduce the spread of infection. Further studies would be of particular value in this regard, particularly those based on whole genome sequencing of archived strains of *M. bovis* and *M. caprae* from different species in Poland.

Conflict of interest

The authors declare no conflict of interest.

Acronyms and abbreviations

| | |
|-----------|--|
| AFB | acid-fast mycobacteria |
| bTB | bovine tuberculosis |
| DRs | direct repeat spacers |
| EMB | ethambutol |
| INH | isoniazid |
| LiPA | Line probe assays |
| LJ | Löwenstein-Jensen |
| MDR | multidrug resistant |
| MIRU-VNTR | mycobacterial interspersed repetitive units-variable number tandem repeats |
| MTBC | <i>Mycobacterium tuberculosis</i> complex |
| OTF | officially tuberculosis free |
| PPD | purified protein derivative |
| PZA | pyrazinamide |
| RFLP | restriction fragment length polymorphism |
| RMP | rifampicin |
| RR | rifampicin resistant |
| SIRE | streptomycin, isoniazid, rifampicin, ethambutol |
| SM | streptomycin |
| TDR | totally drug resistant |
| TB | tuberculosis |
| WGS | whole genome sequencing |
| WHO | World Health Organization |
| XDR | extremely drug resistant |

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