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Standard Review

Current trends in molecular epidemiology studies of *Mycobacterium tuberculosis*

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***Mycobacterium tuberculosis* is one of the most harmful human pathogens worldwide, and there are mass efforts for controlling this pathogen. One of the powerful tools to find out and control this pathogen is molecular epidemiology techniques. Currently, wide ranges of techniques are available to type *mycobacterium tuberculosis*, and choosing the correct technique as a portable and standard method is difficult. IS6110 restriction fragment length polymorphism (RFLP) remains gold standard method on genotyping to date, but it is labor intensive and inefficient on samples which have fewer than six copy numbers of IS6110. In the recent years, some new methods have been introduced for genotyping of *mycobacterium tuberculosis* such as mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) and Spoligotyping. The present review tries to introduce new approaches in molecular techniques for epidemiological investigation of tuberculosis and to illustrate advantages and problems associated with them.**

Key words: Molecular epidemiology, *Mycobacterium tuberculosis*, fingerprinting, transmission, genotype.

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INTRODUCTION

Despite mass BCG vaccination and anti tuberculosis drugs, tuberculosis is one of the major killers among the infectious diseases, causing about eight million cases and two to three million deaths occurs annually (Anh et al., 2000). Current trends suggest that these numbers could rise to 12 million cases and 4 million death, by the year 2010 (Bifani et al., 2002). Tuberculosis is the number one cause of death among individuals infected with human immunodeficiency virus (HIV) (Dye et al., 1999). Young children are likely to develop this disease after infection and are significantly more likely to develop extrapulmonary and severe disseminated disease than adults (Walls et al., 2004).

Increasing incidence of coinfection of tuberculosis with HIV, especially in developing countries, decrease children burdened of tuberculosis, emergence of multidrug-resistant tuberculosis, and immigrations caused new trends for molecular epidemiology and DNA fingerprinting of *Mycobacterium tuberculosis*. Also it introduced new molecular epidemiology markers for case finding and studying manner of transmission and reactivation of tuberculosis in regional investigations.

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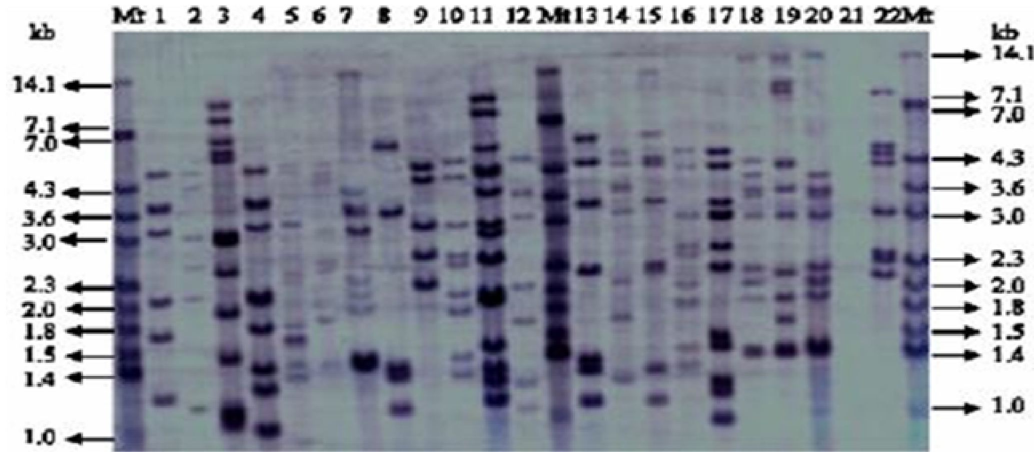

Figure 1. IS6110 fingerprinting in different isolates and reference *Mycobacterium tuberculosis* strain Mt14323. The size of hybridized fragments depends on the distance from this site to the next Pvu II site in DNA.

