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Molecular analysis methods in epidemiological investigations of animal tuberculosis in European bison

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Abstract: This work describes the usefulness of the two molecular methods (spoligotyping and MIRU-VNTR) for bovine tubercle bacilli typing in the epidemiological research of tuberculosis in European bison. Most important factors for tracking the transmission of infection during an epidemiology of infectious diseases, irrespectively the animal species it affects, are the immediate identification of etiologic factor, indication of its source, and prevention a further spread of disease by breaking the chain of transmission. The combination of two PCR-based methods was shown to be useful for determining the relatedness among isolates identified in the population of bison.

Keywords: bovine tuberculosis, transmission, spoligotyping, MIRU/VNTR, European bison, Bison bonasus

Introduction

Most important factors for tracking the transmission of infection during an epidemiology of infectious diseases, irrespectively the animal species it affects, are the immediate identification of etiologic factor, indication of its source and prevention a further spread of disease by breaking the chain of transmission.

Bison bonasus has become the species of interest to many research centers in Poland and worldwide (Bengins *et al.* 2004; Krzysiak *et al.* 2014). Diseases that most frequently affect the European bison and consequently lead to diminishing of this animal population are bovine tuberculosis, paratuberculosis, yersiniosis, brucellosis, pasterellosis, anthrax, salmonellosis and colibacillosis (Mackintosh *et al.* 2002). Although outbreaks and importance of these diseases may naturally fluctuate in the

world, wild animals' populations are still considered the main reservoirs of these microorganisms (van Campen and Rhyan 2010).

Despite the fact that since 2009 Poland has been officially declared as tuberculosis-free country, the number of wild animals infected with BT bacillus increases every year (Welz 2010; Krajewska *et al.* 2014; Orłowska *et al.* 2014; Orłowska 2015).

The implementation of modern methods of molecular biology combined with the observation of new outbreaks of bovine tuberculosis in European bison are the basis of a strategy aimed at eliminating the spread of disease (Krajewska *et al.* 2015). The case detection ability is a key component of tuberculosis eradication program.

The standardization of the used methods allows to compare patterns of genetic strains isolated around the world and thus to track their global spread. The knowledge provided by molecular epidemiology of tuberculosis supplemented by a detailed medical history allows to track environmental factors favoring the transmission.

Identifying such epidemiological chain is very important in terms of free-living animals, for which there is no standardization of the tuberculin skin test reading, and immobilization of the animal in order to set up such a test or perform other intravital test (interferon-gamma release assay) is often difficult if not impossible.

Aim of the study

Evaluation of two selected mycobacterium molecular typing methods (spoligotyping, MIRU-VNTR) for tracking the transmission of bovine tuberculosis in European bison.

Material and molecular analysis methods

Molecular typing of *M. tuberculosis* complex isolates is a useful tool for epidemiological studies at different levels. In the last decade, the application of different DNA fingerprinting techniques has contributed significantly to our understanding of the transmission of human and animal tuberculosis.

Tissue samples were collected from a herd of European bison ("Górny San") from the Bieszczady (Southern Poland). Material for the research consisted of 18 strains of *M. caprae*. In our study we used and evaluated the usefulness of the two methods: spoligotyping (spacer oligonucleotide typing) and MIRU-VNTR (mycobacterial interspersed repetitive units – variable-number tandem-repeat.

Spoligotyping, is a rapid, polymerase chain reaction (PCR), a basic method for genotyping strains of the *M. tuberculosis* complex (MTBC).

Spoligotyping of isolates was performed as described by Kamerbeek *et al.* (1997). In short, PCR-amplified biotin-labeled DR locus is hybridized against an array of 43 different immobilized DR spacers in a Miniblotter MN45 apparatus. The resulting hybridization signals are revealed by chemiluminescence, and are visualized as

a profile of discrete dots. The profiles were compared to SITVIT2 – an international spoligotype database (Demay *et al.* 2012).

Spoligotyping data can be represented in absolute terms (digitally), and the results can be readily shared among laboratories, thereby enabling the creation of large international databases. Since the spoligotype assay was standardized, tens of thousands of isolates have been analyzed, giving a global picture of MTB strain diversity. The method is highly reproducible, and has been developed into a high-throughput assay for large molecular epidemiology projects. Spoligotyping, a PCR-based reverse-hybridization technique, targeting the genetic diversity of the direct repeat (DR) locus, has been proven useful for the clinical laboratory and for molecular epidemiology and evolutionary and population genetics.

The strengths of this method include its low cost, its digital data results, the good correlation of its results with other genetic markers, its fair level of overall differentiation of strains, its high-throughput capacity, and its ability to provide species information. Although spoligotyping is a convenient and rapid method for preliminary screening, it overestimates the number of epidemiologically linked cases. Consequently, spoligotyping has been proposed as a first-line test in a two-step strategy for M. tuberculosis typing, to be followed by another PCR-based fingerprinting method with a higher discrimination level (Varma-Basil *et al.* 2011).

In the present study, spoligotyping was combined with MIRU-VNTR (15 loci) method.

This method is based on the variable-number tandem-repeats of mycobacterial interspersed repetitive units (MIRU-VNTR) scattered throughout the genome, and each isolate is typed based on the number of copies of repeated units. Implementation of the large number of loci is expected to achieve a high discrimination. This relatively new method, which requires only basic PCR and agarose electrophoresis equipment, was shown with different strain samples to possess a higher discriminatory power than that of spoligotyping.

