Molecular Epidemiology of Drug Resistant Strains of Tuberculosis in Bangladesh

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DEPARTMENT OF MICROBIOLOGY
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Molecular Epidemiology of Drug Resistant Strains of Tuberculosis in Bangladesh

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Certification

This is to certify that the thesis entitled **"Molecular epidemiology of drug resistant strains of tuberculosis in Bangladesh"** was carried out by Md Khurshid Alam Hyder, Registration No. 135, Session 2012-2013, for the fulfillment of the degree of Doctor of Philosophy from the Department of Microbiology, Faculty of Biological Sciences, University of Dhaka, Bangladesh.

This work was carried out under our supervision and the style and contents of the thesis have been approved and recommended for the award of the Ph.D. degree.

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Dedicated to…

To the people who are fighting against Tuberculosis……

Acknowledgement

With my full submission to the Holy Creator of the Universe, I start by saying that, it was His divine blessings that has allowed me to complete the task.

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Abbreviation

aDSM	Active *TB* drug-safety monitoring and management
AFB	Acid-fast bacilli
BCG	*Bacillus Calmette-Guérin*
CO_2	Carbon dioxide
DF	Damien Foundation
DNA	Deoxyribonucleic acid
DOT	Directly observed treatment
DPR Korea	The Democratic People's Republic of *Korea*
DR	Direct repeat
DST	Drug-susceptibility testing
DVR	Direct Variant Repeat
GLI	Global Laboratory Initiative
HIV	Human immunodeficiency virus
HIV/AIDS	Human immunodeficiency virus infection and acquired immune deficiency syndrome
IRD	Interactive Research and Development
MDR	Multidrug resistant
MIRU	Mycobacterial Interspersed Repetitive Units
MIRU-VNTR	Mycobacterial interspersed repetitive units-variable number of tandem repeats
MTBC	M. tuberculosis complex
MTC	*Mycobacterium tuberculosis complex*
NIDCH	National Institute of Diseases of Chest and Hospital
NSP	The National Strategic Plan
NTP	National TB Programme
NTRL	*National Tuberculosis Reference Laboratory*
PCR	Polymerase chain reaction
PMDT	Programmatic Management of Drug-resistant TB
RR/MDR-TB	Rifampicin-resistant/Multidrug resistant
SDGs	Sustainable Development Goals
SDS PAGE)	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEA	South East Asia
SOPs	Standard operating procedures
SRL	Supra-national Reference Laboratory
TB	Tuberculosis
USAID	*United States Agency for International Development*
VNTR	Variable Number of Tandem Repeats
WHO	World Health Organization
XDR- TB	Extensively drug-resistant TB

Abstract

Tuberculosis (TB) is one of the top 10 causes of death worldwide. According to the Global TB Report globally in 2015, there were an estimated 10.4 million incident cases of Tuberculosis (TB) of which 5.9 million (56%) were among men, 3.5 million (34%) among women and 1.0 million (10%) among children. In 2015, the gap between notifications of new cases and the estimated number of incident was 4.3 million (missing cases) reflecting a mixture of undetected cases and under-reporting of detected TB cases. Bangladesh is one of the world's most densely populated countries, with 161 million people. In recent years, Bangladesh has made significant progress in child and maternal mortality reduction, life expectancy and poverty reduction. However, Bangladesh still faces serious health challenges and Tuberculosis is one of them. According to WHO Global TB report 2016, with annual occurrence of 362,000 new Tuberculosis cases Bangladesh is one of the world's 30 high TB burden countries. About 73,000 people die annually due to Tuberculosis. The National TB Programme (NTP) of Bangladesh and its partners have maintained good "basic TB control services", with reasonable case detection and excellent treatment outcomes. The Government of Bangladesh, the Global Fund, USAID and other partners have kept their financial commitment to TB control during recent years, enabling NTP to consolidate its activities and address challenges of TB/HIV, multidrug resistant (MDR) TB, intensified case-finding in high-risk groups and vulnerable populations, and use of new technologies. There has been consistent increase in case notification especially among new and relapse cases since 2013. Bangladesh is the first country in the Region to introduce a shorter treatment regimen for MDR-TB and is achieving high cure rate for MDR-TB patients (75%). This study was aimed to early diagnosis of susceptible TB and prevents drug-resistant TB and improves the overall understanding of genotpypic diversity of MTBC strains circulating in Bangladesh. The research was conducted in NTRL, NIDH, Mohakhali and Department of Microbiology, University of Dhaka, Dhaka, Bangladesh and total 371 sputum samples from

