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Mohamed S Kamel, Cairo University

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Molecular Epidemiology of *Mycobacterium bovis* in Humans and Cattle

A. El-Sayed1, S. El-Shannat1, M. Kamel1-2, M. A. Castañeda-Vazquez3 and H. Castañeda-Vazquez3

1 Laboratory of Molecular Epidemiology, Faculty of Veterinary Medicine, Cairo University, Cairo, Egypt
2 Faculty of Veterinary Medicine, Freie Universität Berlin, Berlin, Germany
3 Laboratory of Mastitis and Molecular Diagnostic, Department of Veterinary Medicine, Division of Veterinary Sciences, University of Guadalajara, Guadalajara, Mexico

**Impacts**

- Tuberculosis is a serious re-emerging disease worldwide. The problem of antibiotic resistance is becoming increasingly common. The rate of childhood TB increases annually.
- *Mycobacterium bovis* is responsible for great economic losses and represents a severe threat to public health.
- The purpose of this review was to highlight the seriousness of *M. bovis* for both humans and animals, and to focus on the importance of molecular epidemiological studies of *M. bovis* and the recent tools used in such studies.

**Keywords:**
Molecular Epidemiology; *Mycobacterium bovis*; spoligotyping; tuberculosis

**Correspondence:**
A. El-Sayed. LME, Faculty of Veterinary Medicine, Giza Square, 11451 Giza, Egypt.
Tel.: +201097770926; Fax: +2035725240; E-mail: aaelsayed@cu.edu.eg

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**Summary**

Bovine tuberculosis (bTB), caused by *Mycobacterium bovis* (*M. bovis*), is a serious re-emerging disease in both animals and humans. The evolution of the Multi- and Extensively drug-resistant *M. bovis* strains (MDR-TB and XDR-TB) represents a global threat to public health. Worldwide, the disease is responsible for great economic losses in the veterinary field, serious threat to the ecosystem, and about 3.1% of human TB cases, up to 16% in Tanzania. Only thorough investigation to understand the pathogen’s epidemiology can help in controlling the disease and minimizing its threat. For this purpose, various tools have been developed for use in advanced molecular epidemiological studies of bTB, either alone or in combination with standard conventional epidemiological approaches. These techniques enable the analysis of the intra- and inter-species transmission dynamics of bTB. The delivered data can reveal detailed insights into the source of infection, correlations among human and bovine isolates, strain diversity and evolution, spread, geographical localization, host preference, tracing of certain virulence factors such as antibiotic resistance genes, and finally the risk factors for the maintenance and spread of *M. bovis*. They also allow for the determination of epidemic and endemic strains. This, in turn, has a significant diagnostic impact and helps in vaccine development for bTB eradication programs. The present review discusses many topics including the aetiology, epidemiology and importance of *M. bovis*, the prevalence of bTB in humans and animals in various countries, the molecular epidemiology of *M. bovis*, and finally applied molecular epidemiological techniques.

**Introduction**

Although many researchers use the expression ‘molecular epidemiology’, its actual meaning is sometimes unclear. The most accepted definitions of molecular epidemiology in simple terms are the use of molecular techniques (tools) to define disease and its pre-clinical state by identifying the presence of susceptible genes (Shpilberg et al., 1997), or the science that utilizes molecular biology to define the distribution of disease in a population (descriptive epidemiology) and relies heavily on integration of traditional (or analytical) epidemiological approaches to identify the
aetiological determinants of this distribution (Snow, 2011). It can also be defined as the combination of standard classical epidemiological approaches with molecular techniques to track specific strains of pathogens in order to understand the distribution of disease in populations (Skuce and Neill, 2001).

Molecular epidemiological studies aim in general to provide full characterization of infectious agents in order to determine the physical sources, route of transmission, virulence genes, vaccine-relevant antigens and drug resistance (Levin et al., 1999). In practice, in TB specialized laboratories, molecular epidemiology helps to determine the source of infection and outbreaks, recognize the relationship between different outbreaks and detect wild animal reservoirs of M. bovis. In addition, they can provide insight into the risk factors for bTB transmission by allowing the identification of the disease’s dynamics (Garnier et al., 2003; Zumarraga et al., 2005; Ramos et al., 2014). This can be achieved through the application of various advanced molecular-based techniques such as multilocus VNTR analysis (MLVA), Spacer Oligonucleotide typing (Spoligotyping) and Restriction Fragment Length Polymorphism (RFLP) analysis in strain typing. These techniques provide important epidemiological information about isolates of M. bovis that may help in the control of bovine TB by indicating possible links between diseased animals, detecting and sampling outbreaks, and even demonstrating cases of laboratory cross-contamination between samples, as reported by Méndez et al. (2006), who solved the problem of laboratory cross-contamination by spoligotyping. In addition, these techniques can provide clear data about the identity of single epidemic strains and distinguish them from epidemiologically unrelated isolates. Additional data about the role and distribution of certain incriminated genotypes can only be obtained through the genetic characterization of pathogens isolated from outbreaks. Genotyping is increasingly becoming a standard tool for disease control and eradication programs, as it provides significant insight into the sources of infection, identification of ecosystem, transmission routes between livestock and wildlife or humans, and transmission routes of bTB within migrating livestock (Medeiros Ldos et al., 2010; Schiller et al., 2010; Ramos et al., 2014).

