Molecular typing of *Mycobacterium bovis* isolates: A review

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Abstract

*Mycobacterium bovis* is the main causative agent of animal tuberculosis (TB) and it may cause TB in humans. Molecular typing of *M. bovis* isolates provides precise epidemiological data on issues of inter- or intra-herd transmission and wildlife reservoirs. Techniques used for typing *M. bovis* have evolved over the last 2 decades, and PCR-based methods such as spoligotyping and mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) have been extensively used. These techniques can provide epidemiological information about isolates of *M. Bovis* that may help control bovine TB by indicating possible links between diseased animals, detecting and sampling outbreaks, and even demonstrating cases of laboratory cross-contamination between samples. This review will focus on techniques used for the molecular typing of *M. bovis* and discuss their general aspects and applications.

Key words: tuberculosis, bovine, diagnosis, genotyping, *Mycobacterium bovis*.

Introduction

Bovine tuberculosis (BTB) has been detected in cattle throughout the world. According to disease timelines available in the Worldwide Animal Health Information Database (OIE, 2009), 109 countries reported the presence of *Mycobacterium bovis* infections and/or clinical diseases in their cattle herds at some time in 2005-2010. In developed countries that have a tradition of cattle farming, the prevalence of BTB has reached very low levels because of strict control policies. In several of these countries, the disease has been eradicated. Conversely, in developing countries, despite recently implanted control measures, considerable economic losses consistently occur in regions such as Brazil and Argentina, where there is intense cattle breeding (Ruggiero et al., 2007).

The first attempt to differentiate *M. bovis* isolates based on DNA sequence polymorphism was done by Collins and de Lisle in 1985 (Collins and De Leslie, 1985). They performed restriction digestion and agarose gel electrophoresis of genomic DNA. Identification of insertion sequences in *M. tuberculosis* and *M. bovis* genomes led to the development of the restriction fragment length polymorphism (RFLP) typing method (Thierry et al., 1990), a technique still in use nowadays. Knowledge of the genomic sequence of *M. bovis* and *M. tuberculosis* has facilitated the development of high-throughput molecular typing techniques that allow greater insight into the epidemiology, evolution, and population structure of *M. bovis*. Polymorphic GC Repeat Sequence (Roring et al., 1998; Ross et al., 1992), direct repeats (DR) regions (Hermans et al., 1991), and variable number of tandem repeats (VNTR) (Supply et al., 2000) have all been exploited as typing methods.

These tools can help to determine the source of infection and outbreaks, understand the relationship between different outbreaks, and identify wild animal reservoir of *M. bovis*. In addition, they can provide insight into the risk factors for BTB transmission by allowing identification of the dynamics of this disease (Brosch et al., 2002; Garnier et al., 2003; Zumarraga et al., 2005). In this review, we describe the main techniques used for genotyping and their application in characterizing *M. bovis* isolates.
Restriction Fragment Length Polymorphism (RFLP)

Strain differentiation by using RFLP analysis has proven to be a very useful tool for epidemiologic studies of tuberculosis. RFLP based on the presence of the insertion sequence *IS6110* has been widely used as a genetic marker (Otal *et al.*, 1991). *IS6110* fingerprinting via RFLP has been standardized by using *Pvu*II as the restriction enzyme of choice to digest mycobacterial genomic DNA (Figure 1) (Brosch *et al.*, 2002; Gutierrez *et al.*, 1995; Thierry *et al.*, 1990). After electrophoresis of digested DNA on agarose gel, Southern blotting is carried out. Polymorphic banding patterns is revealed after hybridization by using a fragment of *IS6110* as a probe (Durr *et al.*, 2000). This insertion sequence is present in up to 20 copies in *M. tuberculosis*, thus enabling the application of *IS6110*-RFLP as the gold standard genotyping technique for this organism. In contrast, only 1-5 copies of *IS6110* are found in *M. bovis*, which limits the ability of this element to discriminate between different *M. bovis* strains (Aranaz *et al.*, 1996; Aranaz *et al.*, 1999; van *et al.*, 1994; van, 2001).

One additional limitation of the RFLP-based typing systems is that they require a well-grown culture for DNA extraction. The time lag between isolation of *M. bovis* from a clinical sample and the growth of a mycobacterial culture is often too long. This problem can be circumvented with the use of several complementary biomarkers such as those based in the polymorphic *IS6110* region, and by using direct repeats (DR) and polymorphic GC-rich repeat sequences (PGRS) as probes (Cousins *et al.*, 1998; van Embden *et al.*, 1996).