tuberculosis reference laboratory were collected. We have conducted two state-of-the-art molecular techniques: i) Spacer oligotyping/spoligotyping and ii) MIRU-VNTR (mycobacterial interspersed repetitive units-variable number of tandem repeats). In this study we found that most prevalent tuberculosis clade in Bangladesh is the Beijing clade and constitutes about 33.15% which is identified through Spoligotyping. Two different spoligotypes of Beijing clade including SIT 941 (9.19%) and SIT 1 (23.78%) was found. Evidence of presence of SIT 941 indicates its recent rapid spread in Bangladesh and it might be due to the extensive coverage of BCG vaccines. Moreover, two different types of MIRU-VNTR patterns were observed for the same spoligotype. This is possible as discriminatory power of MIRU-VNTR technique is more than spoligotyping technique. Although, MIRU-VNTR results could not be imparted with spoligotyping data, the spoligotyping technique alone was sufficient to identify predominant clades like Beijing, T1 and EAI in Bangladeshi population.

Chapter 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Tuberculosis (TB) is one of the top 10 causes of death worldwide. (http://www.who.int/mediacentre/factsheets/fs310/en/index1.html) .According to the first Global TB Report globally in 2015, there were an estimated 10.4 million incident cases of Tuberculosis (TB) of which 5.9 million (56%) were among men, 3.5 million (34%) among women and 1.0 million (10%) among children. Six countries including India, Pakistan, Indonesia, China, Nigeria, and South Africa accounted for 60% of the global new cases. In 2015, the gap between notifications of new cases and the estimated number of incident was 4.3 million (missing cases) reflecting a mixture of undetected cases and under-reporting of detected TB cases. Ten countries accounted for 77% of this estimated gap including India, Indonesia and Bangladesh in South East Asia (SEA) Region (www.who.int/entity/mediacentre/factsheets)

The World Health Organization (WHO) South-East Asia (SEA) Region bears the highest burden of TB in the world with a human, economic and social impact that is devastating (WHO South-East Asia Annual TB Report 2017 http://intranet.searo.who.int). The Region has nearly half the global burden1 in terms of new cases appearing (incidence), and close to 40% of the burden in terms of deaths due to TB (mortality) – while about 26% of the global population lives in the Region (Figure 1.1). The disease takes an unconscionably high toll in human lives and economic development.

In 2015, there were approximately 800 000 deaths from TB and TB- HIV, and 4.7 million new cases of TB disease in the countries included of this Region (http://www.who.int). India and Indonesia alone have 37% of the global TB burden. Timor- Leste and DPR Korea are among the top 10 countries in the world in terms of proportions of TB cases emerging (incidence rates). In terms of mortality rate in the Region, Timor-Leste (100 per 100 000 population) is followed by DPR Korea (61), Myanmar (49), Bangladesh (45) and Indonesia (40). The total number of new cases that were notified to national TB programme of the SEA Region was about 2.56 million in 2015 or only about 54% of estimated incidence.

TB is inseparably linked to the region's development agenda – it is a disease of poverty, under-nutrition and overcrowding, the largest killer in the 15-49 age group and the largest cause of work days lost.

End TB targets, aligned to Sustainable Development Goals (SDGs), if not met here, cannot be met globally. This region bears a disproportionately higher TB burden, with nearly half the global TB burden. The performance of TB programs here will, thus, directly determine global TB trends.

