Molecular typing of *Mycobacterium bovis* isolated in the first outbreak of bovine tuberculosis in the Azores Islands: a case report

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ABSTRACT: Twenty *Mycobacterium bovis* isolates from a first reported outbreak in the Azores Islands were differentiated into four spoligotypes: SB0119 (45%), SB0121 (40%), SB1264 (10%) and SB1090 (5%) and into two MIRU-VNTR allelic profiles using eight selected *loci*. The isolates seem to constitute a clonal lineage from a common source of infection. The limited diversity among the analysed Azores strains isolates could be due to the close contact of animals and use of common pastures and all herds can be seen as one epidemiological unit. The population structure of these strains with its low diversity differs from the Portugese mainland indicating a recent infection with accompanying evident founder effect.

Keywords: spoligotyping; MIRU-VNTR typing; *Mycobacterium bovis*

Bovine tuberculosis (bTB) caused by *M. bovis* is a zoonotic disease with a complex epidemiological pattern. Transmission can occur within and between farm animal and wildlife populations. Genotyping methods, such as spoligotyping and MIRU-VNTR, applied to clinical strains have proven to be valuable tools for bTB epidemiological studies and disease control (Allix-Beguec et al., 2008).

The tuberculin test has been applied since 1990 to 15% of existing cattle in the Azores Islands, located in mid-Atlantic and belonging to the insular Portuguese territory. In 2004, a surveillance and control plan for bTB was introduced in this region, with unknown reports of positive reactions. However, in 2007, an outbreak of bTB was noticeable in six herds from the island of Sao Miguel. The aims of this study were to elucidate the possible sources and routes of this infection through the genotyping of *M. bovis* isolates by spoligotyping and MIRU-VNTR, and comparison of the resulting profiles with those previously reported for *M. bovis* from mainland Portugal (Duarte et al., 2008).

MATERIAL AND METHODS

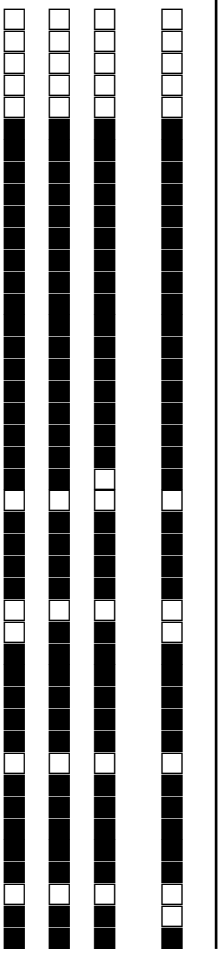
Among the 52 tested animals from eight different herds on the Island of S. Miguel, Azores, 20 gave positive serological results to tuberculin and gamma-interferon tests. Positive animals were slaughtered and tissue samples with suspected tuberculosis lesions were collected. Samples were submitted between October 2007 and February 2008 to the National Reference Laboratory (LNIV) in Lisbon and were analyzed to confirm bacteriological and molecular diagnosis of bovine tuberculosis. Bacterial isolation was performed according to the OIE Manual (2008). For DNA extraction, bacterial pellets from BACTEC liquid medium or colonies on Lowenstein-Jensen or Stonebrink media were suspended in 50–100 ml TE (1mM Tris-HCl, pH 7.6; 0.1mM EDTA) and heated at 95°C for 45 min. *Mycobacteria* isolates were identified by an in-house PCR-REA system targeting the *gyrB* gene adapted from Niemann et al. (2000). Isolates were analysed by spoligotyping

according to Kamerbeek et al. (1997). *M. tuberculosis* H37Ra ATCC 25177 and *M. bovis* ATCC 19015 strains were used as positive controls. The spoligotypes were named according to the *M. bovis* Spoligotype Database website (www.mbovis.org). MIRU-VNTR analysis was performed as a second line genotyping technique by characterisation of eight selected *loci*: VNTR3232, ETR-A, ETR-B, ETR-C, QUB11a, QUB11b, MIRU26 and MIRU4 which were amplified using the recommended PCR conditions (Allix et al., 2006). Primers were used according to Frothingham and Meeker-O'Connell (1998) for ETR-A and ETR-B; Skuce et al. (2002) for QUB11a and QUB11b; Supply et al. (2001) for MIRU26 and MIRU4; and Skuce et al. (2002) for VNTR3232 (AFBI primers). Amplicon sizes were estimated by electrophoresis on a 3% agarose gel at 120 V during a maximum of 5 h, using 100-bp ladder (Promega). The number of repeats present in each *locus* (allele) was determined by correlation with the amplicon size according to previously published tables (Supply, 2006).

