

Molecular characterisation of the *Mycobacterium bovis* causing bovine tuberculosis outbreaks in Poland

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Abstract

Introduction: Since 2009, Poland has been recognised as a country officially free of bovine tuberculosis (bTB), although in each year of the last five there were from 8 to 18 outbreaks of the disease. In 2008–2016, the largest number of cattle infected with bovine mycobacteria were eliminated in the Masovian Province (the central region of Poland) and the largest number of outbreaks of this zoonosis were recorded in this area. The close proximity of farms where bTB was found led to the suspicion that tuberculosis could have been transmitted between the affected herds. The aim of the study was the molecular characterisation of the pertinent *M. bovis/caprae* strains and determination of the epidemiological relationship of various bTB outbreaks. **Material and Methods:** The material for microbiological tests came from 119 cattle (*Bos taurus*) from nine herds located in five provinces, neighbouring the Masovian Province. **Results:** Laboratory tests of tissue material gave results confirming tuberculosis in 54 (45%) animals. All strains belonged to the *Mycobacterium bovis* species. A two-step analysis of genetic affinity allowed 50 strains to be identified as phylogenetically closely related and separated between three genetic clusters consisting of 2 to 27 strains. **Conclusion:** Based on the results of genotyping, bTB outbreaks were found in three herds, and three transmission chains were identified among these herds.

Keywords: cattle, bovine tuberculosis, *Mycobacterium bovis*, Poland.

Introduction

Bovine tuberculosis (bTB) is a slow progressive infectious disease caused by the acid-resistant bovine mycobacteria *Mycobacterium bovis* and *Mycobacterium caprae*, which belong to the *Mycobacterium tuberculosis* complex (MTBC) (30). Bovine mycobacteria may also be the aetiological factor of tuberculosis in other farm livestock species as well as humans and free-living animals (17, 22, 26). The disease imposes significant economic and health burdens, which justifies rigorous epidemiological studies on the monitoring and control of this zoonosis. The implementation of molecular tests to track the transmission of infections both on farms and in the

natural environment facilitates the design of programmes for control and eradication of tuberculosis in cattle in many countries (8).

It is mandatory to take action against tuberculosis in cattle. Currently, this obligation is primarily imposed by the Veterinary Inspection Act, concerning the duties of veterinary practitioners employed in the State Inspectorate. The legislation relating directly to the control of infectious diseases is the Act on the Protection of Animal Health and Combating Animal Infectious Diseases (2). The most important Polish legislation on bovine tuberculosis is the Ordinance of the Polish Minister of Agriculture and Rural Development of November 23, 2004, defining Polish procedure for disease suspicion, confirmation and

eradication of outbreaks of bovine tuberculosis (25). In summary, cattle with confirmed bovine tuberculosis, based on positive tuberculin tests or comparative tuberculin tests, are removed from the herd and then slaughtered. Subsequently the lymph nodes mentioned in the instructions of the Polish General Veterinary Inspectorate and organs with visible lesions are sent to the National Reference Laboratory of Bovine Tuberculosis, which is located in the Department of Microbiology of the National Veterinary Research Institute (NVRI) in Puławy, Poland.

In 2009, Poland attained the status of a country free from this zoonosis, but despite this fact 12 to 33 outbreaks of tuberculosis in cattle have been recorded each year of the last five (18). In 2003–2018, the largest number of cattle infected with bovine mycobacteria were eliminated and the largest number of outbreaks of this zoonosis were recorded in the Masovian Province (the central region of Poland) (Fig. 1). The close vicinity of farms in which bTB was found led to the suspicion that tuberculosis could have been transmitted between the affected herds. According to data from the literature, horizontal infection of animals is fostered by the trading of infected asymptomatic carriers of mycobacteria, use of common pastures, and lack of knowledge about infectious diseases in people involved in animal husbandry (29, 6, 11).

