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Mycobacterium tuberculosis and Molecular Epidemiology: An Overview

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Abstract Tuberculosis is a disease of grave concern which infects one-third of the global population. The high incidence of tuberculosis is further compounded by the increasing emergence of drug resistant strains including multi-drug resistant (MDR). Global incidence MDR-TB is ~4%. Molecular epidemiological studies, based on the assumption that patients infected with clustered strains are epidemiologically linked, have helped understand the transmission dynamics of disease. It has also helped to investigate the basis of variation in *Mycobacterium tuberculosis* (MTB) strains, differences in transmission, and severity of disease or drug resistance mechanisms from across the globe. This has helped in developing strategies for the treatment and prevention of the disease including MDR.

Keywords Mycobcaterium tuberculosis, Molecular epidemiology, Drug resistance

"If the importance of a disease for mankind is measured by the number of fatalities it causes, then tuberculosis must be considered much more important than those most feared infectious diseases, plague, cholera and the like. One in seven of all human beings dies from tuberculosis. If one only considers the productive middle-age groups, tuberculosis carries away one-third, and often more." Robert Koch, March 24, 1882

1. Introduction

More than 130 years after the discovery of its causative agent, tuberculosis (TB) is still a major killer disease worldwide. It is estimated that today one third of the world population is infected with TB. According to World Health Organization (WHO) estimates in 2012, > 8 million people developed TB and 1.3 died due to the disease [1]. The incidence of TB ranges from less than 10 per 100,000 in North America to 100 to 300 per 100,000 in Asia and Western Russia to over 300 per 100,000 in Southern and Central Africa [1]. With the introduction of antituberculosis therapy (ATT) in1950s and the use of Bacille Calmette Guerin (BCG) vaccine, many experts calculated that TB would be eradicated in few years. However, TB generally and drug resistant TB especially is increasing worldwide since 1990. WHO declared TB a global emergency in 1993. Although, globally the prevalence and mortality rate has fallen by 37% and 45% respectively since 1990, the number of TB cases and deaths are still high. WHO has identified 22 highly endemic countries, which contribute about 80% to the global TB burden. These countries are labeled as being high burden countries (HBCs) of TB.

Drug resistant MTB is a major obstacle for the control and successful treatment of tuberculosis (TB). Inappropriate use of first and second line drugs leads to emergence of multidrug-resistant tuberculosis (MDR-TB) and extensively drug resistant tuberculosis (XDR-TB). MDR-TB is caused by isolates which are resistant to at least two first line drugs i.e. rifampicin (RIF) and isoniazid (INH). XDR-TB is caused by MDR-TB isolates which are also resistant to any fluoroquinolone, and to one of the three injectable drugs, capreomycin, kanamycin, and amikacin. WHO estimates that among the patients who were reported to have TB in 2011, there were 310,000 incident cases of MDR-TB and more than 60% of these were from Asia, Africa and Russia [2, 3]. According to WHO estimates 3.6% of new TB cases and 20.2% of previously treated cases in 2012 had MDR-TB globally. WHO further estimates that globally MDR-TB has led to 170 000 deaths in 2012 [1]. Thus drug-resistant TB (DR-TB) is a major threat for global TB control. In addition human immunodeficiency virus (HIV) epidemic has also played a key role in increasing the number of new TB cases [1, 4, 5].

Thus global increase in TB, particularly of drug resistant strains emphasize the need for rapid detection and drug susceptibility testing of MTB in clinical samples. Such rapid detection is necessary for adequate antituberculous therapy and containment of resistant strains. Here, molecular epidemiology has greatly assisted in the analysis of disease transmission and rapid and correct diagnosis of drug resistant MTB strains which in turn may result in decreased transmission of sensitive and drug resistant MTB strains in a population.

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2. Molecular Epidemiology of Tuberculosis for MTB Strain Typing

Molecular epidemiology is an integration of molecular biology with epidemiology. Recent developments in molecular biology have resulted in techniques that allow rapid identification and tracking of specific strains of *M. tuberculosis* as they spread through the population [6-9]. While, previous methods, such as colony morphology, comparative growth rates, susceptibility to antibiotics, and phage typing were useful but did not provide sufficient information regarding TB epidemiology. There is increasing evidence that the genetic difference of MTB is strongly associated with specific geographical locations [9-16]. Thus molecular epidemiological studies in a high TB incidence country may provide unique insights into dissemination dynamics and virulence of the pathogen.

In the early 1990s, different molecular methods were described to discriminate between MTB isolates [17, 18]. Most of these techniques use DNA polymorphism based on repetitive DNA elements of *M. tuberculosis* as genetic markers. Each of these methods results in strain specific genetic profiles (fingerprints). Identical strain fingerprints are called clusters, which are usually associated with recent transmission. While strains with unique fingerprints represent remote transmission or infection acquired in past.