RESULTS AND DISCUSSION

All twenty *Mycobacterium* isolates were identified as *M. bovis* by the in-house PCR-REA system targeting the *gyrB* gene. These isolates were differentiated into four spoligotype patterns: SB0119 (nine isolates – 45 %), SB0121 (eight isolates – 40%), SB1264 (two isolates – 10%) and SB1090 (one isolate – 5%). The four spoligotypes have been previously identified on the Portugese mainland (Duarte et al., 2008), with SB0121 and SB0119 being the two most common spoligotypes identified among 472 *M. bovis* isolates (Duarte et al., 2008 and unpublished results). These two spoligotypes are also highly frequent in Spain and in other European countries (Rodriguez et al., 2010). Spoligotype SB1090 has only been reported in Portugal until now, in 20 *M. bovis* cattle isolates obtained from five different regions of mainland Portugal and the Azores (this study and Duarte et al., 2008). The two spoligotypes SB0119 and SB1090 each differ from SB0121 by the absence of one spacer sequence, specifically, spacers 15 and 22, respectively (Table 1). The SB1264 pattern was previously identified in six cattle and one red deer in four different regions of mainland Portugal (unpublished results) and it differs from the predominant spoligotype SB0119 by the absence of spacer 2 (Table 1).

Table 1. Molecular analysis of the panel of 20 *M. bovis* isolates from Azores using spoligotyping and MIRU-VNTR techniques

Spoligotype pattern (1)	Spoligotype (2)	MIRU-VNTR allelic profile								Number of isolates
		VNTR 3232	ETR-A	ETR-B	ETR-C	QUB11a	QUB11b	MIRU4	MIRU26	
	SB0119	5	6	4	2	11	2	3	2	9
	SB0121	5	6	4	2	11	2	3	2	8
	SB1090	?	6	4	2	11	2	3	2	1
	SB1264	5	6	4	2	11	2	3	2	1
		5	5	4	4	11	2	2	2	1

(1) ■ = indicates the presence of a spacer, □ = indicates the absence of the spacer
(2) Spoligotype as defined in the *M. bovis* Spoligotype Database website(www.mbovis.org)
? = indicates a double band

All isolates presented identical MIRU/VNTR patterns, except one isolate from SB1264 which had variations in *loci* ETR-A, ETR-C and MIRU4 (Table 1). This strain was isolated from a calf from a different herd, that was born in one of the first herds to be infected. All the other strains were isolated from adult animals with different age ranges, used for milk or meat production and reproduction.

The molecular and epidemiological analysis of the *M. bovis* population from the Azores outbreak suggests that the twenty isolates examined are a clonal lineage from the same source of infection. However, this source remains uncharacterised and the official records are not consistent with the introduction of infected animals into an island which is mainly a cattle exportation region. Our hypothesis is that spoligotypes SB0119 and SB1090 could have evolved from SB0121 by a single deletion event of spacers 15 and 22, respectively and, subsequently, spoligotype SB1264 could have evolved from SB0119 by a single deletion event of spacer 2. The population structure of *M. bovis* strains in the Azores is totally different from mainland Portugal (Duarte et al., 2008), where spoligotyping showed a great diversity of strains. Six of the eight infected herds belong to the same owner and, in fact, they can be considered as one single epidemiological unit, since the animals are housed in close contact, share common pastures and move between herds. The other two herds are contact herds and the infected calf was born in one of the initially infected herds. The spoligotype of the strain isolated from this calf (SB1264) was also detected in one cow from the initial herd.

Spoligotyping and MIRU-VNTR typing appear to be useful genotyping methods in situations where infection has been established for some time (Duarte et al., 2009). We hypothesise that the *M. bovis* infection described here is recent with an apparent manifestation of a founder effect.

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