The current level of decline of annual incidence of TB is only 1.5-2%, which is not sufficient to achieve the SDG targets towards ending TB unless additional efforts are being taken to reduce at least 10-15% annually (http://intranet.searo.who.int SEAR TB Annual Report 2017)

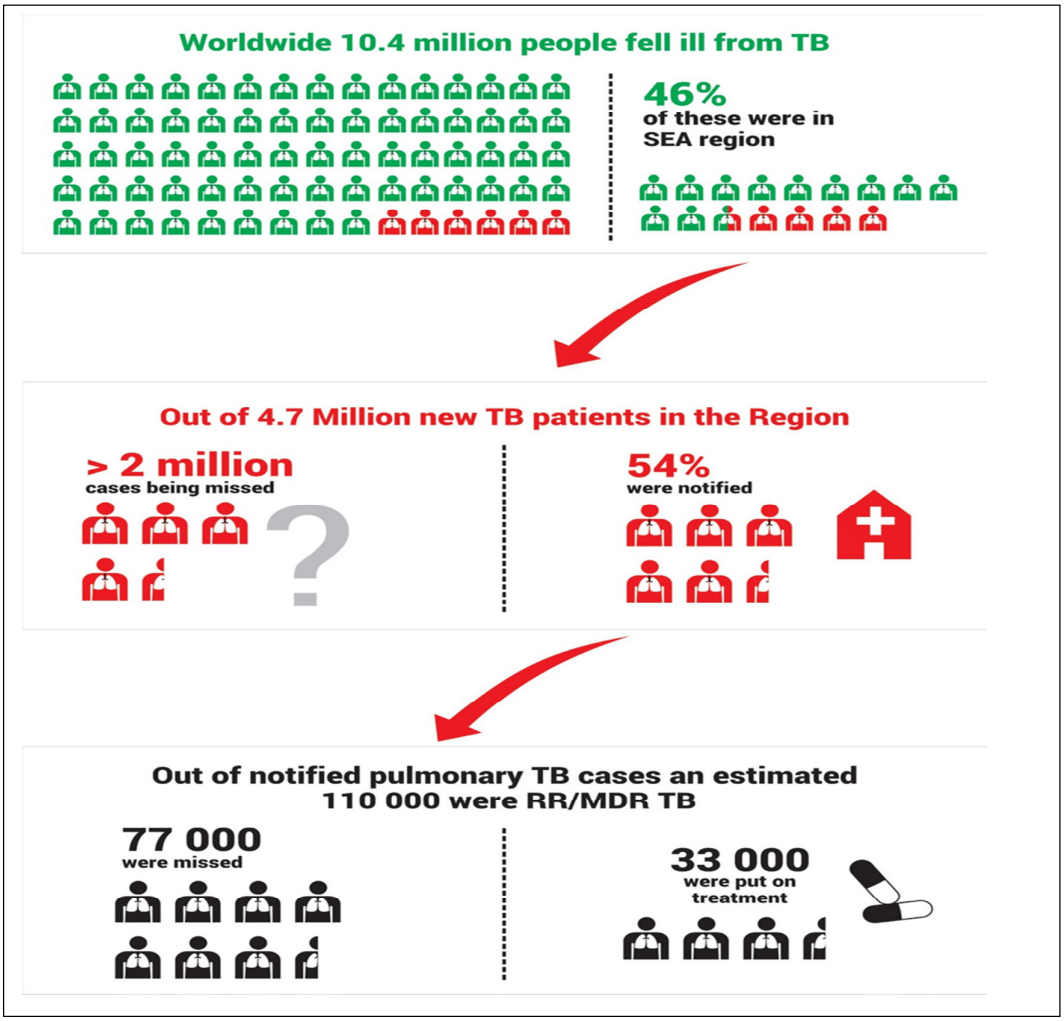


Figure 1.1: TB burden and progress in the WHO South-East Asia Region (2015)

The overall success rate of tuberculosis treatment in the WHO South- East Asia Region stood at 79% in 2015, the lowest in the last 5 years, largely because India's private sector health-care system accounts for a large proportion of TB patients that did not get reported earlier to the national TB programme.