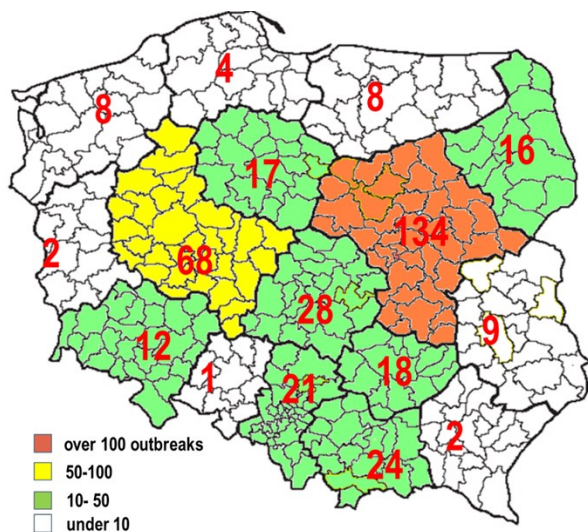


Fig. 1. Number of bovine tuberculosis outbreaks in Poland in 2003–2018

Material and Methods

The material for microbiological tests came from 119 cattle (*Bos taurus*) from nine herds located in five provinces (Podlaskie, Lublin, Masovian, Kuyavian-Pomeranian, and Łódź). The animals were sent for slaughter due to suspicion of bovine tuberculosis, based on a positive result in an intradermal tuberculin test. In compliance with the Ministry Ordinance, the following

lymph nodes were sent for microbiological tests for tuberculosis: retropharyngeal, bronchial, mediastinal, supramammary, mandibular, and mesenteric, and additionally so was a fragment of the lungs. All tissue materials were collected *post mortem*, prepared and cultured on media as stipulated by the State Veterinary Inspectorate's Instructions for Laboratory Diagnosis of Bovine Tuberculosis.

Bacteriological culture. The sediment obtained from each prepared sample was plated on three Stonebrink, and three Petragrani solid media and incubated at 37°C ($\pm 2^\circ\text{C}$) for 4–6 weeks. All media for *M. bovis* culture were prepared by the NVRI in Puławy.

Mycobacteria growth indicator tube (MGIT) identification test. A commercial test, the MGIT TBC Identification Test (Becton-Dickinson, Poland), was used to identify strains grown on Stonebrink media. It is an immunochromatographic test used to detect the MPT64 protein fraction secreted by mycobacteria belonging to the *Mycobacterium tuberculosis* complex (MTBC) during culture.

Niacin test. A niacin test (BD BBL Taxo TB Niacin, Becton-Dickinson, Poland) was used to differentiate between *M. tuberculosis* and *M. bovis* species. Mycobacterial culture was suspended in sterile distilled water and placed in a test tube with a BBL Taxo TB Niacin Test Strip (Becton-Dickinson). According to the manufacturer's protocol, a test with the BBL Taxo TB Niacin Test Control should be performed for each tested sample. *M. tuberculosis* produces the largest amounts of niacin, therefore unlike *M. bovis*, it causes a yellow coloration of the strips.

DNA isolation. Bacteria grown on Stonebrink medium were suspended in Tris-EDTA buffer, inactivated at 85°C for 20 min and then lysed with proteinase K. The obtained lysate was extracted with a cetyltrimethylammonium bromide (CTAB)-NaCl solution and purified with a chloroform-isoamyl alcohol mixture. Precipitation of the DNA was carried out in isopropanol at 20°C for 30 min. The preparation was then centrifuged and the obtained sediment was washed with 70% ethanol, dried, and suspended in 20 μL of water. The DNA obtained was then used for further analysis.

Identification of strains for the MTBC species. Species identification was made with the commercial GenoType MTBC test (Hain Lifescience, Germany), which uses DNA-STRIP technology. The test is based on the polymorphism of the *gyrB* gene. Each test strip contains three control areas: a conjugate control (CC) which means control of conjugate binding and reagent activity; a universal control area (UC) detecting the presence of all mycobacteria and G + bacteria with high content of G + C pairs in the genome in the tested DNA sample, and a control area confirming the presence of *M. tuberculosis* complex bacteria.

Genetic profile of strains. The strains were subjected to genetic analysis in a later stage of the study. Two genotyping methods were used:

spoligotyping and mycobacterial interspersed repetitive units-variable number tandem repeats (MIRU-VNTR). The spoligotyping method (spacer oligonucleotide typing) detects the polymorphism of the chromosomal DR (direct repeat) region found only in the genome of *M. tuberculosis* complex members (15).

In the presented studies, spoligotyping of strains was carried out using a commercial Isogen kit (Isogen Bioscience, the Netherlands). The obtained hybridisation patterns were compared to the patterns registered in the international databases Spol DB4 (<http://www.pasteur-guadeloupe.fr/tb/spolddb4.pdf>), SITVIT WEB (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/), and *Mycobacterium bovis* (<http://www.mbovis.org/>) (31).