Aetiology, Epidemiology and Zoonotic Importance

*Mycobacterium bovis* is one of the members of the *Mycobacterium tuberculosis* complex (MTBC) and is the causative agent of bovine tuberculosis (bTB) (Smith et al., 2006; Palmer, 2007; Hershberg et al., 2008; Wirth et al., 2008; Miller and Sweeney, 2013; Kamel et al., 2014). To date, the known members of the *Mycobacterium tuberculosis* complex consist of: *M. tuberculosis*, *M. canetti*, *M. africanum* subtypes I and II, *M. bovis*, *M. caprae*, *M. microti*, *M. pinnipedii* and the attenuated *M. bovis* BCG (Bacillus Calmette-Guérin) vaccine strain (OIE, 2014), in addition to *M. oryges* (van Ingen et al., 2012), *M. mungi* (Alexander et al., 2010), *Dassie bacillus* (Parsons et al., 2008) and *Chimpanzee bacillus* (Coscolla et al., 2013). All members of the MTBC exhibit a 99.9% sequence similarity and their 16S rRNA are also conserved, with the exception of *M. canetti* (Dos Vultos et al., 2008) and are capable of causing a serious disease with similar pathology (Rodriguez-Campos et al., 2014). Like other mycobacteria, *M. bovis* is very robust in the environment and can survive under extreme environmental conditions (Courtenay et al., 2006; Fine et al., 2011). It can be isolated for about 6 weeks from carcasses and 4 weeks from faecal matter, depending on weather conditions, but its survival can be prolonged up to 58 days in contaminated water, up to 2 years in covered dungs and up to 5 months under diffuse sunlight conditions. *Mycobacterium bovis* bacilli in game parks can also be spread by rains to contaminate grazing areas, making cattle more susceptible to infection from wildlife. However, the amount required for indirect transmission of the disease is clearly higher than that needed for direct or aerosol transmission (Cosivi et al., 1995; Tanner and Michel, 1999; Sweeney et al., 2007; Fine et al., 2011). Once bTB is established in a herd, it spreads via aerosols, suckling, direct contact between animals and sharing of water and feed. The bTB infection in maintenance hosts can persist through horizontal transfer in the absence of any other source of *M. bovis* and can be transmitted to other susceptible hosts (Biet et al., 2005).

Although *M. bovis* is the main causative agent of bovine tuberculosis, it has the broadest host range among all MTBC members. Although it was observed in 1898 that the mycobacteria isolated from humans and cows vary in their infectious power in different animal species, the two species were first separated in 1970 (Karlson and Lessel, 1970). *Mycobacterium bovis* can infect farm animals such as cattle, farmed buffalo, goats and farmed deer. While goats are considered reservoir hosts of *M. bovis* (Kamel et al., 2014), other animal species such as pigs, cats, dogs, horses and sheep are considered spillover hosts. In addition, bovine tuberculosis has been reported worldwide in wild ruminants, with very similar symptoms to those of domestic ruminants (Biet et al., 2005). At present, cases of *M. bovis* infections have been reported in more than 40 free-ranging wild animal species, such as equines, camels, wild boars, deer, antelopes, foxes, mink, ferrets, rats, primates, llamas, kudus, elands, tapirs, elks, elephants, sitatungas, oryxes, addaxes, rhinoceroses, possums, ground squirrels, otters, seals, hares, moles, raccoons, coyotes and several predatory felines including lions, tigers, leopards and lynx (OIE, 2014). Other wildlife species are major reservoirs of *M. bovis* infection in different countries including...
White-tailed deer (USA), wild boar (Spain), brush tail possums (New Zealand), African buffalo (South Africa), in addition to badgers (Sweeney et al., 2007; Fitzgerald and Kaneene, 2012; Hardstaff et al., 2014; Pesciaroli et al., 2014).

The pathogenesis of bovine tuberculosis is not as well understood as that of human tuberculosis. Advances in the field of human tuberculosis have been made using several small animal models of M. tuberculosis infection. Extrapolations of findings from small animal models have been applied to bovine tuberculosis, but these extrapolations may not always be relevant as there are clear differences in host susceptibility, host immune response, environment, host ecology and behaviour (Palmer and Waters, 2006). Despite significant variations in size, appearance and distribution of the tuberculous lesions in different species, the majority of lesions in affected wildlife species closely resemble those of domestic cattle (Zanella et al., 2008; Drewe et al., 2009). The severity of the disease and the nature of the clinical symptoms depend on the species susceptibility, invading genotype and on the prevalence of bTB (Waters et al., 2014). It is even believed that some breeds of cattle and deer could be resistant to M. bovis infections (Delahay et al., 2001; Michel, 2002; Brotherstone et al., 2010; Rodríguez Campos, 2013; Bermingham et al., 2014).

