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Mycobacterium bovis infections in Portugal: Spoligotyping and MIRU-VNTR typing of animal isolates

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Introduction

Tuberculosis is still one of the most important infectious diseases worldwide. Although human cases due to *Mycobacterium bovis*, the aetiological agent of **Bovine tuberculosis** (bTB), considerably decreased in most industrialized countries, the possible zoonotic health hazard and the considerable economic losses it brings justify significant efforts to eradicate the disease in several countries. In Portugal, although a systematic slaughter policy of tuberculin reactive animals has been ongoing for several years, eradication is far from being achieved. In order to clarify possible infection sources and transmission routes, **molecular typing** of Portuguese *M. bovis* animal strains was undertaken. Two typing methods directed on potentially polymorphic genomic regions were chosen: **Spoligotyping**, a reverse line blot hybridization technique that evaluates the presence or absence of 43 oligonucleotide sequences in the Direct Repeat genomic region and **MIRU-VNTR** (Mycobacterial Interspersed Repetitive Units- Variable Number Tandem Repeats) typing of 8 selected minisatellite like *loci*.

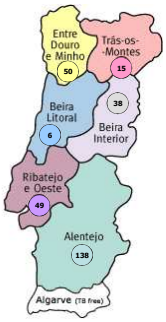

Figure 1: Number of *M. bovis* isolates typed per geographical region

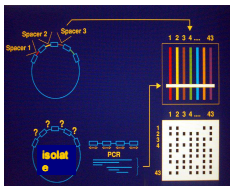

Figure 2: Schematic representation of spoligotyping procedure. *M. bovis* isolates invariably show absence of spacers 3,9,16,39-43

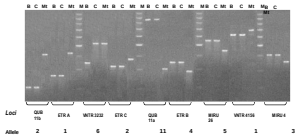

Figure 3: MIRU typing results of M. bovis SB0295 cattle and wild boar isolates. M= 100 bp molecular weight marker, C= cattle isolate, B= Boar isolate, Mt= M. tuberculosis ATCC reference strain

Materials and Methods

M. bovis strains: Two hundred and ninety-four (N=294) *Mycobacterium bovis* strains were isolated between 2002 to early 2007 from tuberculous lesions of cattle (n=258), goats (n=8), wild boar (*Sus scrofa*, n=6) ,red deer (*Cervus elaphus*, n=21) and domestic rabbit (n=1) from 6 geographical regions of Portugal (fig.1), and identified by *gyr* B PCR-REA (5).

Strain typing: Spoligotyping was performed (1,4) using traditional 43 spacers home-made membranes (fig. 2) . MIRU-VNTR typing (6) was performed characterizing 8 selected *loci* (VNTR 3232, ETR A, ETR B, ETR C, QUB 11a, QUB 11b, MIRU 26 and MIRU 4) for strains belonging to the 3 major spoligotype defined clusters: SB0121, SB0886 and SB0140. *M. tuberculosis* H37RV ATCC 25177 and *M. bovis* ATCC19015 were used as reference strains. The discriminatory power of each typing method was calculated by the Hunter-Gaston Index (h) (2).

Results

- **Twenty-nine** different spoligotypes were found (h=0,9). SB0121 was clearly the most frequent as 24,15% of isolates showed this spoligotype. **Ten** spoligotypes patterns were exclusively found in Portugal (tab.1).

- MIRU-VNTR typing of the 3 major spoligotypes provided 53 patterns (h=0.96), subdividing spoligotype **SB0121** cluster into **38 MIRU types**, **SB0886** into into **7 MIRU types** and **SB0140** into **8 MIRU types**. Locus VNTR3232 was the most discriminatory one (h=0.765) for our set of strains.

- Nine spoligotype patterns were common to domestic and feral animals. Spoligotyping and MIRU-VNTR typing results allowed us to strongly suspect **transmission between domestic and feral** species in 2 cases (Fig.3):

  - A **cattle** isolate and a **wild boar** isolate in AL region (spoligotype SB0295, MIRU profile 6,1,4,2,11,2,5,3)
  - A **cattle** isolate and a **deer** isolate in BI region (spoligotype SB1174, MIRU profile 6,4,5,4,11,4,5,3)

- The presence of more than one spoligotype was recorded in **10** outbreaks, suggesting the possibility of **more than one infection source**.