IS6110

IS6110 was first described by Thierry et al. (1990). Its standard approach to genotyping *M. tuberculosis* isolate was based on restriction-fragment-length polymorphism (RFLP) analysis (van Embden et al., 1993; Asgharzadeh et al., 2006).

IS6110 is a novel Mycobacterial insertion element formed the basis of a reproducible genotyping technique. Mycobacterial DNA is digested with the restriction enzyme PvuII (Asgharzadeh et al., 2007a). The IS6110 DNA probe was prepared by *in vitro* amplification of 245-bp fragment using the polymerase chain reaction and was labeled by digoxgenin. Fragments after digestion with PvuII were transferred from gel to positively charged nylon membrane. And then Hybridization was performed with 245bp probe of insertion sequence and detected by colorimetric system (Asgharzadeh et al., 2007b; van Embden et al., 1993; van Soolingen et al., 1994). The IS6110 probe hybridizes to IS6110 DNA to the right of the Pvu II site in IS6110. The size of hybridized fragments depends on the distance from this site (PvuII digested) to the next PvuII site in DNA (Figure 1). Advantage of this method is by discriminatory power on detecting epidemiological relations, whereas patients with epidemiological links have similar pattern of RFLP and it can reveal source of infection as a reactivated infection (Small et al., 1993a; van Rie et al., 1999) or transmission (Barnes et al., 2003; Bauer et al., 1998; Barnes et al., 1997; Burman et al., 1997; Chin et al., 1998; Dooley et al., 1992; Edlin et al., 1992; Durmaz et al., 2003; Pena et al., 2003; Niobe-Eyangoh et al., 2003). But it has several restrictions like, it is still expensive, requires subculturing the isolates for several weeks for obtaining sufficient DNA, labour intensive, discriminatory and polymorphism of isolates decrease and strains with fewer than six IS6110 insertion sites (Asgharzadeh et al., 2006; Asgharzadeh et al., 2007c; Bauer at al., 1999; Cowan et al., 2002; Goguet de la Salmoniere et al., 1997; Kremer et al., 1999; Lee et al., 2002; Mazars et al., 2001).

In order to estimate the stability of IS6110 RFLP patterns, studies have examined serial isolates collected from patients with persistent disease (de Boer et al., 1999; Niemann et al., 1999; Warren et al., 2002) and have demonstrated that the IS6110 banding pattern may change over time in a subset of these patients. When survival analysis was applied to the RFLP data collected from these patients, de Boer et al. (1999) calculated that the half life of the IS6110 banding pattern was on the order of 3.2 year. In another study, demonstrated Changes were observed in 4% of strains, and half-life ($t_{1/2}$) of 8.74 year was calculated (Rhee et al., 2000).

The early rate of Change in RFLP pattern probably reflects the change that occurred during active growth prior to therapy, while the low rate may reflect change occurring during or after treatment.

IS6110-based typing is the most widely applied genotyping method in the molecular epidemiology of *M. tuberculosis* and the gold standard to which other currently described method (van Embden et al., 1993; Rhee et al., 2000). Probably if overcome obstacles of IS6110-based genotyping, it can be incorporated into, routine, prospective and population-based method in identifying unsuspected outbreaks.

IS1081

In order to overcome the problem of absence or low copy number, an alternative molecular markers have been identified by van Soolingen et al. (1993). IS1081, first time identified by Collins and Stephens (1991). This insertion element is a 1324-bp sequence found in *M. tuberculosis* complex. It had some restrictions in compar-

ing with IS6110; it has a lower degree of polymorphism than IS6110, and can be due to its low transpositional activity (van Soolingen et al., 1993; van Soolingen et al., 1992; Kanduma et al., 2003). The copy number in IS1081 is lower than that of IS6110, which limits its use in epidemiological studies (Kanduma et al., 2003). Also it cannot be used to differentiate BCG from the other members of *M. tuberculosis* complex (van Soolingen et al., 1993).