Several molecular epidemiological studies of tuberculosis have been carried out using various polymorphic repeat sequences i.e. insertion sequence (IS), direct repeat (DR) and tandem repeats (Figure 1). IS elements are small DNA segment that can be inserted at multiple sites. These elements show high level of genetic polymorphism and widely used for studying the genetic variability in MTB species. IS*6110*-RFLP typing method, based on IS*6110* copy numbers and positions, has been used worldwide as the gold standard TB epidemiology because of reproducibility of the results [18]. However, there are certain limitations of this technique. Firstly, around 20% of MTB isolates contains few or no copies of IS6110 element and the method is unreliable for typing such strains. Secondly, it needs around 4.5µg of DNA which takes several weeks to culture enough viable organisms. In addition, the method is labor intensive, technically demanding and expensive (van Soolingen et al, 1995).

Various PCR based techniques, which target polymorphic loci other than IS6110, have also been used in epidemiological studies of TB. Spacer oligonucleotide typing (spoligotyping) based on the polymorphism in DR locus is a widely used molecular typing method for epidemiological studies worldwide [19]. The 36 bp size DRs are interspersed by unique spacer DNA sequences of 35-41 bp. Spoligotyping identifies the presence or absence of 43 spacer DNA sequences between the variable direct repeats using PCR in a particular MTB strain. Spoligotyping is simple, rapid and highly reproducible. However, Spoligotyping has less discriminatory power as it targets less than 0.1% MTB genomic area as compared to IS*6110* based typing which examines the entire genome (Figure 1). Therefore, it cannot totally replace *IS6110*-RFLP typing because of its lower discriminatory power, except for in strains with low copy numbers of IS*6110* (van Soolingen et al, 2001).

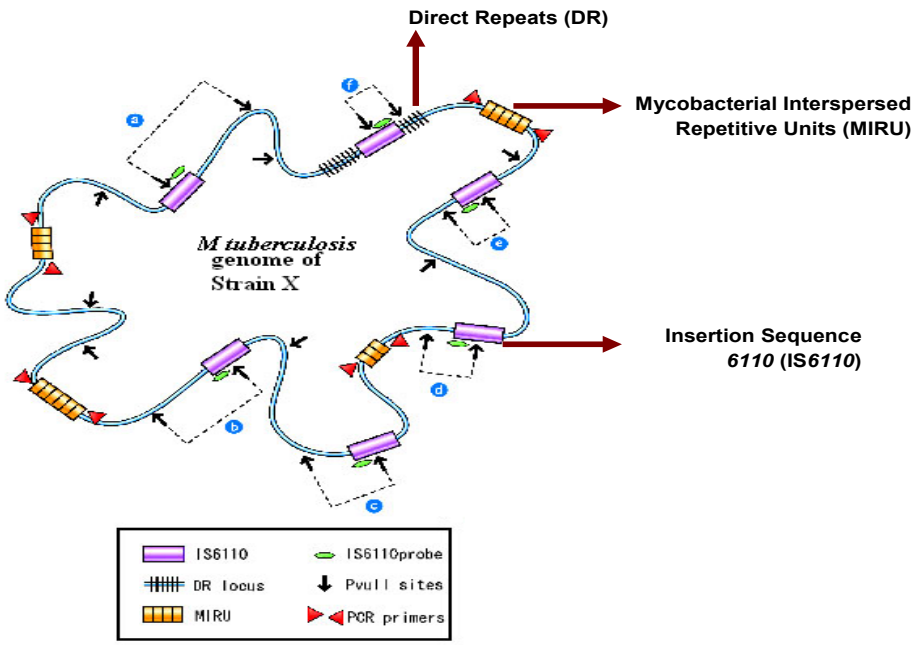

Figure 1. Hypothetical genome of MTB strain X and polymorphic repetitive sequences such as IS6110, DR and MIRUs for genotyping (adapted from Reference [38])

VNTR-MIRU is another PCR based genotyping method which has higher discriminatory power than spoligotyping. MIRU-VNTR is based on detection of independent mini satellite like loci scattered throughout the MTB genome and has been shown to be a reliable and reproducible typing method for studying the MTB population structure in different geographical locations [20-23]. The typed strains are expressed by a 12-digit numerical code, corresponding to the number of repeats at each locus [12, 24].