Each of the 15 MIRU-VNTR loci was amplified individually with primers specific for sequences flanking the MIRU units as described by Supply *et al.* (2001; 2006) The amplicons were evaluated on the 2% standard agarose gels. The H37Rv strain was run as an additional control of the performance of the method. The apparent advantage of the MIRU-VNTR approach is its portability due to easy digitalization of the generated profiles and hence easy interlaboratory exchange, as well as easy creation and maintenance of the databases.

Results

In 18 cases, soligotyping method showing spoligo pattern 2000037777377400, not registered in an international database. In MIRU-VNTR all strains showed the same genetic patterns or similar (MIRU-VNTR pattern differed at most in 1 of the 15 locci analyzed).

Among the 18 strains analyzed by MIRU-VNTR (tab.1):

- 11 strains – 453552362412223,
- 5 strains – 453552362413223
- 1 strain – 423552342411223
- 1 strain – 423552362412223

Isolates are grouped in one cluster if they belonged to one spoligo pattern and also had identical MIRU-VNTR pattern or belonged to one spoligo pattern and also had the pattern of MIRU-VNTR differed at most in 1 of the 15 loci analyzed.

Table 1. The epidemiological characteristics of isolates from bison belonging to the cluster

	Animal species	**Identification**		
		Mycobacterium species	**Spoligotype**	**MIRU-VNTR**
1.	European bison	*M. caprae*	2000037777377400	453552362412223
2.				453552362412223
3.				453552362412223
4.				453552362412223
5.				453552362412223
6.				453552362412223
7.				453552362412223
8.				453552362412223
9.				453552362412223
10.				453552362412223
11.				453552362412223
12.				453552362413223
13.				453552362413223
14.				453552362413223
15.				453552362413223
16.				453552362413223
17.				453552362413223
18.				453552362413223

In conclusion, a combination of two PCR-based methods, spoligotyping and MIRU-VNTR, was shown to be useful for determining the relatedness among isolates identified in the population of European bison.

Summary

European bison in Poland have been straggling with bovine tuberculosis over more than 20 years now (Żórawski and Lipiec 1997; Welz *et al.* 2005; Krajewska *et al.* 2015). The first case of tuberculosis in the European bison in Poland was reported 19 years

ago from Bieszczady. Based on the growth parameters obtained on Stonebrinck solid medium, positive results of bioassays and considering results of niacin test, the authors described the isolated strain as *M. bovis* (Żórawski and Lipiec 1997). Over the years the identification of mycobacteria based on a number of labor-intense and time-consuming biochemical tests and only the results they supplied made the classification of the separated strains possible. First commercially available assay intended for the differentiation of members of the MTBC and identification of *M. bovis* BCG was Genotype MTBC®; Hain Lifescience GmbH, Nehren, Germany. Within *M. bovis* the assay is capable to discern two subspecies *M. bovis* ssp. *bovis* and *M. bovis* ssp. *caprae* which are now, according to present taxonomy, reckon separate species of mycobacteria – *M. bovis* and *M. caprae* (Aranaz *et al.* 2003). The identification of insertion sequences or some specific genes in the genome of mycobacterium appeared to be essential for establishing individual genetic profiles – fingerprints of *M. tuberculosis* strains studied.

The selection of adequate typing technique that allows an unambiguous discern of mycobacterium molecular patterns and determines their genetic affinities is essential for successful molecular investigations of tuberculosis.

Research conducted in Poland have identified four outbreaks of bovine tuberculosis in Polish population of European bison in the following locations – (1) an already-non-existing herd „Górny San", that lived in Bieszczady (Brewczyński and Welz 2011; Salwa *et al.* 2011, Krajewska *et al.* 2015); (2) Warsaw Zoological Garden; (3) European bison enclosure at Borki; (4) European bison Breeding Centre at Smardzewice.

Based on this research, molecular characteristics of *M. caprae* strains isolated from European bison from Bieszczady, revealed a common source of transmission in this herd.

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Metody analizy molekularnej w epidemiologicznych dochodzeniach gruźlicy bydlęcej u żubrów

Streszczenie: W artykule opisano przydatność dwóch metod molekularnych (spoligotyping i MIRU-VNTR) w epidemiologicznych dochodzeniach gruźlicy bydlęcej u żubrów. W epidemiologii chorób zakaźnych wszystkich gatunków zwierząt, najistotniejszymi czynnikami w śledzeniu transmisji choroby jest jak najszybsze znalezienie czynnika etiologicznego, wskazanie źródła zakażenia i zapobieganie dalszemu rozprzestrzenianiu się choroby poprzez przerwanie łańcucha transmisji. Wdrożenie nowoczesnych metod biologii molekularnej oraz obserwacja nowych ognisk gruźlicy bydlęcej u żubrów jest podstawą strategii mającej na celu eliminację rozpowszechniania się tej zoonozy. Połączenie dwóch metod opartych na PCR okazało się przydatne do określenia pokrewieństwa pomiędzy szczepami *M. caprae* wyizolowanymi z tkanek żubrów pochodzących z jednego stada „Górny San" w Bieszczadach.