The aerosol transmission of M. bovis is the most frequent form of transmission between herd members, and spillover to other species occurs via different means of transmission. Although humans, dogs and cats can contract the infection through direct contact with infected herds, they are more susceptible to the digestive form of the disease. Drinking infected milk, feeding on infected carcasses or even coming into contact with pus secreted from draining fistulae of lymph nodes can be the means of disease transmission (Michel et al., 2006, 2007). However, it was suggested that dogs are more likely to be infected with M. tuberculosis following exposure to infected humans (Birn et al., 1965; Greene and Gunn-Moore, 2012; Macpherson, 2012).

Not only is M. bovis the principal cause of re-emerging TB in free-living or captive wildlife, domestic livestock and non-human primates, but it also has zoonotic potential to cause human infections that are indistinguishable from those produced by the human type (M. tuberculosis). Therefore, identification of the TB causative agent at the subspecies level is important for public health. Mycobacterium bovis strains are, in contrast to M. tuberculosis, not transmitted effectively between humans (Runyon, 1959; CDC, 2011; Gonzalo-Asensio et al., 2014) and are usually associated with extrapulmonary TB in humans (Malama et al., 2014; Han et al., 2015). However, the isolation of M. bovis from human sputum in some reports may indicate its potential for human-to-human infection, especially in closed populations such as prisons (Romero et al., 2006; Gumi et al., 2012). The human-to-human transmission can be differentiated from animal-to-human transmission by the application of fingerprinting tools (Pérez-Lago et al., 2014).

For a long time, due to the close relationship between Mycobacterium bovis and mycobacterium tuberculosis, it was believed that M. bovis was the evolutionary precursor to M. tuberculosis. Then, recent molecular epidemiological studies suggested that M. bovis evolved in parallel with M. tuberculosis, possibly through the independent evolution of both types from another precursor mycobacteria likely related to M. canetti (Smith, 2003). However, the M. bovis genome was sequenced in 2003, and its size was clearly found to be smaller than that of M. tuberculosis, which indicates that its evolution from an M. tuberculosis-like common ancestor most likely originated from an RD9 deleted strain such as M. africanum subtype I, in contrast to M. tuberculosis, which is more closely related to M. africanum subtype II (Mostowy et al., 2004, 2005; Huard et al., 2006).

**Epidemiological Prevalence of Bovine Tuberculosis**

The exact prevalence of M. bovis infections in humans is unknown and underdiagnosed, especially in various African countries (Cosivi et al., 1998). It is commonly assumed that M. bovis is less virulent and has less transmission potential among human populations than M. tuberculosis. This could be attributed to the presence of three mutations affecting the virulence regulation system PhoPR in M. bovis and the closely related M. africanum lineage 6 (Gonzalo-Asensio et al., 2014). Nevertheless, M. bovis remains a serious opportunistic infection in HIV-infected persons. Therefore, it is important to public health policy makers to be able to differentiate human infections caused by M. bovis from those due to M. tuberculosis (Cosivi et al., 1998; Wedlock et al., 2002). The free movement of cattle within and between countries and even continents certainly facilitated the worldwide distribution of bovine tuberculosis and the spread of newly evolved antibiotic-resistant strains (Sechi et al., 2001). For this reason, great efforts are being made to understand the population structure of M. bovis through the use of recent molecular techniques that allow for the identification of the dominant M. bovis strain in larger geographic locations and for the tracing of the source of introduction (Kamerbeek et al., 1997; Lindstedt, 2005; Müller et al., 2009). The re-emergence of bovine tuberculosis among animals and humans is a serious worldwide concern, especially in developing countries. Mycobacterium bovis infections in cattle herds were detected in 109 countries worldwide between 2005 and 2010 (Ramos et al., 2014). People living in close contact with infected cattle or
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children who drink unpasteurized milk are at risk of infection. On the other hand, tuberculosis caused by M. tuberculosis was also reported in animals living in close contact with humans (Cadmus et al., 2006; Srivastava et al., 2008). It was estimated that about 3.1% of human TB cases worldwide are caused by M. bovis. While <1% of human infections are caused by M. bovis in Spain, the prevalence in Africa ranges from 3.9% in Nigeria, up to 7% in Uganda, and even 16% in Tanzania (Romero et al., 2006; Malama et al., 2014). It was reported that in some developing countries, M. bovis is responsible for 5–10% of all human TB cases and 30% of all TB cases in children (Wedlock et al., 2002). Mycobacterium bovis could be isolated from approximately 3.9–5% of human tuberculosis patients in Nigeria (Idigbe et al., 1986; Cadmus et al., 2006; Mawak et al., 2006) and 0.6–1.85% of patients with pulmonary tuberculosis in Burkina Faso. The pathogen was also detected in 26.5% of unpasteurized milk samples (Sanou et al., 2014). In Zambia, the spoligotyping of 49 M. bovis strains with bovine and human origin revealed a high homogeneity among the existing genotypes and even identical MIRU-VNTR genetic profiles. The same genetic profile characterized by the absence of spacers 3, 9, 16 and 39–43, SB 0120/ST 482 was reported in tubercular cattle in Algeria, Brazil, France and Zambia, and in human patients from Italy and Germany. This profile differs from that observed in East African and Southern African countries (Malama et al., 2014); however, similar observations were reported in Ethiopia and in Spain, where M. bovis isolates from human and cattle shared the same genetic profile even in multidrug-resistant M. bovis (MDR) strains. Nevertheless, some spacers and certain MIRU-VNTR, which are not used in the current spoligotyping system, could differentiate between M. bovis of both human and cattle origin. They differ in a copy at ETR-A and at QUB-3232 (Romero et al., 2006; Gumi et al., 2012).

