Tuberculosis (TB) is an infectious disease with a relatively high death rate. About 30 percent of the world’s population is latently infected with Mycobacterium tuberculosis. The annual incidence of all forms of TB is currently about 1.7 million globally, and is increasing. TB causes more health problems than any other disease caused by a single infectious agent. The increasing global TB burden is much more serious than in previous decades. This is probably due to multiple factors including changing demographic situations, deterioration of TB control programmes, co-infection with Human Immunodeficiency Virus (HIV), medical practitioners who have insufficient knowledge of the disease, limited health provisions especially for those living in poor socioeconomic conditions, increasing immigration from countries endemic for the disease, and the emergence and spread of multi-drug resistant (MDR)-TB. The increase of drug-resistance, and especially MDR-TB, presents a serious threat to tuberculosis control. Consequently, MDR-TB has led to the development of more rapid and efficient methods of diagnosis and identification of resistance phenotypes as well as new epidemiological markers.

The standardised and most frequently used molecular fingerprinting technique for detecting M. tuberculosis complex isolates is IS6110 Restriction Fragment Length Polymorphism (RFLP). Spoligotyping was developed as a PCR-based macroarray technique for the simultaneous detection and differentiation of M. tuberculosis complex bacteria. This technique has several advantages compared with other amplification and genotyping methods. Detection and genotyping can be carried out without first culturing the bacteria, and genotyping is not only much faster than with methods using RFLPs, but can also differentiate more strains. This article reviews the technology and its applications and speculates on future developments.

Figure 1. Structure of the DR locus in the genome of M. tuberculosis H37Rv and M. bovis BCG P3. The green rectangles depict the 36 bp Direct Repeat (DR).

Figure 2. The DNA sequence of the DR region has been determined for the reference species M. tuberculosis H37Rv and M. bovis BCG P3. Ninety-four (1 - 94) variable spacers have been identified, pieces of DNA of the variable spacers have been synthesised and 43 of them have been introduced vertically onto the membrane used for traditional spoligotyping. The conserved direct repeats (DR) serve as targets for the primers and thus are the basis for amplification. These products can be added to the membrane of a species or samples containing a species of an unknown genotype and belonging to the M. tuberculosis complex after PCR has been carried out. After hybridisation and detection, the spoligo pattern can be read from the horizontal axis of the various clinical isolates belonging to the M. tuberculosis complex, such as M. tuberculosis, M. bovis subsp. caprae comb. nov., M. microti and M. canettii. Thirdly, genotyping can be performed much more quickly than with RFLP techniques, and fourthly it is a more sensitive method for strains with one or two IS6110
Background to spoligotyping

Spoligotyping based on PCR to detect TB specific DNA was first described in the mid 1990s [1]. Spoligotyping is based on DNA polymorphisms at one chromosomal locus that is characterised by the presence of a high number of conserved direct repeats, and which has been designated the Direct Repeat (DR) region. Direct repeats are 36 bp long and are interrupted by DNA spacers of 35 to 41 bp. When the DR regions of several isolates were compared it was noted that the order of spacers was nearly the same in all isolates, but that many deletions or insertions occurred in different strains. The presence or absence of 43 individual spacers can be detected using the spoligotyping method. Because spoligotyping is a PCR driven technique, only small amounts of DNA are required for analysis, so the method is particularly suitable for the analysis of slowly growing mycobacteria. It also permits the comparison of isolates which are not reculturable after prolonged storage. Typing can be absolutely essential, for instance in the case of relapses, when it is necessary to compare new isolates from patients with isolates from former episodes of the disease.