Polymorphic GC-Rich Repeat Sequence (PGRS)

The PGRS method is similar to standardized *IS6110* fingerprinting in that it requires purified DNA for Southern blot hybridization and banding pattern analysis. PGRS fingerprinting has proven to be useful for differentiating strains with fewer than 6 copies of *IS6110* that could not readily be differentiated by *IS6110* fingerprinting (van *et al.*, 1993; Yang *et al.*, 2000).

The PGRS-based RFLP probe is the single most discriminatory of the probes currently available for *M. bovis* strain typing and can be present in up to 30 copies in members of the *M. tuberculosis* complex. PGRS is present in multiple copies interspersed throughout the genome and it exhibits a high level of polymorphism between unrelated isolates. However, the result of a PGRS DNA fingerprint is relatively complex because it contains many bands, making it potentially difficult to interpret. For the same reason,

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![Figure 1](image-url) - Representation of the *M. bovis* chromosome with the *IS6110* region (red), *Pvu*II (arrow) restriction sites and the 245 bp (green) *IS6110* probe used for Southern blotting. Different banding patterns result from the number and position of *IS6110* copies, as well as polymorphism in the adjacent region where the *Pvu*II site is located. Different strains produce distinct banding patterns as shown in this example with strains A and B.
Polymerase Chain Reaction (PCR) Based Techniques

Techniques based on DNA amplification via PCR, such as spoligotyping and MIRU-VNTR (Burgos et al., 2004), have become tools for epidemiological studies of bovine tuberculosis transmission, and have given prominence to a modern field of research known as molecular epidemiology. The agility and speed in detecting *M. bovis* can be decisive in the choice of these methods, which differentiate the species and different isolates of the same species at the DNA level.

Spoligotyping

Spoligotyping (from “spacer oligotyping”) is based on the direct repeat region (DR), a DNA polymorphism present in a particular chromosomal locus that was first described by Hermans et al... (1991). This chromosomal region contains a large number of DRs of 36 bp each, interspersed with a spacer DNA of 35-41 bp in length. When DR regions of several isolates are compared, it is observed that the order of the spacers is about the same in all isolates; however, deletions and insertions of DRs occur (Figure 2). Polymorphisms in various isolates comprise the presence or absence of spacers of known sequence. This characteristic is used to determine genetic similarity among strains (Kontsevaya et al., 2011). Spoligotyping can easily distinguish between *M. tuberculosis* and *M. bovis*, can be used with DNA extracted from a bacterial culture as well as directly from a specimen, and has been used to identify the clonal nature of the isolates (O’Brien et al., 2000; Zanini et al., 2001; Zumarraga et al., 1999).

Spoligotyping is reportedly useful for identifying sources of infection, transmission of tuberculosis (TB) between species, and the stability of tuberculosis strains for long periods of time in closed populations, indicating that *Mycobacterium* is clonal (Cousins et al., 1998). In an ecological setting, spoligotyping is a rapid and inexpensive option that can be used to search for a relationship between strains (Zumarraga et al., 2012). Because strains of *M. bovis* from cattle usually contain few copies of *IS6110*, *IS6110*-RFLP is not the best method for distinguishing strains of *M. bovis* (Allix et al., 2006). Spoligotyping has proven to be a practical and discriminatory method for large-scale studies of the epidemiology of *M. bovis* as well as for the differentiation of *M. bovis* from *M. tuberculosis*, because the former lacks spacers 39-43 (Kamerbeek et al., 1997). Furthermore, there is an international database holding over 1900 spoligotype patterns from around the world (Lazzarini et al., 2012).

The main disadvantage of spoligotyping is that all genetic polymorphisms are restricted to a single genomic locus, the DR cluster, which limits the resolution. While having the advantages of being considerably faster and less labor-intensive than RFLP analysis, spoligotyping alone does not usually provide sufficient discrimination among strains of *M. bovis* to be used as a sole typing method, and is thus often combined with supplementary techniques (Costello et al., 1999; Cousins et al., 1998; McLernon et al., 2010; Roring et al., 1998).