Of 55 African and 36 Asian countries, only seven African and seven Asian countries apply adequate disease control measures; the remaining 77 countries control the disease inadequately or not at all. About 94% of cattle and 99% of buffalo populations in Asia are either partly controlled for bTB or not controlled at all. Therefore, about 85% of African cattle and 82% of the human population in Africa, and 94% of the human population in Asia live at high bTB risk (Cosivi et al., 1998). In Latin America, about 24% of cattle and 60% of the human population live in areas where there is no control or only limited control for bovine TB (Cosivi et al., 1995; Muller et al., 2013). This is thought to be a result of wildlife reservoirs that spread the infection to farmed animals, especially cattle. Such reservoirs make eradication more complicated and extremely difficult, and necessitate special programs to control the disease (Cosivi et al., 1998; Renwick et al., 2007; More et al., 2015).

Molecular epidemiological tools are now applied to provide novel information about the spread of the disease and to analyse its transmission dynamics (Small and Van Embden, 1994; Savine et al., 2002). In Africa, epidemiological studies of TB are difficult to perform due to cost and the lack of information and trained teams. However, some primary data are available from certain African countries. In Nigeria and Cameroon, the spoligotyping profiles of some isolated M. bovis strains offer some information about their distribution among human and animal populations in West Africa (Müller et al., 2009). Molecular epidemiological typing of the isolates based on spoligotyping, RDAf1 deletion and MIRU-VNTR typing was performed and resulted in detection of 12 spoligotypes, and the obtained data grouped the 33 investigated M. bovis isolates into seven clusters, including isolates exclusively from cattle (5) or humans (1) or from both (1). These isolates were shown to belong mainly to the African 1 (Af1) clonal complex (81.8%) and, to a lesser extent, to the putative African 5 (Af5) clonal complex (18.2%) according to (Sanou et al., 2014). With the aid of spoligotyping, M. bovis strains of bovine origin isolated from Mali could be divided into two groups. The first group is marked by the distinct loss of spacer 30 and accounts for 65% of the strains detected and resembles strains detected in Chad, Cameroon and Nigeria. This spoligotype is closely related to the BCG-like type which dominates in France, suggesting that M. bovis could have been imported to this region during the French colonial period, with 12/13 isolates lacking spacer 6 in addition to spacer 30. The second group (6/7 isolates), lacking spacers 4 and 5, resembles spoligotype patterns previously identified in France, Spain and North Africa (Muller et al., 2008). However, a strong relationship between North African (mainly Algerian) and French isolates was expected due to the importation of live animals from Europe to Algeria during the French colonial period (1830–1962) and later (Sahraoui et al., 2009). In West Africa, African 1 (Af1) M. bovis clone spoligotype pattern SB0944 was found to dominate only in Chad, Nigeria, Mali and Cameroon, indicating a separate ancestor for this clone in the aforementioned countries. They are closely related and characterized by specific chromosomal deletion (RDAf1) and the absence of spacer 30 in the spoligotype patterns. On the other hand, a different clone (African 2; Af2) was detected only in East Africa (Ethiopia, Burundi, Uganda and Tanzania), which might be less virulent than Af1 due to the entire lack of mce2 operon as a consequence of the RDAf2 deletion in Af2 clone, in addition to the mce3 operon which is already absent in all M. bovis strains. However, the Af2 strains are capable of inducing typical tubercle lesions in cattle. This clone is defined by a specific chromosomal deletion (RDAf2) with the absence of spacers 3 to 7 in their patterns. It is possible that all Af2 spoligotype
patterns were derived from an ancestral spoligotype pattern equivalent to that of the vaccine strain BCG (SB0120, missing spacers 3, 9, 16 and 39 to 43), with the additional deletion of spacers 4 to 7 (SB0133). While the strains of the Af2 clonal complex were detected only in cattle reared in East Africa, the Af1 clonal complex is reported only in cattle from West-Central Africa (Berg et al., 2011). In Mozambique and South Africa, non-Af1–non-Af2 clones are reported, most likely the European clone (European 1; Eu1) characterized by a specific deletion called RDEu1 and the loss of spoligotype spacer 11 (spacers 4 to 7 are usually present) according to (Berg et al., 2011). The Eu1 clone is rarely reported in Africa with the exception of South Africa. Nearly 99% of bTB found in England and Ireland belong to the Eu1 characterized by the deletion of chromosomal region RDEu1 and absence of spacer 11 in their pattern. This clone also dominates in locations of former trading partners and English speaking colonies of the UK. This may be attributed to the massive export of Hereford beef cattle in the 18th century from the UK to its former colonies, which would be the vehicle for the spread of this clone. However, the Eu1 is present in 14% of French, Portuguese and Spanish isolates belonging to this profile and is rare in other European countries. Meanwhile, with the exception of Brazil, the Eu1 clonal complex dominates in both North and South America, especially in Argentina, Chile, Ecuador, Mexico and North America. The Eu1 clone was even reported in Korea and Kazakhstan but not in Africa except South Africa (Ameni et al., 2010; Berg et al., 2011; Mwakapuja et al., 2013; Rodríguez Campos, 2013).