Multidrug resistant and rifampicin-resistant TB (MDR/RR-TB) is a key threat. Its estimated incidence in the Region was 200 000, of which just 32 648 or 16% were started on treatment in 2015. Of those started on treatment in previous years (2013), less than half were successfully treated. Six SEAR nations reported extensively drug-resistant TB (XDR- TB) by 2015.

An estimated 227 000 cases (4.7%) of the 4.7 million incident cases were HIV positive. An estimated 74 000 people died of human immunodeficiency virus (HIV)-linked TB in 2015. About 0.18% of the new TB cases are HIV positive in Bangladesh as HIV prevalence is low (www.who.int/tb)

Furthermore, Bangladesh share a large amount of border with India and have close proximity to Pakistan and China, which are among the top TB burden countries in the world. Despite having high TB burden in this region, very limited data is available on prevalent *Mycobacterium tuberculosis* strains that are circulating in Bangladesh.

Bangladesh is one of the world's most densely populated countries, with 161 million people. In recent years, Bangladesh has made significant progress in child and maternal mortality reduction, life expectancy and poverty reduction. However, Bangladesh still faces serious health challenges and Tuberculosis is one of them.

According to WHO Global TB report 2016, with annual occurrence of 362,000 new Tuberculosis cases Bangladesh is one of the world's 30 high TB burden countries. About 73,000 people die annually due to Tuberculosis (www.who.int/tb).

The National TB Programme (NTP) of Bangladesh and its partners have maintained good "basic TB control services", with reasonable case detection and excellent treatment outcomes (NTP Annual Report 2016). The Government of Bangladesh, the Global Fund, USAID and other partners have kept their financial commitment to TB control during recent years, enabling NTP to consolidate its activities and address challenges of TB/HIV, multidrug resistant (MDR) TB, intensified case-finding in high-risk groups and vulnerable populations, and use of new technologies. There has been consistent increase in case notification especially among new and relapse cases since 2013. Bangladesh is the first country in the Region to introduce a shorter treatment regimen for MDR-TB and is achieving high cure rate for MDR-TB patients (75%). National Strategic Plan for TB Control 2018–2022 is being updated.

In 2015, 59.3% of all TB notifications were male for a male to female (M: F) ratio of 1.42. In 2015, the ratio of males among the retreatment categories is 1.85 and 1.43 among new cases. Among the bacteriologically confirmed pulmonary TB cases initiated on treatment in 2014, 94% of new cases, 87% of relapse cases and 86% of retreatment cases were cured or completed treatment (http://intranet.searo.who.int). Cases treated after having previously been lost to follow-up had a lower cure rate (74.1%) for the reason that predictably; a greater proportion of these cases went on to be lost to follow-up again (11.6%). The death rate was lowest among the new cases (3.3%) and highest among the relapse cases (5.8%). Treatment failure was highest among cases who had previously failed (2.4%).

To identify key interventions that will most likely contribute to an improved performance of the NTP, a gap analysis was performed describing current gaps in NTP performance for key programmatic areas. The analysis drew on the results of the Joint Monitoring Mission that was completed in November 2016. The mission provided an updated assessment of the status of TB control in Bangladesh, as well as a detailed description of achievements and deficiencies in key programmatic areas. The programmatic areas that were considered for the gap analysis are listed below:

- Case detection including intensified case finding and new approaches
- Quality assured laboratory network
- Treatment (including patient support and DOT)
- Management of anti-TB medicines and supplies including registration of new drugs
- Supervision
- Recording, reporting and surveillance
- Paediatric TB
- MDR-TB including aDSM and infection control
- Involving all care providers