Hosts	Number of *M. bovis* isolates	Regions were isolated	Spoligotype[1]	spoligotype frequency
CATTLE, DEER	71	AL-BI-BL-EDM-RO-TM	SB0121	24,15%
CATTLE	27	AL-EDM-RO-TM	SB0886	9,18%
CATTLE, DEER	26	AL-BI-EDM-RO	SB0140	8,84%
CATTLE , DEER, BOAR	21	AL-BI-RO	SB0119	7,14%
CATTLE, DEER, RABBIT	18	AL-BI-EDM-TM	SB1095	6,12%
CATTLE, BOAR	17	AL-BI-EDM-RO	SB0295	5,78%
CATTLE	16	AL-EDM-RO-TM	SB0124	5,44%
CATTLE	13	AL-EDM-RO	SB1090	4,42%
CATTLE	12	AL-RO	SB1172	4,08%
CATTLE, DEER	12	AL-BI-TM	SB1174	4,08%
CATTLE, DEER, BOAR, GOAT	10	AL-BI	SB0265	3,40%
CATTLE, DEER	8	AL-BI-EDM-TM	SB0120	2,72%
CATTLE, DEER	6	AL-BI	SB0122	2,04%
CATTLE	4	BI	SB0848	1,36%
CATTLE, DEER	4	AL-BI-RO	SB1167	1,36%
CATTLE	3	EDM-TM	SB0130	1,02%
CATTLE, BOAR	3	AL-BI	SB1230	1,02%
CATTLE	2	RO	SB0833	0,68%
CATTLE	2	BI	SB1173	0,68%
CATTLE	1	BI	SB1191	0,34%
CATTLE	1	EDM	SB1175	0,34%
DEER	1	BI	SB1091	0,34%
CATTLE	1	AL	SB0867	0,34%
CATTLE	1	RO	SB0849	0,34%
CATTLE	1	BL	SB0332	0,34%
CATTLE	1	AL	SB0334	0,34%
CATTLE	1	BL	SB1093	0,34%
DEER	1	BI	SB1267	0,34%
CATTLE, BOAR, GOAT	10	AL-BI-EDM	SB0157 [2]	3,40%

Table 1- Spoligotyping results. AL= Alentejo, BI= Beira Interior, BL= Beira Litoral, EDM= Entre Douro e Minho, RO= Ribatejo e Oeste, TM= Trás-os-Montes . [1]allocated by database http://www.mbovis.org [2]Isolates identified as *Mycobacterium caprae* by *gyr*B PCR-REA

Spoligotype described for the first time in Portugal

Discussion/conclusions

- Spoligotyping and MIRU-VNTR typing are proven to be useful molecular typing tools, for our set of *M. bovis* strains, as they both presented a good discriminatory power index (greater than 0,9);

- MIRU-VNTR typing showed a higher discriminatory power than spoligotyping, with the additional benefit of being based in independent genomic regions, allowing a more confident linkage of isolates for epidemiological purposes. We suggest spoligotyping should be used as a first screening typing method to assess strain distribution within major geographical regions and patterns evolution over time. MIRU-VNTR typing is likely to be an essential tool in outbreak investigations, when identical spoligotype patterns are present ;

- SB0121, the predominant spoligotype in this study, is also the most prevalent in Spain in a recent study (3). SB0140 is surprisingly frequent (8,9%) in Portugal: it is predominantly found in UK and Ireland were it represents more than 1/3 of *M. bovis* strains. Future comparison with MIRU-VNTR profiles of British Islands SB0140 strains is likely to provide additional data to on their possible relatedness;

- Although several spoligotypes were common to domestic animals and wildlife, MIRU typing only provided strong evidence of transmission in two occasions. Furthermore, *M. bovis* isolated from deer from a same hunt, presented different spoligotypes (data not showed). These results indicate that wildlife is probably not a major bTB reservoir in Portugal and deer and wild boar could be spillover hosts. Nevertheless, due to the possibility of transmission, a cautious monitorizing of *M. bovis* infection in wildlife should be implemented as they are not the subject of any disease control policy as in cattle;

- These results emphasize the need of a continued molecular typing of Portuguese *M. bovis* strains to complement traditional epidemiological surveys, providing further knowledge on bTB dynamics in Portugal and additional scientific basis for more efficient and focussed future eradication measures.

References

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