PGRS

Ten percent of the *M. tuberculosis* genome consists of the genes that encode *M. tuberculosis* specific PE, PPE and PE-PGRS proteins, the PE family proteins are involved in iron acquisition, which is a critical process for *M. tuberculosis* survival (Ahmed et al., 2004).

PE-PGRS (polymorphic GC-rich sequences family represents an extension of the PE protein family with multiple repeats of glycine–glycine alanine asparagine) motifs (Choudhary et al., 2003; Chakhaiyer et al., 2004). There are numerous copies of PGRS repetitive element in the *M. tuberculosis* complex (De wit et al., 1990; Ross et al., 1992; Poulet sand Cole et al., 1995). It consists of many tandem repeats of 96 bp GC rich consensus sequence. PGRS elements are present in 26 sites of *M. tuberculosis* chromosome (Poulet sand Cole et al., 1995). PGRS – based genotyping involve *sma I* digestion of DNA and probing for a 32bp oligonucleotide (Chaves et al., 1996). PGRS is similar to the standardized IS6110 fingerprinting in which it requires purified DNA for southern blot hybridization and banding pattern analysis (Yang et al., 2000). PGRS fingerprinting has proven to be useful for differentiating *M. tuberculosis* strains with fewer than six copies of IS6110 that could not be readily differentiated by IS6110 fingerprinting (Barnes et al., 1997; Braden et al., 1997; Burman et al., 1997; Chaves et al., 1996). A better correlation between DNA fingerprinting data and the results of conventional epidemiology was found when a combination of the IS6110 and PGRS fingerprinting was applied than when IS6110 was used alone (Burman et al., 1997; Chaves et al., 1996; Yang et al., 2000). However the difficulties in computerizing the analysis of PGRS fingerprints is due to complexity of fingerprint patterns that have limited the wide use of PGRS fingerprinting (Yang et al., 2000).

Spoligotyping

The direct repeat locus in *M. tuberculosis* contains 10 to 50 copies of 36-bp direct repeat, that are separated from one another by spacer which have different sequences.

However, the spacer sequences between any two specific direct repeats are conserved among strains.

Because strains differ in terms of the presence or absence of specific spacer, the pattern of spacers in a strain can be used for genotyping (spacer oligonucleotide typing) or spoligotyping (Barnes et al., 2003; Filliol et al., 2000; Kamerbeek et al., 1997; Groenen et al., 1993; Hermans et al., 1991).

Spoligotyping is a PCR-based technique (Kamerbeek et al., 1997; Hermans., 1992). With using one set of primers it is possible to simultaneously amplify all the unique nonrepetitive sequences, spacers or between the direct repeats. The presence or absence of spacers is then determined via southern hybridization (Figure 2). Individual strains are distinguished by the number of spacers missing from the complete spacer set that was defined by sequencing this region from a large number of *M. tuberculosis* strains (Burgos et al., 2002).

This method have same advantage and by comparing with IS6110 RFLP method, it can be performed by small amount of DNA and a little time after inoculation of bacteria to liquid culture can be perform(Barnes et al., 2003; Kamerbeek et al., 1997). Also spoligotyping is useful for discrimination between isolates of *M. tuberculosis* with few copy number of IS6110 (Goyal et al., 1999; Goguet et al., 1997). This technique is specific for *M. tuberculosis* and other atypical strains dose not have any signal in analyzing with spoligotyping (Burges et al., 2002).

Usually the results of spoligotyping are expressed as positive or negative for spacers. Therefore, it can be expressed in a digital format (Dale et al., 2001). This method is simple, rapid, robust and economical mean for typing *M. tuberculosis* complex (Kremer et al., 1999; Kanduma et al., 2003; Barnes et al., 2003; Burgos et al., 2002; Goyal et al., 1999). However, the differentiating power of spoligotyping is less than IS6110 typing when high copy number strains are being analyzed (Kremer et al., 1999; Kamerbeek et al., 1997; Goyal et al., 1999; Diaz et al., 1998; Doroudchi et al., 2000). This method is a candidate for use in resource-poor situations (Burgos et al., 2002).