The use of modern molecular epidemiological tools enabled the identification of three clonal lineages of M. bovis, namely African 1, African 2 and European 1. Members of each group have a distinct spoligotype signature and are characterized by a unique deletion present in each member of the complex. The African 1 and African 2 groups are restricted to central-west Africa and eEast Africa, respectively, and were never detected in cattle outside of these regions, while the European 1 members are globally distributed. It is thought that this global distribution resulted from the exportation of modern cattle breeds, such as Herefords bred in the UK in the 18th Century, to the former British colonies. The cows acted as a vehicle for the global spread of this closely related group of strains (Smith, 2012).

**Molecular Epidemiology of Mycobacterium bovis**

**Overview of the most commonly used molecular epidemiological techniques**

Although different molecular epidemiological studies have been performed to enable better traceability of M. bovis infections and to detect the source of outbreaks (Haddad et al., 2001), there are still several problems in the development of effective and standardized genotyping systems of M. bovis to create widely accepted standard genotyping protocols (Durr et al., 2000b). Thus, different techniques have been developed for the genotyping of M. bovis, including whole genome techniques (e.g. restriction endonuclease analysis [REA]), the restriction fragment length polymorphism /RFLP, pulsed-field gel electrophoresis/PFGE, whole genome sequencing and whole genome microarray) and the partial genome techniques such as specific repeated sequences (e.g. tandem-repeated sequences and non-tandem repeated sequences); random sequences (e.g. random amplified polymorphic deoxyribonucleic acid /RAPD analysis); house-keeping genes (e.g. multilocus sequence typing /MLST); regions of difference (direct repeats /DR typing) and single nucleotide polymorphism (SNP typing). The Tandem Repeated Sequences include the VNTR typing and IS6110 amplification while the Non-tandem-repeated sequences include the IS6110-RFLP, IS1081-RFLP, PGRS-RFLP, DR-RFLP and the spacer oligonucleotide typing (Spoliogotyping) (Kremer et al., 1999; Durr et al., 2000a,b; Gormley et al., 2014; Jagielski et al., 2014).

RAPD, MTPR and PGRS techniques

Not all of these techniques are in use for various reasons, mainly the lack of reproducibility, high cost and complexity. Among the first PCR-based genotyping techniques was the Random Amplified Polymorphic Deoxyribonucleic Acid Analysis (RAPD). This technique is rarely used now due to its lack of reproducibility and poor discrimination (Jagielski et al., 2014). Similarly, there is Ampliprinting of the IS6110 targeting the Major Polymorphic Tandem Repeat (MPTR). The MPTR region resembles the DR region and consists of 10-bp direct repeats separated by 5-bp spacers. The MPTR also lacks reproducibility and therefore is not used as a routine genotyping method (Kremer et al., 1999; Collins, 2011). Because other regions in the genome of Mycobacteria contain up to 80% GC nucleotides, these Polymorphic GC Rich Sequences (PGRS) can be used for molecular typing; however, they achieve only high discriminatory power in strains with a high copy number of IS6110. For this reason, IS6110-RFLP became the gold standard for both human and bovine tuberculosis over many years. However, its use in bovine tuberculosis is less common due to the lower copy number of IS6110 in M. bovis (Collins, 2011). Although Single Nucleotide Polymorphism (SNP) typing has a clear discriminatory power, it is not commonly used because it is a two-step technique (PCR then sequencing, or PCR then restriction enzyme), which makes it expensive and time-consuming (Abadia et al., 2010; Schurch et al., 2011; Rodríguez Campos, 2013; Jagielski et al., 2014).
Restriction fragment length polymorphism

