Restriction Fragment Length Polymorphism (RFLP)—Application for Mycobacteria Typing

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INTRODUCTION

Outbreaks of infectious diseases often result from exposure to a common source of the etiologic agent. Generally, the etiologic agent involved in an outbreak of disease is derived from a single cell whose progeny are genetically identical or closely related to the source organism. In epidemiological terms, the organisms involved in the outbreak are clonally related; that is, they have a common origin. Clonally related organisms are members of the same species that share virulence factors, biochemical properties, and genomic characteristics. However, there is sufficient diversity at the species level that organisms isolated at different times and in different geographical areas may be differentiated or classified into subtypes or strains.

OVERVIEW

The process of subtyping is epidemiologically important for recognizing outbreaks of diseases, detecting the cross-transmission of nosocomial pathogens, determining the source of infection, and in some cases recognizing particularly virulent strains of organisms.

Subtyping or strain classification has been accomplished by a number of different approaches. In recent years, the development and extensive use of high-resolution molecular typing systems based on direct analysis of genomic polymorphism have greatly improved the understanding of the epidemiology of infectious diseases.[1,2] These molecular typing methods can be applied to answer a number of different questions, such as:

In an outbreak, what is the extent and mode of transmission of the epidemic clone(s)?

In long-term surveillance, what is the prevalence over time and the geographic spread of epidemic and endemic clones in the population?

A large number of molecular typing methods are available for a wide range of microorganisms and can provide a good epidemiological tool. However, the rapid diversification and incomplete comparative evaluation of some of these methods leave the microbiologist and the epidemiologist faced with a number of questions dealing with selection of appropriate typing system(s), to address a particular problem, as well as a lack of consensus about interpretation and communication of results.

Several criteria have been proposed for evaluating the performance of typing systems.[1,2] These criteria include typeability, reproducibility, stability, and discriminatory power.

- *Typeability*. Typeability refers to the proportion of isolates that can be scored in the typing system and assigned to a type.
- *Reproducibility*. Reproducibility refers to the ability of the typing system to assign the same type on repeated testing of the same strain.
- *Stability*. Stability is based on the biological features of clonally derived isolates to express constant markers over time and generations.
- *Discriminatory power*. This is a key characteristic of typing systems, as it estimates the probability that isolates sharing identical or closely related types are truly clonal and part of the same chain of transmission.

Additional comparative studies are needed to establish the relative value of systems currently used for typing microbial pathogens. Moreover, there are important variations in the performance of any given method depending on the species and on modifications of the procedure as applied by different investigators.

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SOUTHERN BLOTTING AND RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) METHODS

Technical Description

Southern blotting has been used for many years to detect and locate genomic sequences from a variety of prokaryotic and eukaryotic organisms. For gene detection, whole chromosomal DNA is digested with a restriction enzyme, and the fragments are separated by electrophoresis through an agarose gel. The separated fragments are transferred from the agarose gel to either a nitrocellulose or a nylon membrane by Southern blotting (Fig. 1). The membrane-bound nucleic acid is then hybridized to one or more labeled probes homologous to the gene to be examined. Probes can be labeled with a number of detectable moieties, including colorimetric and chemiluminescent enzymes to bus with the appropriate substrates. This classical method has been adapted to differentiate bacterial strains on the basis of the observation that the location of various restriction enzyme recognition sites within a particular genetic locus of interest can be polymorphic from strain to strain, resulting in gel bands that differ in size between different strains. Thus the name restriction fragment length polymorphism (RFLP) refers to this polymorphic nature of the locations of restriction enzyme sites within defined genetic regions. Only the genomic DNA fragments that hybridize to the probes are visible in RFLP analysis, which greatly simplifies the analysis. Different types of nucleic acid probes can be used for typing: 1) genes encoding metabolic, virulence or resistance functions; 2) multicopy elements, including insertion sequences (IS) and transposons. Insertion sequences typing technique using insertion sequences as a probe are in general very reproducible and can provide a highly discriminating typing tool. Discrimination is related to the presence of multiple copies of these elements at diverse locations in the chromosome. However, RFLP is a slow and labor-intensive technique requiring specialized equipment and expertise. Besides, careful selection and optimization of probe sequence, restriction endonucleases, electrophoresis, and hybridization conditions need to be developed for each species or pathovar to be typed. Furthermore, large-scale application of RFLP analysis requires international standardization of the technique, reagents, type strains, and nomenclature. In the field of *Mycobacterium tuberculosis* this was established by several public health reference laboratories for IS*6110* RFLP-fingerprinting, which integrates standard computer analysis of patterns and a common database, and it is now widely applied for large-scale surveillance of tuberculosis.[3]