This numerical code is easy to compare and exchange at inter-, and intra-laboratory level. The discriminatory power of MIRU-VNTR analysis is proportional to the number of loci evaluated. Twelve loci based MIRU-VNTR analysis has been used in a number of molecular epidemiologic studies and to elucidate the phylogenetic relationship of clinical isolates [22, 25-28]. The discriminatory power of standard twelve loci based typing only slightly lower than that of the IS*6110*-RFLP, which is currently the gold standard for MTB genotyping [23]. However MIRU-VNTR has several advantages over gold standard IS*6110*-RFLP method; it requires little culture growth for DNA and provides comparable, numerical data by using standard gel electrophoresis or by automated analysis using fluorescence tagged PCR primers and sequencer. MIRU typing is also a method of choice for MTB strains with 0-5 copies of IS*6110* element**,** as have been reported from south Asian countries [12].

Using these molecular tools molecular epidemiological studies have identified several MTB strains so far including Beijing family [29], Haarlem family [30], Delhi [31], the Cameroon family [32], the Latin American Mediterranean(LAM) family, the East African Indian (EAI) clade [9, 33-35] etc.

Molecular epidemiological investigations to study transmission dynamics by exploring genetic diversity of MTB strains are of prime importance. These help to evaluate the patterns and dynamics of TB transmission in a defined geographical location. When this technique is applied similar MTB strains can be identified, which can lead to important clues about the pattern and dynamics of transmission. Two of the earliest such studies conducted in the United States, one in San Francisco and one in New York City, investigated MTB isolates with matching DNA fingerprints with the assumption that they were epidemiologically related and represented recent transmission of TB among the patients [36]. This finding led the authors to conclude that as many as 40% of TB cases in these two cities were the result of recent transmission and that TB control practices in San Francisco and New York were not effectively decreasing MTB transmission. Thus these data were used to implement effective interventions to stop such outbreaks.

Molecular epidemiological studies have also assisted in unraveling the differences in drug susceptibility related to bacterial genetic background [37-40]. Such as Beijing strain (W strain) have found to have a greater association with drug resistance and have also been found to be less protective against BCG vaccination in infection as compared to other MTB isolates [41].

3. Drug Resistance in MTB and Molecular Testing

The resistance of MTB strains to anti-tuberculosis drugs was noted when streptomycin (S) was first used as monotherapy for TB in the 1940s. In the subsequent years with the addition of RMP, pyrazinamide (PYZ) and ethambutol (E), multiple antituberculous therapy was implemented to combat emergence of single drug resistance amongst MTB strains [42, 43]. WHO and International Union against Tuberculosis and Lung Diseases (IUATLD) recommends using two terms: drug resistance among new cases and drug resistance among previously treated cases. Drug resistance among new cases is the presence of drug-resistant strain of *M. tuberculosis* in a newly diagnosed patient who has never received anti-tuberculosis drugs or has received them for less than 1 month. Drug resistance among previously treated cases is that found in a patient who has previously received at least 1 month therapy with anti-tuberculosis drugs [44].

Despite of implementation of multiple antituberculous therapies, a steady increase in the frequency of TB with single and multiple drug resistant MTB strains has been reported throughout the world [45]. In early 1990s outbreaks of MDR-TB received global attention [46]. Nosocomial outbreaks of MDR-TB have been reported in the USA, France and other countries [47, 48]. MDR and XDR-TB can give rise to potentially untreatable form of the disease. MDR and XDR-TB treatment require use of second-line drugs (SLDs) that are less effective, more toxic and more costly than the first line based treatment [49, 50]. In addition mortality is significantly higher among persons infected with MDR and XDR strains than of those infected with sensitive strains. Moreover, patients with MDR and XDR-TB remain infectious for longer time increasing the risk of disease transmission [51, 52].

The mechanisms of drug resistance in MTB are chromosomal, caused by accumulation of one or more mutations in independent genes (Table 1). Accumulation of a number of drug resistance mutations result in multiple drug resistance [53]. Such as resistance to RMP is well characterized and more than 95% of the RMP resistant have mutations in an 81bp hot spot region (codon 507-533), or Rif Resistance Determining Region (RRDR) of the 3534bp *rpoB* gene [54, 55]. RMP interferes with the transcription and elongation of the RNA by binding to the DNA dependent RNA polymerase [55, 56]. In contrast to RMP, INH resistance is controlled by a more complex genetic system, involving several genes such as *katG, inhA, kasA, oxyR* and *ahp*C [57-59]. Although the frequency of mutation at these loci varies between different population, studies show that 70-80% of INH resistance is mostly associated with mutation in codon 315 of *katG* and *inhA* genes [58, 59]. Similarly S, PYZ, E and Fluoroquinolones resistance have been linked with mutations in *rrs*, *pnc*A, *emb*B and *gyr*A genes

respectively (Table 1).