MIRU-VNTR

Variable Number Tandem Repeat (VNTR) typing is an invaluable tool for genotyping and provides data in a simple and format based on the number of repetitive sequences in so called polymorphic micro – and mini satellite regions (Mazars et al., 2001; Asgharzadeh et al., 2007c). VNTR introduced for *M. tuberculosis* which named Mycobacterial Interspersed Repetitive Unit-Variable Number Tandem Repeat (MIRU-VNTR) (Magdalena et al., 1998; Mazars et al., 2001). Out of 41 different loci in the genome of *M. tuberculosis* have been identified by supply et al (Supply et al., 2000).

Twelve loci were identified as hypervariable repetitive units (Magdalena et al., 1998; Supply et al., 2000; Supply et al., 2001). These fragments should be accurately sized for determining the number of repeats at each locus. The repeated units are 52 to 77 nucleotides in length and therefore power of this method may be com-

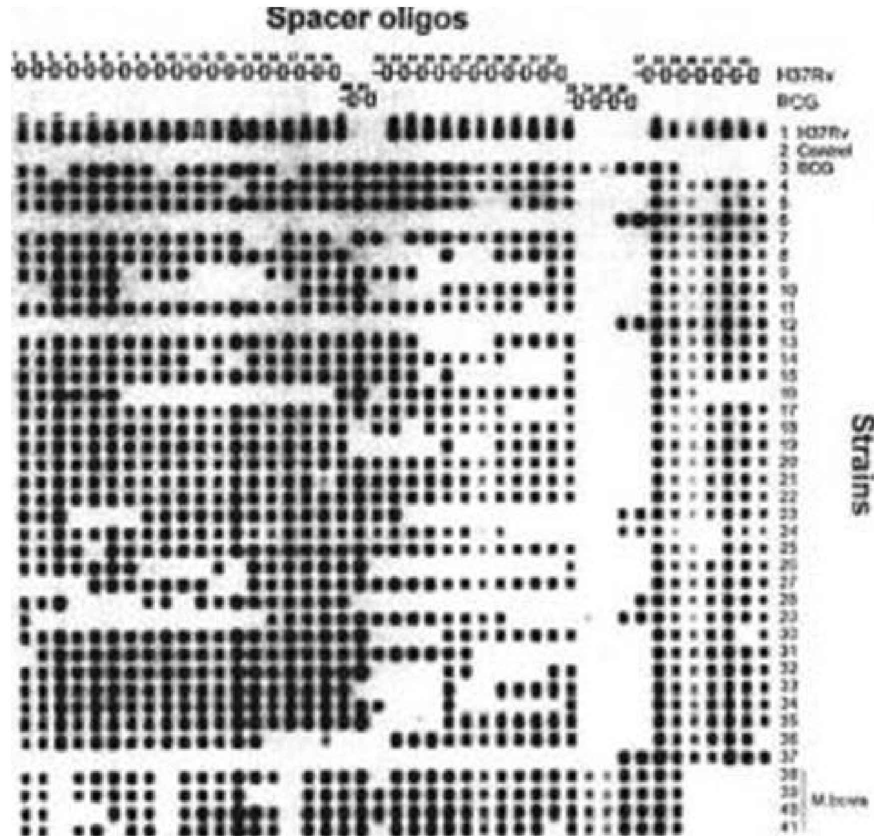

Figure 2. Spoligotyping, strains differ in terms of the presence or absence of specific spacer, the pattern of spacers in a strain can be used for genotyping (spacer oligonucleotide typing).

parable to that of IS6110- RFLP.