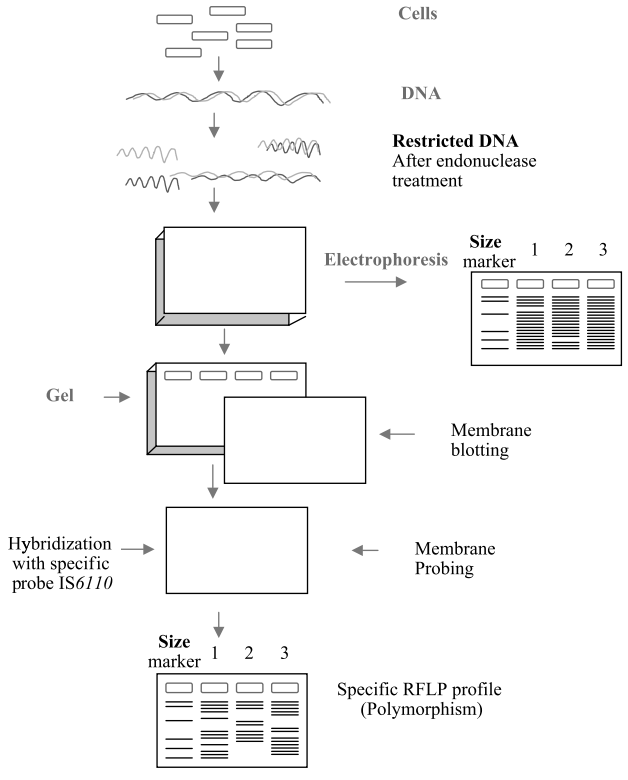

Fig. 1 Schematic representation of RFLP technique. (*View this art in color at www.dekker.com.*)

Example of Application of RFLP (*Mycobacterium tuberculosis*)

The most commonly used typing system for *M. tuberculosis* is IS*6110*-based RFLP,[4] so far considered the gold standard for TB typing. IS*6110* is an IS3-like element, also known as IS*986* or IS*987*. It occurs at various locations in the genome of *M. tuberculosis* in variable copy numbers.[5,6] As its sequence is apparently invariant,[7] it is an ideal target sequence. The copy number of the element ranges from 1 to 25 copies per genome, although rare *M. tuberculosis* cultures have been reported with no inserts. To visualize IS*6110* RFLP patterns, DNA is extracted and purified from bacterial culture. Thereafter, the DNA is digested with the restriction enzyme *Pvu*II, the restriction fragments are separated on an agarose gel and transferred to a DNA membrane. A peroxidase-labeled probe with a DNA sequence complementary to the IS*6110*-DNA sequence is added in order to visualize the IS*6110* containing restriction fragments, by chemiluminescence, which is initiated by adding two substrates. Restriction fragment length polymorphism

