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Multiple sampling and discriminatory fingerprinting reveals clonally complex and compartmentalized infections by *M. bovis* in cattle

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ABSTRACT

The combination of new genotyping tools and a more exhaustive sampling policy in the analysis of infection by *Mycobacterium tuberculosis* has shown that infection by this pathogen is more complex than initially expected. Mixed infections, coexistence of clonal variants from a parental strain, and compartmentalized infections are all different modalities of this clonal complexity. Until recently, genotyping of *Mycobacterium bovis* in animal populations was based on spoligotyping and analysis of a single isolate per infection; therefore, clonal complexity is probably underdetected. We used multiple sampling combined with highly discriminatory MIRU-VNTR to study compartmentalized infections by *M. bovis* in a low-tuberculosis prevalence setting. We spoligotyped the *M. bovis* isolates from two or more anatomic locations sampled from 55 animals on 39 independent farms. Compartmentalized infections, with two different strains infecting independent lymph nodes in the same animal, were found in six cases (10.9%). MIRU-VNTR analysis confirmed that the compartmentalization was strict and that only one strain was present in each infected node. MIRU-VNTR analysis of additional infected animals on one of the farms confirmed that the compartmentalized infection was a consequence of superinfection, since the two strains were independently infecting other animals. This same analysis revealed the emergence of a microevolved clonal variant in one of the lymph nodes of the compartmentalized animal. Clonal complexity must also be taken into consideration in *M. bovis* infection, even in low-prevalence settings, and analyses must be adapted to detect it and increase the accuracy of molecular epidemiology studies.

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Bovine tuberculosis (bTB) generates a considerable economic and health burden, thus justifying strict epidemiological surveys for monitoring and control. Studies of the molecular epidemiology of infection by *Mycobacterium bovis* have increased our knowledge of the transmission dynamics of this pathogen (Costello et al., 1999; McCluskey et al., 2014) on farms, in the natural environment, and between both settings and have facilitated the design of control programs.

The application of molecular fingerprinting to the analysis of *Mycobacterium tuberculosis* (MTB) infections has shown that the infection by this pathogen is more clonally complex than initially expected. Infections by more than one MTB strain and compartmentalized infections with different strains at independent anatomical sites in the same patient have been described (Muwonge et al., 2013; Navarro et al., 2011). We recorded these findings after improving our sampling schemes and applying MIRU-VNTR (mycobacterial interspersed repetitive units-variable number of tandem repeats), which is particularly sensitive to detect coinfecting strains, even if they are under-represented (Garcia de Viedma et al., 2005). Accurate identification of infections involving more than one strain or clonal variant and its potentially asymmetric distribution in different tissues has enabled us to more accurately track the dynamics of MTB transmission and increase our knowledge of the complex patterns of MTB infection.

However, the issue of clonal complexity has received little attention in animals infected by *M. bovis*. Standard molecular epidemiology studies of *M. bovis* are based on DVR-spoligotyping (Cunha et al., 2012; Mwakapuja et al., 2013), a hybridization-based approach that is unable to identify mixed infections. This limitation is aggravated by the common habit of genotyping isolates from a pool of tissues from a single animal. Both limitations could explain why clonal complexity is underdetected in infection by *M. bovis*.

Our aim was to apply the multiple-sampling with refined analysis by MIRU-VNTR strategy, used to analyze clonal complexity in MTB infections, to animals infected by *M. bovis* in order to identify and analyze clonally complex events associated with this pathogen.

1. Materials and methods

1.1. Study samples

The study sample comprised 154 animals diagnosed with bTB (based on the intradermal tuberculin test) between January 2007 and December 2008 from 74 cattle farms in the autonomous community of Asturias in northern Spain. Samples were processed according to the pattern of macroscopic lesions found in each carcass. When lesions were observed, the involved lymph node/s (mediastinal, tracheobronchial, and/or retropharyngeal) and/or the lungs were taken and cultured separately. When lesions were not observed, each lymph node and a pool of the lymph nodes (mix) were cultured separately.

Animals with $\geq$ two positive cultures from different samples were selected for the study of clonal complexity.

1.2. Bacterial procedures and DNA extraction

Lymph nodes were homogenized separately, decontaminated, and cultured in Coletsos, Lowenstein-Jensen with pyruvate or MGIT (Mycobacterial-Growth-Indicator-Tube) media. Pelleted cultures were boiled to extract the DNA and identify the isolates as *M. bovis* by spoligotyping.

For MIRU-VNTR typing, purification of the extracted DNA from boiled specimens was needed. We used a column-based purification method (QIAamp DNA Mini Kit protocol; Qiagen, Courtaboeuf, France) without the proteinase K step. Samples were finally eluted in 70 μl of buffer AE.

1.3. Genotyping analysis

1.3.1. DVR-spoligotyping

The isolates were spoligotyped following the protocol described by Kamerbeek (Kamerbeek et al., 1997). Spoligotyping profiles were assigned according a standard nomenclature (www.mbovis.org) (Smith and Upton, 2012).

1.3.2. MIRU-VNTR typing

Standard panel of nine loci: the nine MIRU-VNTR loci (VNTR 580 [MIRU 4], VNTR 2996 [MIRU 26], VNTR 3192 [MIRU 31], VNTR 2163b [QUB-11b], VNTR 2461 [ETR-B], VNTR 4052 [QUB-26], VNTR 2163a [QUB-11a], VNTR 3232 [QUB-3232], VNTR 2165 [ETR-A]) defined as optimum for typing of *M. bovis* (Rodriguez-Campos et al., 2013) were selected for analysis using multiplex PCR (Navarro et al., 2014). Five microliters of non-diluted DNA were added to a final reaction mixture of 50 μl. MIRU-VNTR types were obtained by sizing amplicons in an ABI Prism 3100 genetic analyzer (Applied Biosystems, NLLab Centraal B.V., Haarlem, The Netherlands) and assigning allelic values using GeneMapper 4.0 (Applied Biosystems, Foster City, CA, USA).

Extended panel of 24 loci: in cases showing differences in the nine MIRU-VNTR analysis, we continued the analysis to complete the 24 loci in the extended set, using simplex PCR and agarose electrophoresis (Navarro et al., 2011).

2. Results

2.1. Screening of clonal complexity by multiple sampling and spoligotyping

We diagnosed bTB in 154 animals from 74 cattle farms. In 64 animals (41.5%), lesions were observed at $\geq$ two locations and were therefore independently sampled and cultured. The cultured isolates were from lymph nodes (mediastinal [51 animals], tracheobronchial [42 animals], retropharyngeal [15 animals]) and lung tissue (six animals). Spoligotyping of $\geq$ two isolates from the same animal was performed in 55 cases. The spoligotypes obtained were as follows: SB0120, SB0121, SB0130, SB0134, SB0140, SB0265, SB0329, SB0339, SB0828, SB0869, SB0882, SB1019 and SB1665.

Comparison of the spoligotypes obtained for the isolates cultured from independent nodes revealed differences in six animals (10.9%), each from a separate farm (Fig. 1).