Moreover, MIRU typing showed its usefulness in studying the population structure of *M. tuberculosis* (Sola et al., 2003; Supply et al., 2003). MIRU-VNTR can also be used in global databases as each typed strain is assigned a 12-digict number corresponding to the num-ber of repeats at each MIRU-VNTR loci (Mazars et al., 2001). Various studies demonstrated the importance of MIRU-VNTR method for tracking epidemiological key events, such as transmission or relapse and provide non ambiguous data (Mazars et al., 2001), and, which are highly portable between different laboratories (Mazars et al., 2001; Frothingham et al., 1998; Supply et al., 1997).

Approximately 20 million possible combinations of 12 loci alleles are possible (Mazars et al., 2001; Supply et al., 2001). Most important advantage of this method is its simpler performance than IS6110, applying directly to *M. tuberculosis* cultures without DNA purification (Barnes et al., 2003). Based on high discriminatory power (Mazars et al., 2001; Asghaerzadeh et al., 2007c) and when comparing MIRU-VNTR with IS6110-RFLP or spoligotyping, MIRU-VNTR produced suitable discriminatory power (Mazars et al., 2001; Supply et al., 2001; Barlow et al., 2001; Cowan et al., 2002). Hardship of this method is associated with accurate sizing of multiple small fragments (Burgos et al., 2002). This can be partly overcome by combining multiplex PCR with a fluorescence-based DNA analyzer (Mazars et al., 2001). A population-based study indicated that the use of 12- locus-based MIRU-VNTR typing combined with spoligotyping as a first-line approach provided an adequate discrimination in most cases for large-scale genotyping of M. tuberculosis in the United States (Cowan et al., 2005). In a recent study based on a worldwide collection of tubercle bacillus isolates defined an optimized set of 24 MIRU-VNTR loci, including a subset of 15 discriminatory loci proposed to be used as a first-line typing method. These 15- and 24-locus sets reliably improved the discrimination of *M. tuberculosis* isolates compared to the original 12-locus set (Supply et al., 2006; Oelemann et al., 2007) (Table 1).

RAPD

Random amplification of polymorphic domains (RAPD) is a PCR-based method for genotyping *M. tuberculosis* (Palitta et al., 1993). This method is based on amplify-cation of the spacer region between the genes coding for 16S and 23S rRND of M. tuberculosis and digestion by

Table 1. Clustering results by genotyping methods.

Methods	No. of unique isolates	No. of clustered isolates	No. of clusters	No. of distinct types	Clustering rate (%)
Spoligotyping	56	98	16	72	53.2
MIRU-VNTR 12 old	84	70	20	104	32.5
MIRU-VNTR 12 old + Spoligotyping	106	48	14	120	22.1
IS6110-RFLP	115	39	13	128	16.9
MIRU-VNTR 15	115	39	12	127	17.5
MIRU-VNTR 24	117	37	11	128	16.9
MIRU-VNTR 15+ Spoligotyping	120	34	11	131	14.9
MIRU-VNTR 24 + Spoligotyping	120	34	11	131	14.9

Table 1. Brought from Oelemann et al., 2007. 154 isolates investigated for Genotyping by IS6110 DNA fingerprints and MIRU-VNTR typing with 12, 15 and the full set of 24 loci. 24 loci MIRU-VNTR made a slightly better resolution than IS6110. However, both methods yielded identical clustering rates of 16.9% due to minor differences in cluster compositions. The use of the discriminatory subset of 15 MIRU-VNTR loci only marginally affected the resolution, with a clustering rate of 17.5%. Spoligotyping increased the resolution of MIRU-VNTR typing based on the 24 loci and 15 loci to 14.9%.

restriction enzymes (Abed et al., 1995).

Patterns generated this method can be easily analyzed, have discriminatory power, have satisfactory results and may serve as rapid screening test for typing a large number of clinical isolates into clusters for further subtyping by more sophisticated methods (Vrioni et al., 2004). But in some studies reproducibility and the final discriminative power of the RAPD-based method was found to be limited (Frothingham et al., 1995; Glennon et al., 1995).