The typing of TB isolates using IS6110 restriction fragment length polymorphism (RFLP) analysis was first described in 1991. It quickly became the standard tool for molecular epidemiological investigations (van Soolingen et al., 1991) and is even considered to be the current ‘gold standard’ and the most widely applied typing method of the M. tuberculosis complex (van Soolingen et al., 1991). The technique detects the variation in both the number and genomic position of IS6110 and by so doing, it generates reproducible strain-specific patterns (Hayward and Watson, 1998). However, the use of molecular typing of M. bovis in England based on RFLP indicated that most strains in the UK contain only one copy of IS6110, which limits the usefulness of this method (Smith et al., 2006). Further, it has a low discriminatory power, especially for strains with a low number of IS6110 copies (Kremer et al., 1999; Serraino et al., 1999).

Among all RFLP techniques for M. bovis typing, PGRS-RFLP has the best discriminatory power. However, in contrast to all RFLP techniques, which require DNA extraction, large amounts of DNA and high technicality, and which have problems with their reproducibility and are time consuming, spoligotyping is not only rapid, simple and reproducible but can also be carried out on cell lysates or clinical specimens (Dvorska et al., 2001; Haddad et al., 2001; Jagielski et al., 2014).

Spoligotyping

The introduction of ‘spacer oligonucleotide’ or ‘spoligotyping’ for molecular typing of MTBC started in 1997 (Kamerbeek et al., 1997). It is a PCR-based method like other techniques, but it avoids the technical difficulties facing IS6110 RFLP typing. At the beginning, spoligotyping showed limited value for epidemiological transmission when used alone, so it was combined with IS6110 RFLP analysis (Kremer et al., 1999). Spoligotyping is now increasingly used in molecular epidemiology of MTC as well as in species and strain differentiation. This technique depends on the genetic differences between strains due to loss of spacers at the direct repeat (DR) region in MTC (Kamerbeek et al., 1997). Spoligotyping is more discriminating than IS6110 RFLP, but equally as discriminating as DR-RFLP, which identifies genome polymorphism in the DR region. Nevertheless, spoligotyping remains the preferred technique as it is easy to do and to interpret (Durr et al., 2000b).

As the introduction of any mutation (e.g. deletion) in a strain will be inherited by all of its descendants, it can be used to trace back clonal complexes. On these bases, spoligotyping was developed. Spoligotyping is a simple and rapid tool for molecular typing of M. bovis that does not require purified DNA. Spoligotyping is a mixture of PCR and hybridization techniques. In 1993, Clustered Regularly Interspaced Palindromic Repeats (CRISPRs) were discovered in the MTBC. This direct repeat (DR) region consists of multiple 36-bp direct repeats separated by spacers ranging in length from 25 to 41 bp. Meanwhile, a direct variant repeat (DVR) consists of a single direct repeat (DR) and its adjacent spacer. The typing of the strains depends on the presence or absence of the different spacers. The use of spoligotyping depends on the detection of 43 of 104 identified spacers. For spoligotyping, the DRs are targeted by PCR oligonucleotides to be amplified and blotted to detect the present spacers. The data can be digitalized and stored as binary code, and every spoligotype pattern becomes a unique identifier when submitted to the international bank of spoligotype patterns at www.Mbovis.org. The code used includes the prefix SB followed by one to four digits in which 0 and 1 stand for the absence or presence of spacers, respectively. This enables the comparison and tracing of the evolutionary relationship among strains, as the spacers can only be lost but cannot be regained. The spacers are frequently lost independently in different lineages (homoplasies) (Mueller et al., 2008; Sahraoui et al., 2009; Berg et al., 2011; Rodriguez Campos, 2013).

Spoligotyping is considered to be the most common epidemiological molecular-typing tool for M. bovis. The application of spoligotyping for the spatial (geotyping) and temporal (chronotyping) traceability of outbreaks is practical and helpful (Haddad et al., 2001). This depends on the identification of the polymorphisms in the spacer units of the direct repeat (DR) region of the chromosome. This region consists of multiple, virtually identical direct variant repeat (DVR) units of 36-bp sequences interspersed with DNA spacers of a similar size. The DR region may contain more than 60 DVR units. Nevertheless, 43 of these units were chosen from the genome of the M. tuberculosis strain (H37RV) and the M. bovis BCG strain (P3) to be used as standard for spoligotyping of all members of M. tuberculosis complex. However, the amplification of some of the spacers (14, 15, 18, 39 and 40) was found to be problematic due to sequence mutations (Abadia et al., 2010; Sahraoui et al., 2009; Berg et al., 2011; Rodriguez Campos, 2013).