Detection of drug resistance is performed by culturing MTB in the presence of antibiotics. More recent methods are based on liquid media including the BACTEC radiometric and the Mycobacterial Growth Indicator Tube methods (MGIT) [60]. However, due to the long time period necessary to obtain results and laboriousness of these methods molecular approaches have been proposed.

Molecular methods are based on the genetic determinants of drug resistance rather than phenotypic resistance. These including DNA sequencing, real time PCR, microarrays and kit based line probe assays has enabled detection of drug resistance in MTB from several weeks to a few days [61, 62]. These methods include amplification of targeted gene segment correlating with drug resistance by polymerase chain reaction (PCR), sequencing, solid-phase hybridization, real-time PCR assay or microarray technique [8].

Molecular methods have an important role in identifying prevalent drug resistance mutations amongst MTB population in a particular geographical location. Molecular tools and genetic determinants of drug resistance has made timely diagnosis of MTB drug resistance and adequate antituberculous therapy possible [61, 62]. It is important to understand and study the drug resistance mutations in a particular MTB strain types to develop tests for rapid detection of resistance among MTB strains.

Knowledge of type and frequency of mutations amongst drug resistant strains of prevalent genogroup within a defined geographical location is essential for the development of tools for early diagnosis and control of drug resistant MTB strains [64, 65].

Different molecular assays have been proposed for the detection of mutations associated with resistance to anti-TB drugs beside DNA sequencing which is considered gold standard for molecular detection of drug resistance. These include real time PCR, microarrays and kit based line probe assays [61, 62]. Commercially available methods for rapid molecular based detection of drug resistant MTB i.e. MTBDR*plus*, MTBDR*sl* (Hain Lifescience) and Xpert MTB/RIF (Cepheid) assays [61, 66, 67] target common mutations associated with drug resistance in the hyper variable or hot-spot regions of the genes i.e., single nucleotide polymorphisms (SNPs) associated with RIF, INH, flouroquinolone, aminoglycoside and ethhambutol resistance [61, 66, 67]. The Xpert MTB/RIF assay (Cepheid, USA) detects RIF resistance by targeting the *rpo*B gene [67]. MTBDR*plus* (Hain Lifescience GmbH, Germany) assay detects rifampicin (*rpo*B gene) and isoniazid (*kat*G and *inh*A genes) resistance. MTBDR*sl* (Hain Lifescience GmbH, Germany) detects fluorquinolone (*gyr*A), aminoglycoside (*rrs)* and ethambutol (*emb*B*)* resistance respectively [66]. A number of studies have evaluated the diagnostic accuracy of these methods for the detection of drug resistance in diverse geographic settings with promising results for rapid detection and control of drug resistant tuberculosis [1, 68-70].

Table 1. MTB genes associated with resistance to antituberculous agents

Antituberculous agents	Gene	Size (bp)	Product	Mutation frequency among resistant MTB isolates (%)
Rifampicin	*rpoB*	3,534	β subunit of RNA polymerase	> 95
Isoniazid	*katG*	2,205	Catalase-peroxidase	60-70
	oxyR-ahpC	585	Alkylhydroreductase	~20
	inhA	810	Enoyl-ACP reductase	<10
	kasA	1,251	β-Ketoacyl-ACP reductase	<10
Streptomycin	*rpsL*	372	Ribosomal proteinS12	~60
	rrs	1,464	16s rRNA	<10
Ethambutol	*embCAB*	1,164	Arabinosyltransfer- ases	~70
Pyrazinamide	*pncA*	560	Amidase	70-100
Ethionamide	*inhA*	810	Enoyl-ACP reductase	<10
	ethA	1467	Flavoprotein monooxygenase	NA*
Kanamycin	*rrs*	1,464	16s rRNA	~65
Fluoroquinol-one	*gyrA*	2,517	DNA gyrase α subunit	>90
	gyrB	2,142	DNA gyrase β subunit	NA

*data not available (Adapted from references [54, 63])

4. Conclusions

Molecular methods could enhance understanding of transmission dynamics of tuberculosis disease and could be used to improve current control programs locally as well as globally. Genotyping information of MTB strains together with epidemiologic investigations may provide important information about the spread of MTB strains by identifying factors related to transmission and progression to tuberculosis disease. This in turn could greatly assist in formulating strategies for control of tuberculosis. Moreover, rapid detection of drug resistance using molecular methods in MTB isolates of TB patients in conjunction of routine susceptibility testing could further assist in timely and adequate use of anti-tuberculosis therapy which could play a pivotal role in treatment and containment of sensitive as well as drug resistant tuberculosis. As Ian Sutherland noted:"one man's cure is many men's prevention" [71].

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