Variable Number Tandem Repeat (VNTR)

Tandemly repeated sequences are dispersed by thousands of copies in virtually all higher eukaryote genomes. Loci with short sequence repeats of 1 ± 13 bp are generally referred to as microsatellites, and those with 10 ± 100 bp sequence repeats as minisatellites (Figure 3). Many of these

![Figure 2](image-url) - (A) Structure of the DR locus in the mycobacterial genome. Multiple DRs in the chromosomes of *M. tuberculosis* and *M. bovis* (depicted as rectangles) are interspersed with unique spacers varying in length from 35 to 41 bp. The (numbered) spacers used correspond to 37 spacers from *M. tuberculosis* H37Rv and 6 from *M. bovis* BCG. The site of integration of insertion element *IS6110* is depicted. (B) Principle of in vitro amplification of the DR region by using PCR. Any DR in the DR region may serve as a target for these primers; therefore, the amplified DNA is composed of a mixture of a large number of different-size fragments (Kamerbeek et al., 1997).
loci show hypervariability in their repeat numbers in humans and in animals, and are therefore called VNTR loci (Supply et al., 2000). VNTR sequences are also found in bacteria, and have been used to genotype many species. Polymorphism at a tandem repeat (TR) locus can occur either as a result of nucleotide sequence changes between individual repeat units or as a result of variations in the number of repeat units, thereby creating allelic variants. VNTR typing is based upon repeat number polymorphisms within these tandemly arranged repetitive DNA sequences.

There are several VNTR loci in the genome of *M. bovis*, and hence VNTR typing provides a greater resolution than spoligotyping alone (Roring et al., 2002). Many of these TR loci display hypervariability, enabling their exploitation for strain typing in numerous bacterial species. Originally, 6 VNTR loci, described as exact TR A through F (ETR-A, -B, -C, -D, -E, and -F), were reported and applied to *M. tuberculosis* isolates (Frothingham and Meeker-O’Connell, 1998).

However, the level of discrimination found in *M. tuberculosis* or *M. bovis* isolates using the 5 ETRs (A through E) was not as good as that achieved with either spoligotyping or IS6110-RFLP typing (Collins et al., 1994; Good and Duignan, 2011). Therefore, another set of polymorphic repeats, termed as mycobacterial interspersed repetitive units (MIRU-VNTR) (Roring et al., 2002; Supply et al., 2000; Supply et al., 2006), has been proposed. Initially, these involved a 12-loci set, which was considered efficient for epidemiological purposes (Supply et al., 2001a), but some limitations were found with regard to the discriminatory power (Garcia, V et al., 2006; Scott et al., 2005). Furthermore, a 15- or 24-loci subset has been shown to ensure better discrimination (Supply et al., 2006).

The challenge is to compile standardized molecular fingerprinting patterns originating from highly networked, multi-centric, genotypic analysis in databases for inter-laboratory use and for further references. Rapid genotyping methods are needed to overcome low reproducibility, not proven application-less discriminatory methods such as MIRU-VNTR (Viedma et al., 2011). By analyzing the intended purpose and possibilities for each method (Table 1), it is possible to use a combination of typing methods and thus accurately identify differences among strains.

**Implications of Molecular Typing in Epidemiology**

Within the last 10 years, many techniques have been developed or adapted for typing *M. tuberculosis* complex isolates. In this review, we have presented an overview of the main techniques used for the differentiation of *M. bovis* isolates. For the majority of them, a specific and polymorphic genetic region is involved. In comparison to the majority of other bacterial groups, the genome of mycobacteria has a high GC content (GC% is 65%), and its polymorphism is very limited compared to its genome size (4.4 Mb). However, some regions are highly polymorphic, either due to variations in number and/or position, or because of variations in primary structure. These areas of higher polymorphism appear to correspond essentially to segments of genes encoding proteins where variability provides a selective advantage to the bacteria, such as antibiotic-resistance proteins, antigens involved in escaping the immune response, or non-coding sequences (insertion sequences or repeated sequences) that are probably involved in inducing variability in neighboring genetic areas.

Implementation of molecular methods for *M. bovis* typing requires an analysis of their advantages and disadvantages with regard to tuberculosis surveillance and control programs. Their use enables the detection of epidemiological links among samples (Pheiffer et al., 2005), and contributes to better understanding of BTB transmission dynamics. Some studies have shown that laboratories should determine the discriminatory power for each molecular method to enable better selection, implementation, and combination according to specific conditions found in each
Table 1 - Molecular typing methods for Mycobacterium bovis.