Each spoligotype pattern has a given identifier and an international name (e.g. ‘type 9’ in UK and SB0140, respectively) according to (Smith et al., 2006). The technique delivers digital data which can be easily analysed and shared between laboratories with a high distinguishing power between the MTBC members. It can also define M. tuberculosis clades and families in phylogenetic studies (Brudey et al., 2006). For the detection of the present spacers, Reverse Line Blotting step (RLB) is required. The blotting of spoligotyping can be performed on a hybridization
membrane or a commercial spoligotyping membrane (Luminex platform). The latter was found to be faster and easier and to increase the reproducibility (Jeffries et al., 2009).

A major disadvantage of spoligotyping is its inability to deal with the mixed infection as the delivered data will be from the present spacers of all existing strains. The VNTR, in contrast, can be applied even if a mixed infection is suspected, as this will be visualized as a double band. In addition, and opposite to spoligotyping, variable number tandem repeats typing (VNTR) also known as multilocus variable number tandem repeat typing (MLVA) targets polymorphic loci of tandem repeats distributed all over the genome (Rodríguez Campos, 2013).

Different Spoligotypes Reported from Different Countries

The comparison of genetic profiles between animal and human isolates of M. bovis enables us to detect the source(s) of infection and the route(s) of transmission, and to predict the future picture of disease progression. It is worth mentioning that the discriminatory power of the different typing techniques available for M. bovis varies by country, and M. bovis genotypes of human origin parallel those of livestock within the same country, which supports the presence of an epidemiological link between human and animal M. bovis TB cases (Lari et al., 2011). When comparing both human and bovine isolates from Tuscany, Italy, the isolates with the spoligotype profile ST482/SB0120 dominated in both species. They were detected in 63.8% and 54.6% of human isolates and infected herds, respectively (Bonìotti et al., 2009; Lari et al., 2011). The same spoligotype dominates among bovine isolates from France, Germany and Spain (Aranaz et al., 1996; Haddad et al., 2001; Kubica et al., 2003), but never in the United Kingdom or Ireland. Other profiles were also reported in M. bovis isolates from humans in Italy including the ST482/SB0120 spoligotype, which was also detected in 20% of German patients and 1% of British patients, the profile ST665/SB0134, present in 5.8% of bovine isolates, the profile SB0934 in 1.3% of bovine isolates and finally the less significant profiles SB0927, SB0950 and SB0867, which were present in <1% of the herds (Aranaz et al., 1996; Kubica et al., 2003; Lari et al., 2011).

The total number of spoligotypes in France is very high, indicating more strain heterogeneity when compared to other regions such as the UK, Australia, Cameroon and Tanzania, and leading to the low prevalence of the dominant types among the M. bovis population. In France, the most common spoligotype is the BCG-like group, which resembles the vaccine strain M. bovis BCG isolated in France in the 19th century. However, the prevalence of the two main spoligotypes (BCG-like and GB54) in France is low compared to the prevalence of the two major spoligotypes present in the UK (70% of isolates), Ireland (52% of isolates), Italy (66.6%), Spain (46%) and Australia, Canada and Iran (88%) (Haddad et al., 2001).

It is remarkable that the spoligotypes in France differ from those detected in the UK; the predominant spoligotypes in the UK were detected at a very low percentage in France and vice versa. On the other hand, the strains that dominate in the UK also dominate in places traditionally linked to the UK, such as commonwealth countries and South American countries. It is also unusual that the BCG-like type which dominates in France has never been reported in the UK, but is frequently detected in other European countries such as Belgium, Italy and Spain, and also in North Africa (Haddad et al., 2001; Smith, 2012; Lamine-Khemiri et al., 2014).

In France, the dominance of the BCG-like spoligotype over decades indicates the high stability of the DR region compared to other markers like IS6110. Meanwhile, the detection of more variants of spoligotypes in the BCG-like group along with the continuous decrease of the prevalence of BCG isolates indicates the progressive evolution of the spoligotypes (Haddad et al., 2001).

Mycobacterial Interspersed Repetitive Unit-Variable Number of Tandem Repeats

Nowadays, most laboratories depend on PCR-based techniques such as multilocus variable analysis (MLVA) for M. bovis typing as they are faster, easier, reproducible and cost-effective (Romero et al., 2006). MLVA analysis, also referred to as mycobacterial interspersed repetitive unit–variable number of tandem repeats (MIRU-VNTR) (Allix et al., 2006) depends on strain variation indicated by allelic diversity at these multiple loci. The use of MLVA analysis for M. bovis transmission research helps in delivering highly informative data when combined with traditional epidemiology (Allix et al., 2006). To increase the discriminatory power of spoligotyping, VNTR typing is used for further subtyping of strain groups initially identified by spoligotyping, as VNTR typing enables greater discrimination than spoligotyping (Muller et al., 2008; Sahraoui et al., 2009). Moreover, spoligotyping allows for species differentiation based on the absence of certain spacers in certain MTB members, for example the absence of spacer number 3, 9, 16 and 39–43 in M. bovis and spacers 1, 3–16, 28 and 39–43 in M. caprae (Dvorska et al., 2001; Rodriguez Campos, 2013).