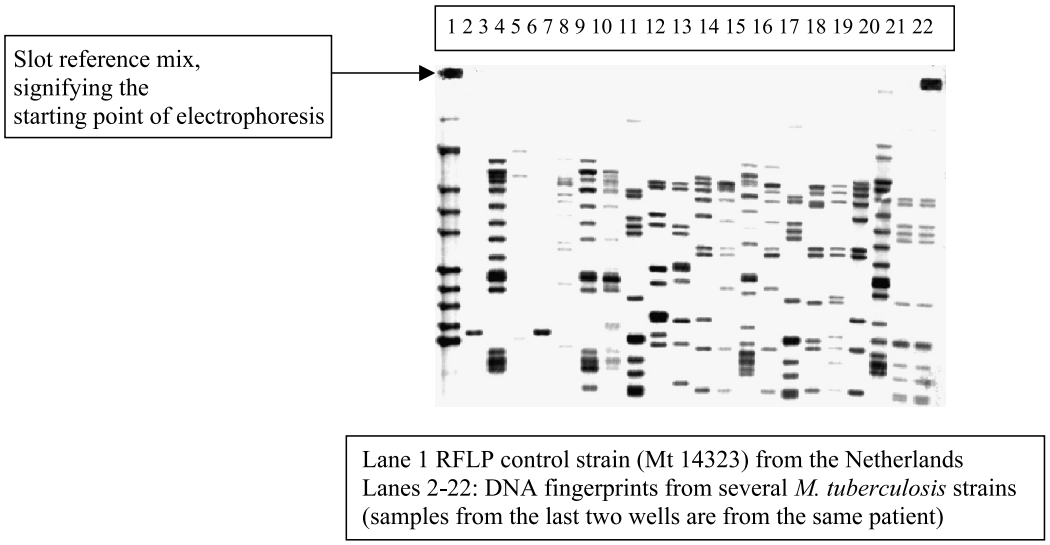

Fig. 2 Representative RFLP profiles of *M. tuberculosis* using IS*6110* as a probe.

patterns are detected by placing a light-sensitive film on the wrapped membrane in a light-blocked cassette[8] (Fig. 2). Restriction fragment length polymorphism has been used to confirm outbreaks of tuberculosis or incidents of suspected laboratory cross contamination,[9] to investigate the question of reinfection vs. reactivation, to examine the existence of multiple infections, and to track the spread of multidrug resistance.[10] DNA fingerprints of *M. tuberculosis* do not change during the development of resistance to various antituberculous drugs.[11] However, there are a number of notable limitations with standard IS*6110* typing. For example, it is labor intensive and thus costly. The typing patterns produced have varying numbers of bands which may also vary in position, so that sophisticated software is needed to analyze large numbers of patterns and to compare results between laboratories.[12] Furthermore, RFLP typing of strains with a low copy number of IS*6110* is not sufficiently discriminatory.[6,13,14] Additional genetic typing method in cases where *M. tuberculosis* isolates contain fewer than five IS*6110* copies is necessary.

In addition to IS*6110*, many other different DNA sequences that have been used as probes for typing *M. tuberculosis* complex strain by RFLP include the major polymorphic tandem repeat (MPTR), the polymorphic GC-rich sequence, the direct repeat region, $(GTG)_5$, repetitive DNA elements, and rDNA (ribotyping).[6,15,16]

Application for Other Organisms

Ribotyping is the most versatile and the most widely used strategy of Southern blot analysis of bacterial genome polymorphism. The evolutionary conservation of ribosomal RNA makes it applicable as a universal bacterial probe. Many important pathogens, including *Enterobacteriaceae*, *Listeria*, *Pseudomonas* spp., and staphylococci have more than five ribosomal operons and thus produce ribotype patterns of 5 to 15 bands.[17] Ribotyping is a robust method that exhibits excellent reproducibility and stability, during the course of the outbreaks. It is commercially available and fully automated and well standardized. However, its discriminatory power is only moderate, and this is related to the fact that ribosomal operons cover less than 0.1% of chromosomal length and tend to cluster in one particular region of the genome. Discrimination of ribotyping depends on the species and on the choice and number of restriction endonucleases used. No consensus has been achieved on optimal procedure and no general rules are available for interpretation of technically problematic results, such as weakly hybridizing fragments.

LOCUS SPECIFIC PCR-BASED RFLP

Technical Description

In recent years, a number of PCR-based strategies have been developed for strain discrimination of microbial pathogens. In PCR *gene* RFLP *typing*, a target sequence, 1 to 2 kb long and known to show polymorphism among strains of species of interest, is amplified at high stringency. The amplified product is cut with restriction endonucleases and isolates are compared by RFLP pattern on an agarose gel after staining with ethidium bromide avoiding the need for Southern blot.