PFGE

Pulsed–field gel electrophoresis (PFGE) has been widely used to type various microorganisms in both outbreak and population based studies and is available in many clinical laboratories (Singh et al., 1999). But, this method is not commonly used in epidemiological studies of *M. tuberculosis*.

Most published PFGE protocols for *M. tuberculosis* are technically challengeable. Biosafety considerations and the unique cell wall composition of the organism have led to the development of protocols that are high complex and difficult to reproduce (Singh et al., 1999). Little has been done for developing PFGE patterns as a standardized method for analyses of M. tuberculosis, and therefore, it is a limited method for population based molecular epidemiologic studies of *M. tuberculosis*. Also there are contradictory reports on the genetic diversity captured by PFGE and on its utility for molecular epidemiology (D'Amato et al., 1995; Miller et al., 1994; Schirm et al., 1993).

Current application of molecular epidemiology

There are several available applications for molecular epidemiology in control of tuberculosis. The first important role of genotyping studies is on intensive use of these studies on trace outbreaks (Bifani et al., 1996; Frieden et al., 1996; Moss et al., 1997; Valway et al., 1994). Combination of genotyping with epidemiological investigations reveal the occurrence of an outbreak and by controlling the manner of disease transmission, also new molecular methods prepared a rapid typing of isolates where an outbreak is suspected in a hospital ward (Kanduma et al., 2003).

Molecular genotyping methods are important in detecting the dominance of transmission or reinfection in a population. In reinfections, was a documented source should be sought. In anti-tuberculosis – treatment trails, it is important to determine whether recurrent tuberculosis is due to relapse of reinfection when the former represents treatment failure.

The transmission index, defined as the mean number of tuberculosis cases resulting from recent transmission of a potential source case, has been used to qualify transmission between different subpopulations (Borgdorff et al., 2000). Finding the exact transmission of index is important in regional and global controlling programs.

Laboratory cross-contamination is a significant problem in detection of tuberculosis and cause abuse of drugs for treatment. The occurrence of cross-contamination is most likely when acid fact smears are negative and only one specimen is culture –positive (Burmen at al., 2000).

Genotyping can be used to confirm the occurrence of cross contamination in the laboratories and have been identified as a useful measure for avoiding false-positive cultures (Small et al., 1993b; Behr et al., 1997; Asgharzadeh et al., 2007d).

Molecular epidemiology studies have an important role of improving disease control in coinfections, exactly coinfection of tuberculosis and HIV. These studies reveal progress and spread of tuberculosis amongst HIV infected patients (Daley et al., 1992). These studies are useful in evaluating the development of drug resistant in patients with relapse or transmission of tuberculosis and investigation in spread of drug resistant strains among

hospitalized patients (Daley et al., 1992; Ritacco et al., 1997; Anastasis et al., 1997; Angarano et al., 1998; Breathnach et al., 1998).

Beijing genotype is a distinct family of tuberculosis which was found first time in Beijing–china. This genotype is prevalent in young individuals and indicates ongoing transmission of tuberculosis (Doroudchi et al., 2000; Anh et al., 2000). These genotypes are more virulent (Anh et al., 2000), and in detection of global epidemiology of this genotype have an important role in controlling tuberculosis.

Conclusion

Several different molecular methods have been made available for epidemiological and evolutionary studies as a result of comparative genomic studies. Now the challenge is to compile standardized molecular fingerprinting patterns originating from highly networked, multi-centric, genotypic analysis in the databases for interlaboratory use and for further references. Rapid genotyping method are needed to overcome low reproducibility, not proven application, less discrimination method such as Mycobacterial interspersed repeat units variable number tandem repeats (MIRU- VNTR). Also A combination of typing methods based on more rapid and slower molecular clacks maybe able to exactly differentiate between the contributions of remote and recent transmission in clustering of infected patient and controlling outbreaks

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