| Method         | Advantages                                                                 | Disadvantages                                                                                     |
|----------------|----------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------|
| IS6110-RFLP    | The number of copies and their positions in the genome may vary from isolate to isolate (van Embden et al., 1993), providing identical profiles of isolates from animals involved in recent transmission chains, and different genetic patterns in isolates from animals not associated with infection (Aramaz et al., 1999). | Requires large amounts of DNA (1-2 µg) and technical skills; it is slow and has little discriminating power in isolates with less than 6 IS6110 copies (Gutacker et al., 2002); there is difficulty in reproducing results and comparing them among different laboratories (Supply et al., 2001b); and the majority of M. bovis isolates have a low number of IS6110 copies-in general, only 1 or 2 (Haddad et al., 2004). |
| PGRS           | Higher discriminating power in isolates with 6 or less IS6110 copies (Rozo and Ribón, 2010); useful tool for confirming the identity of strains matched by IS6110 or to type low-copy number stains (Kanduma et al., 2003); for the majority of M. bovis strains, except those originating from certain animal species like goats, PGRS sequences, in terms of copy number, much more polymorphic than the DR region, which is more polymorphic than the IS6110; it is considered more stable than the IS6110-RFLP patterns and shows 100% reproducibility. | Requires large amounts of high quality DNA (Asgharzadeh and Kafil, 2007; Doroudchi et al., 2000) and technical skills; it has lower discriminating power in isolates with multiple copies (Bauer et al., 1999); the large number of bands produced by this technique makes interpretation of the gels difficult, limiting its application as a primary typing technique (Kanduma et al., 2003). |
| Spoligotyping  | This technique is fast, robust, low cost and can differentiate strains of M. bovis and M. tuberculosis; the results can be fully expressed in a simple, digital format, which facilitates inter- and intra-laboratory results comparisons, as has been demonstrated for VNTR; this method may differentiate better when the location of more spacers is investigated; it can potentially be applied directly to pathological samples (Kamerbeek et al., 1997). | The discriminatory power of this method is lower than IS6110-RFLP typing when high copy number strains are being analyzed. |
| VNTR           | Powerful approach to high-resolution genotyping of isolates (Supply et al., 2001b); it is reproducible, fast and specific for M. tuberculosis complex isolates (Supply et al., 2001b); potential approach to detect and genotype bacteria of the M. tuberculosis complex directly in a range of clinical samples (Roring et al., 2002); the results can be fully expressed in a simple, digital format, which facilitates inter- and intra-laboratory results comparisons; it has shown 100% reproducibility. | The discriminatory power of this method is lower than IS6110-RFLP (for high copy number strains) and spoligotyping (Kremer et al., 1999); detection via electrophoresis is inexpensive, but automated detection involving fluorescence is not. |

Genotyping of bacterial isolates or PCR products is increasingly becoming a standard tool for epidemiological disease control and eradication. Distinguishing M. bovis strains at the molecular level provides important insights into the sources of infection and identification of practices or environments, thereby aiding the spread and maintenance of tuberculosis (Medeiros et al., 2010). More importantly, transmission routes between livestock and wildlife may be identified by strain typing. In addition, transmission routes of BTB within livestock via animal movements become evident; this is a prerequisite for targeted disease control aiming at testing all potentially exposed animals (Schiller et al., 2010).

PCR-based techniques used for strain fingerprinting have proven to be of useful in relating outbreaks of TB to sources of infection. Epidemiologically related isolates have similar fingerprints that differ from those that are epidemiologically unrelated. Therefore, a desirable characteristic for typing is related to its stability within a strain and diversity within a species.

DNA fingerprinting of M. bovis for molecular epidemiology has been used to study transmission of bovine tuberculosis in Latin America and other parts of the world (PARREIRAS et al., 2012). However, there are a few reports in Brazil with regard to characterization of M. bovis (Figueiredo et al., 2011; Rodriguez et al., 2004; Zanini et al., 2005) that revealed the occurrence of a high genetic diversity; however, these studies were conducted using a limited number of isolates (Zumarraga et al., 1999).

Conclusion

Tuberculosis caused by M. bovis is important for public health, animal health, and animal production. Whatever the epidemiological context, the need for techniques permitting differentiation of isolates at the molecular level is evident. These tools would help determine the origin of outbreaks, increase the understanding with regard to the link between different outbreaks, show the relationship between domestic TB and wild TB, and identify the source of infection. No typing technique developed so far can be used on its own. Each technique has its advantages and disadvantages that must be considered when choosing the one to be
implemented in the laboratory. Using spoligotyping in combination with MIRU-VNTR seems to be the best choice as both have the advantages of being PCR-based, with improved discriminatory power when combined. Hopefully, in the future we will have new and improved techniques for typing M. bovis. It is conceivable that a lab-on-a-chip approach will be capable of not only detecting M. bovis from a clinical sample but also typing the pathogen at the same time. Regarding the target locus, it is likely that single nucleotide polymorphisms in specific genes will be used for molecular epidemiology.

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