The spoligotyping method

Spoligotyping is based on a DNA polymorphism present at one particular chromosomal locus, the "Direct Repeat" (DR) region, which is uniquely present in Mycobacterium tuberculosis complex bacteria. This locus was first described and the region sequenced for Mycobacterium bovis BCG, the strain used worldwide to vaccinate against tuberculosis [2]. The DR region in M. bovis BCG consists of direct repeat sequences of 36 base pairs, which are interspersed by non-repetitive DNA spacers, each 35 to 41 base pairs in length. The number of copies of the DR sequence in M. bovis BCG was determined to be 49. In other M. tuberculosis complex strains the number of DR elements was found to vary significantly. The vast majority of the M. tuberculosis strains contain one or more IS6110 elements in the DR region [Figure 1]. In contrast to the DRs, the spacers are usually present only once in the DR region, but occasionally some are found twice, either separated by one or by several DRs and other spacers. One DR and its neighbouring non-repetitive spacer is termed "Direct Variant Repeat" (DVR). When the DR regions of several strains were compared, it was observed that the order of the spacers is about the same in all strains, but deletions and/or insertions of spacers and DRs occur [Figure 2]. With spoligotyping the presence or absence of 43 spacers of known sequence in the DR region can be detected by hybridisation of PCR-amplified spacer DNA to a set of immobilised oligonucleotides, representing each of the unique spacer DNA sequences. The first step in spoligotyping is to amplify the DR region of a given strain by PCR. The primers used are based on the sequence of the DR, and allow the amplification of the spacer(s) between the DR targets. The PCR products obtained differ in length for two reasons. Firstly the product contains several spacers and the DRs in between the primers anneal to DRs which are not next to each other. Secondly, the product itself can act as a primer and become elongated with one or more DRs. A biotin labelled reverse primer is used, so that all the reverse strands synthesised are biotin labelled. Oligonucleotides derived from the known spacers in the DR cluster are covalently linked to an activated membrane in parallel lines. PCR products are hybridised perpendicular to the oligonucleotide lines [Figure 3]. After hybridisation the membrane is incubated in streptavidin-peroxidase or streptavidin-alkaliphosphatase, which binds to the biotin label on the PCR products. Detection of hybridisation signals is performed by enhanced chemiluminescence (ECL) or enhanced chemifluorescence (ECF) [Figure 4]. The peroxidase or alkaliphosphatase present on the streptavidine catalyses a reaction resulting in the emission of light or fluorescence, which can be detected by autoradiography of the membrane or by specific imaging systems developed by Isogen Life Science [Figure 5].
Applications of spoligotyping

In contrast to traditional culturing, ZN or PCR, spoligotyping can distinguish false positives due to interchange of samples or cross-contamination in the laboratory, because different *M. tuberculosis* strains or DNA can yield different spoligotypes. Thus spoligotyping can be used for quality control of microscopy of AFB, cultures or nucleic acid amplification techniques.

Successful spoligotyping with ZN and auramine-stained slides and formalin fixed tissues means that it is possible to demonstrate the presence of *M. tuberculosis* complex bacteria rapidly in non-infected samples without culturing. In this way, it is possible to confirm the diagnosis when no cultures of the samples are available. As to be expected of a DNA amplification technique, it is much more sensitive than microscopy of formalin fixed tissues and can be used for diagnosing TB instead of traditional ZN staining [3, 4].

If no culture is available, the only way to obtain a genotype of *M. tuberculosis* complex strains is by PCR. Spoligotyping can be helpful in tracing sources of infection and obtaining indirect information about anti-tuberculosis drug susceptibility in the absence of mycobacterial cultures. Spoligotyping is especially useful in detecting fragmented DNA because the target is generally present in multiple copies and needs to have a minimal average contiguous size of only about 75 base pairs. Fragmented DNA is present in samples that have been treated with formalin or old samples e.g. Hungarian mummies from the 18th century [5] or bison from 15,000 BC [6].

**Conclusion**

Nucleic acid amplification techniques like spoligotyping have, in recent years, allowed more rapid diagnosis of infectious diseases. Different tests for rapid diagnosis of tuberculosis have been developed and applied directly on clinical samples. The only PCR method with a multiple genotyping target is spoligotyping, a method which offers high sensitivity for genotyping of *M. tuberculosis* complex in clinical laboratory samples.

**References**


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