MIRU-VNTR typing was carried out based on the analysis of selected polymorphic microsatellite sequences dispersed in the genome of mycobacteria. The DNA of each strain was subjected to 15 amplification reactions using 15 pairs of primers. The obtained results are presented in the form of a 15-digit code (MIRU-VNTR), where each digit depicts the number of repetitions of subsequent MIRU-VNTR repetitive sequences.

Results

Tuberculosis was confirmed in 54 (45%) animals and pathological tubercular lesions were observed in 40 of them (74%). Bovine tuberculosis was characterised by the formation of granulomas (tubercles), which were located mainly in the bronchial and mediastinal lymph nodes. In one animal, tuberculosis was detected in the peritoneum and the mediastinal lymph node, which was

four times larger than normal. There were no clinical signs of the infection.

Materials from all examined cows were pooled. A culture test on Stonebrink and Löwenstein-Jensen media was carried out on each sample pool and yielded 54 mycobacterial strains on Stonebrink solid medium. Positive culture results in an MGIT test confirmed the existence of the disease in all cases. A positive niacin test result allowed *M. tuberculosis* to be excluded. A negative niacin test result was treated as the initial identification of *M. bovis* mycobacteria. Molecular identification of the obtained cultures of mycobacteria strains, by the GenoType MTBC test showed that the strains isolated from all 54 animals belonged to the *M. bovis* species.

A two-stage analysis of the genetic relationship of the isolated strains was made. In the spoligotyping method, 52 strains were identified as SB2220 according to the Mbovis.org database (32). The same strains were described as BOVIS 595 according to the Spol DB4 database. Two strains presented the same spoligotype and it was one which had not been previously registered in international spoligotype databases. The MIRU-VNTR results are presented in Table 1. Four strains (7.4%) revealed unique patterns, and 50 (92.6%) were distributed between three clusters which contained from 2 to 27 strains with an identical MIRU-VNTR profile or with patterns differing only at one locus. Cluster I represented strains with identical MIRU-VNTR patterns, cluster II comprised strains differing at locus 31 and cluster III held strains differing at locus 3690. The molecular clusters identified among 50 strains by spoligotypes and MIRU-VNTR patterns are presented in Table 2.

Table 1. Nine MIRU-VNTR patterns identified among 54 strains

No	Spoligotype	MIRU-VNTR type	LOCUS														
			4	10	16	26	31	40	424	577	2165	2401	3690	4156	2163	1955	4052
1	BOVIS 595	424722356422327	4	2	4	7	2	2	3	5	6	4	2	2	3	2	7
2	BOVIS 595	424722357422325	4	2	4	7	2	2	3	5	7	4	2	2	3	2	5
3	BOVIS 595	425532355422434	4	2	5	5	3	2	3	5	5	4	2	2	4	3	4
4	BOVIS 595	424721374422525	4	2	4	7	2	1	3	7	4	4	2	2	5	2	5
5	BOVIS 595	424732357422325	4	2	4	7	3	2	3	5	7	4	2	2	3	2	5
6	BOVIS 595	424731357432215	4	2	4	7	3	1	3	5	7	4	3	2	2	1	5
7	BOVIS 595	424832354422437	4	2	4	8	3	2	3	5	4	4	2	2	4	3	7
8	BOVIS 595	424731374452525	4	2	4	7	3	1	3	7	4	4	5	2	5	2	5
9	BOVIS 595	424731374422525	4	2	4	7	3	1	3	7	4	4	2	2	5	2	5

Table 2. Molecular clusters identified among 50 strains by spoligotypes and MIRU-VNTR patterns

No	Spoligotype	MIRU-VNTR (number of strains)	Number of strains in the cluster	Number of herds with strains in the cluster
I	BOVIS 595 ¹ SB2220 ²	425532355422434 (21)	21	2
II	BOVIS 595 ¹ SB2220 ²	424722357422325 (18) 424732357422325 (9)	27	2
III	Not registered	424731374452525 (1) 424731374422525 (1)	2	2

¹Assigned by Spol DB4²Assigned by www.Mbovis.org

Discussion

In all cases, most of the observed anatomopathological changes confirm the data from the literature (9, 24). However, it is worth mentioning that these lesions must be considered in the differential diagnosis of animal infection by *Rhodococcus equi*, *Mycobacterium avium*, *M. fortuitum*, *M. chelonae*, and *M. peregrinum* (10, 27). The anatomopathological changes were usually observed in the lymph nodes of the chest and were not present in other parts of the body or internal organs. Tuberculosis in cattle can be very difficult to diagnose based only on a clinical assessment. The course of the disease is different, for example, in alpacas: they can quickly lose weight, which leads to death (12).