In general, the most commonly accepted molecular epidemiological tool is variable numbers of tandem repeats (VNTR) typing. It is easy and cheap PCR-based typing that delivers reproducible and accurate discrimination among MTBC isolates. It includes typing of different genomic loci.
such as exact tandem repeats (ETR) (Frothingham and Meeker-O’Connell, 1998), mycobacterial interspersed repetitive units (MIRUs) (Supply et al., 2000; Mazars et al., 2001) and Queen’s University Belfast (QUB) (Skuce et al., 2002). Variable nucleotide tandem repeat (VNTR) typing detects the variation in the numbers of different repeat sets dispersed throughout the genome and is similar to the concept of mini-satellite typing. The loci used are already well characterized. However, no sharp demarcation exists among these classifications, that is some genomic regions are both ETR and MIRU at the same time (Smith et al., 2006). Nonetheless, there are many differences between MIRUs and other VNTRs, like the presence of some regulatory elements in the MIRUs as start and stop codons or consensus sequences, and the fact that MIRUs usually overlap the start and stop codons of the flanking genes (Supply et al., 2000; Romano et al., 2005).

Queen’s University in Belfast (QUB) and exact tandem repeat (ETR) are other sets of VNTR sequence repeats. They consist of many copies of 56- to 60-bp long units and show high reproducibility. However, their major disadvantage is their low discrimination power for strains with a high copy number of IS6110 in comparison with those with a low copy number. In general, VNTR analysis allows for the discrimination of unrelated strains of M. bovis better than spoligotyping (Hilty et al., 2005; Skuce et al., 2005).

Six tandem repeats called exact tandem repeats (ETR) were reported, namely A, B, C, D, E and F. They vary in size from 53 to 79 bp. The first five (A to E) were found to offer enough discrimination in the MIRUs as start and stop codons or consensus sequences, and the fact that MIRUs usually overlap the start and stop codons of the flanking genes (Supply et al., 2000; Romano et al., 2005).

In 2009, this protocol became the new global standard panel for typing (Goyal et al., 1994; HPA, 2012; Rodríguez Campos, 2013). Moreover, this panel system was also found to be useful for the typing of non-M. tuberculosis members of the MTBC (Stone et al., 2012). Different researchers reported that the application of QUB3232, ETR-A, ETR-B, QUB11a, QUB11b and QUB 26 compared to MIRU4, 10, 16, 26 and 31 for M. caprae resulted in the greatest discrimination among different M. bovis isolates (Dvorska et al., 2001; Lari et al., 2011).

Thus far, none of the known typing techniques can be applied on its own. Each tool has both advantages and disadvantages. However, the use of spoligotyping in combination with other MIRU-VNTR improves discriminatory power among the strains (Ramos et al., 2014).

**Novel DNA chip**

In 2014, a new technique was developed by Srilohasin and his team that was seen to be faster and simpler than other techniques already in use. The multipurpose genotyping tool can be efficiently applied for the performance of epidemiological and evolutionary studies of Mycobacterium tuberculosis complex clinical isolates. It is very simple, flexible and quick, and allows for identification within 6 h using a thermal cycler, a hybridization oven and a DNA chip scanner. The technique, named Novel DNA Chip, is based on a Modified DigiTag2 Assay. It depends on a multiplex PCR assay, followed by sizing of the amplicons using capillary electrophoresis and automatic assignation of allelic values (Srilohasin et al., 2014).
Whole genome sequencing

The invention of new generations of sequencers enabled the commercial use of Whole Genome Sequencing (WGS) so that it became available for researchers. The technique offers the potential for extraordinary insight into pathogens. The WGS-delivered data must be corroborated with available epidemiological data in well-described systems. It will likely become the typing method of choice in the future as sequencing costs are reducing markedly (Biek et al., 2012).

Conclusion

The development of molecular tools has added new aspects to the conventional epidemiological approaches of *M. bovis* infections. The data delivered significantly strengthened the classical epidemiology, making the epidemiological data more precise. Thus, understanding the advantages and disadvantages of these epidemiological tools is essential for a more beneficial and accurate epidemiological evaluation of the disease situation. This will be reflected in our understanding of the mycobacteria transmission dynamics and pathogenesis within populations and between hosts, leading to the improvement of MTB control programs worldwide.

Conflict of Interest

The authors declare that they have no conflict of interest. This article does not contain any studies with human participants or animals performed by any of the authors.

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