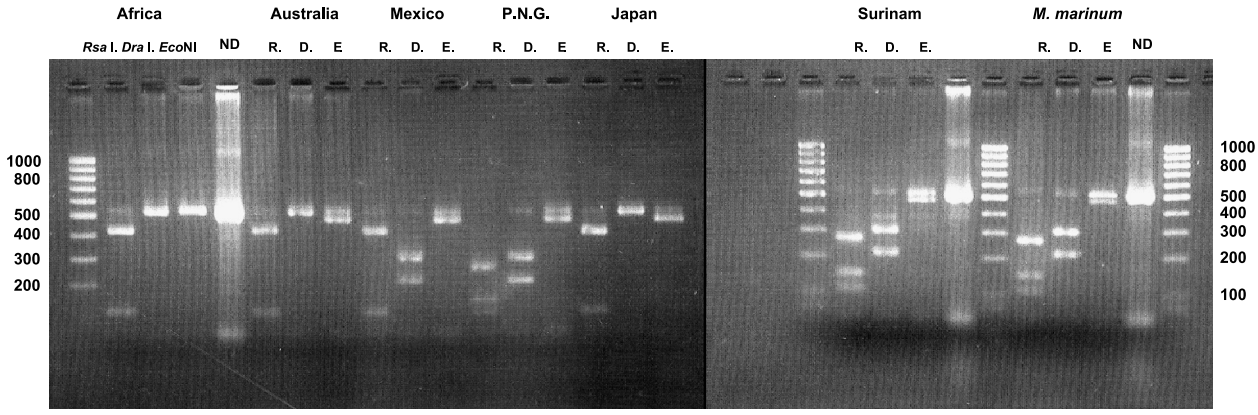

Fig. 3 Examples of PCR restriction profiles obtained from a representative set of *M. ulcerans* (from different geographic areas) and one *M. marinum* strain by using three restriction enzymes, *Rsa*I, *Dra*I, and *Eco*NI. The first and last lanes show the 100-bp ladder. ND, no digested PCR product; R, D, and E, *Rsa*I, *Dra*I, and *Eco*NI, respectively, P.N.G., Papua New Guinea.

Application for Mycobacteria (*M. tuberculosis*, *Mycobacterium ulcerans*, and *Mycobacterium marinum*)

In the field of *M. tuberculosis*, PCR-based RFLP is not so widely applied as the classical RFLP method. In a novel application of locus-specific RFLP, Cockerill et al.[18] have identified point mutations in the *katG* gene of *M. tuberculosis* that correspond to different levels of resistance to isoniazid by comparing the different RFLP banding pattern produced by the amplified gene. However, the discriminatory power is limited compared to other methods.

M. ulcerans and *M. marinum* are slow-growing mycobacterial species with optimal growth temperatures of 30°C to 33°C. These organisms are emerging necrotizing mycobacterial pathogens that reside in common reservoirs of infection and exhibit striking pathophysiological similarities. The interspecific taxonomic relationship between the two species is not clear as a result of the very high phylogenetic relatedness. To help understand the genotypic affiliation between these two closely related species, another approach of RFLP has been performed; namely, PCR restriction profile analysis (PRPA).

By targeting the 3′ end of 16S rRNA gene and by using three restriction enzymes, a set of geographically diverse *M. ulcerans* and *M. marinum* have been investigated.[19] The results (Fig. 3) showed that *M. ulcerans* can be typed at both intra- (three subtypes related to the geographical origin) and interspecific levels (differentiation between *M. marinum* and *M. ulcerans*). However, the major limitation of the technique is related to its discriminatory power as it cannot usually differentiate between these two species (Fig. 3); this may be related to the high degree of conservation of the mycobacterial 16SrRNA genes.

Application for Other Organisms

This new RFLP approach has been applied in a number of situations. Shortridge et al.[20] used the RFLP of the *ureC* gene to demonstrate the genetic diversity of *Helicobacter pylori* strains in the United States. The 16S, 23S, and 16S–23S spacer regions have also been used as targets for locus-specific RFLP.[21] In this variation of ribotyping, the ribosomal DNA is amplified and subjected to digestion with restriction enzyme, and the DNA fragments are visualized following separation by gel electrophoresis avoiding the need for Southern blotting.

Gene-specific probes have been used to subtype *Brucella* species,[22] *Legionella pneumophila*,[23] and *Pseudomonas aerugenosa*.[24] Furthermore, ribotyping has been applied successfully in many studies to differentiate bacterial strains.[25,26] Other applications of locus-specific RFLP found place in epidemiological studies of hepatitis C virus (HCV). By this technique, the virus can be subtyped into six major genetical groups.[27] The RFLP of the 5′ untranslated region has facilitated studies of the geographical distribution of viral genotypes and natural history of the disease.

CONCLUSION

Restriction fragment length polymorphism strategies are undergoing rapid technical improvements to circumvent

the different limitations (labor intensity, rapidity, and cost). Advances in the understanding of biological basis of microbial biodiversity at subspecies levels will improve the conceptual framework required for proper epidemiological interpretation of typing results. Wider application of these systems should shed more light on the epidemiology of hospital- and community-acquired infections, and therefore allow for more effective prevention and control strategies.

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