The strain genotyping results of this study confirmed the transmission of tuberculosis between cattle herds from these different provinces. The transfer of the disease took place among nine examined herds, located in five provinces. In one case, the herds were 240 km away from each other and a common source of infection could not be identified. In the second case, the herds were 100 km apart and the source of the infection could not be determined in this case either. The third transmission of tuberculosis occurred between herds in the same province on farms 8 km apart. In each described infection event, animal owners did not trade cattle between themselves as confirmed by veterinary inspection. If no animal trade caused the transmission of the disease, it happened as a result of the transfer of the mycobacteria mechanically or by wild animals.

The MIRU-VNTR molecular analysis method also indicated that tuberculosis in the herd may have more than one source of infection, as additionally demonstrated by García de Vidma *et al.* (13) and Navarro *et al.* (23). They described cases in which on one farm, tuberculosis in two cows was caused by different strains with dissimilar MIRU-VNTR patterns.

Studies to ascertain if there were more than one source of infection in the same herd were also carried out in Poland and the results confirmed that there can be (19). Researchers involved in the molecular diagnosis of tuberculosis in humans also indicated

various sources of *M. tuberculosis* infection among closely related patients (16, 31). Epidemiological investigations in Polish penitentiary institutions among tuberculosis-suffering prisoners did not prove that the disease had been transmitted within the studied group (7).

Most European countries and some others have their own collections of MIRU-VNTR patterns and on this basis it is possible to follow the transmission of bovine tuberculosis even at the international level. A study of 3,398 isolates from New Zealand, archived in 1982–2008, indicated that *M. bovis* infections in cattle herds were imported directly from the United Kingdom and Australia (28). In Poland, the bovine mycobacteria reservoir is cattle suffering from tuberculosis. Unlike in the United Kingdom, the Polish badger population is not a reservoir of *M. bovis* and is free of infection (21). Identified spoligotypes of Polish strains are commonly found in Europe (5, 18) and the most common spoligotype in these studies, SB2220 according to the Mbovis.org database, was isolated for the first time in Iran in 2013, which is also demonstrative of international transmission. Poland has close contacts with this region of the world through cattle breeding. For many years, Poland has been exporting cattle to Azerbaijan, which is Iran's neighbour. However, it is difficult to establish a common source of infection.

The published epidemiological studies show that *M. bovis* is identified worldwide (1, 3). In most cases, *M. bovis* is transmitted between animals in the same herd in aerosols. Cows may also become infected when they ingest mycobacteria, and the disease is transmitted during close contact between sick and healthy animals (4). Poor care of livestock contributes to the reduction of animal resistance, and poor conditions in the cowshed in which the animals are kept, especially insufficient space and lack of ventilation, can cause the accumulation of mycobacteria excreted by sick cattle and the overwhelming of the resistance of other animals in the herd. The resistance of the mycobacteria to external environmental factors may also contribute to the further occurrence of the disease in the same herd. Bovine mycobacteria can survive in faeces or soil

for several years in a viable state. Therefore, it is extremely important to control the disease by correctly disinfecting affected premises, which is one of the key procedures for elimination of the pathogen (20).

Along with the establishment of the status of Poland as a country free from bovine tuberculosis in 2009, the principles of testing animal populations changed. The period between successive tuberculin tests was extended from three to five years. This longer interval creates greater opportunities for disease transmission in the herd before it is diagnosed. The revised instruction of the Polish Chief Veterinary Officer for the eradication of bovine tuberculosis prevents such a scenario. It grants the possibility to increase the frequency of testing in an area at risk. This allows a flexible response to any prevailing epidemiological situation (14).

As can be seen in Fig. 1, the largest number of outbreaks of tuberculosis in cattle (134) in the last 15 years were found in the Masovian Province, from where the isolated bovine mycobacteria originated. From 10 to 50 outbreaks were found in seven more provinces and up to 10 outbreaks occurred in seven provinces. During the period, there was no province free from bovine tuberculosis in Poland.

Unpublished data testify that the number of animals eliminated as a result of a single outbreak of the disease has significantly increased. In 2018, there were nine outbreaks of tuberculosis in Poland, and the largest number of animals eliminated from one herd was 127.

In conclusion, molecular tests show variety in spoligotype prevalence of *M. bovis* strains on Polish farms and suggest that better elucidation of routes of transmission would be useful. Further research is needed in these areas to underpin measures to reduce the economic losses inflicted by this global pathogen.

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