National Strategic Plan of Bangladesh

The National Strategic Plan (NSP) for TB contains strategies and interventions based on the principles outlined in WHO's End TB Strategy (www.who.int/tb/End_TB_Brochure.pdf) Building on the Strategy's Three Pillars (I. Integrated, Patient-Centred Care and Prevention; II. Bold Policies and Supportive Systems; III. Intensified Research and Innovation) and following the key principles of government stewardship and accountability, strong coalition with civil society organizations and communities, protection and promotion of human rights, ethics and equity, and adaptation of the strategy and targets at country level, the NSP describes key

interventions and activities that would enable the NTP to achieve the End TB Strategy's Milestones for 2025 (75% reduction in tuberculosis deaths and 50% reduction in tuberculosis incidence rate) and targets for 2035 (95% reduction in tuberculosis deaths and 90% reduction in tuberculosis incidence rate).

The NSP has following objectives:

Objective 1: Increase annual case detection of all forms of TB to more than 90% of all incident cases by 2022 (from baseline of 57% in 2015) with childhood TB contribution of 8% by 2022 (from baseline of 4% in 2015)

Objective 2: Further Increase treatment success rate at levels of more than 90% in all forms of detected non-MDR TB cases and ensure quality-controlled treatment services at all implementation sites

Objective 3: Increase annual case detection of MDR- TB to 4,100 with a child MDR case detection of 112 by 2022 (from baseline of 0 in 2015), and improve management of MDR-TB cases through country-wide implementation of the shorter MDR-TB treatment regimen

Objective 4: Ensure that no TB-affected families facing catastrophic costs due to tuberculosis by 2022

Objective 5: Ensure that 100% of TB service facilities receive regular supervision and monitoring with appropriate feedback resulting in remedial actions by 2022

Objective 6: Ensure the long-term availability of 100% of required funding for activities at all program levels through effective planning and partner coordination and the continuing increase of GOB contributions to the NTP budget

Objective 7: Ensure adequate support for operational research to foster innovation

Another important challenge is Multi drug resistance Tuberculosis (MDR TB)- with an estimated 9,700 MDR cases per year.

Drug resistance is becoming a more important barrier to effective treatment of TB, and threatens the effectiveness of NTPs (WHO SEA Regional Programmatic Management of Drug Resistant TB Mission Report 2009). NTP Bangladesh has successfully conducted its first nationwide drug resistance survey in 2010 and 2011 at NTRL under technical support of WHO and funding support by Global fund and USAID. This survey reveals MDR-TB rate was 1.4% and 28.5% among new & retreatment cases respectively14. NTP Bangladesh started PMDT from 2008 and accordingly there were a pooling of MDR cases from the past among retreatment cases especially in large metropolitan city.

According to the First Drug Resistant Survey conducted in 2014, MDR rate in new and previously treated case was1.4% and 28.5% respectively. NTP diagnostic algorithm did not include diagnosis of MDR among new cases, except some sporadic intervention. In 2015 by testing of 36 836 TB cases 954 RR/MDR-TB cases were documented. Among these 880 (92.2%) were put on treatment. The treatment success rate of MDR/ RR TB cases by WHO regimen is 75%. The Bangladesh regimen (shorter regimen is running in Damien Foundation (DF) area before starting the Programmatic Management of Drug Resistant TB (PMDT) of NTP. This regimen was also evaluated in other countries and endorsed by WHO. The success rate of shorter regimen is about 84.5% for the cohort 2005-2011.

National TB Reference Laboratory (NTRL) at National Institute of Diseases of Chest and Hospital (NIDCH) are gradually developing capacity both qualitatively and quantitatively and are regularly passing proficiency testing conducted by Supra-national Reference Laboratory (SRL), Antwerp, Belgium through WHO and the UNION. All TB and MDR-TB diagnostic methods are available at NTRL Bangladesh, including the diagnosis of Extremely Drug Resistant TB (XDR-TB). Presently NTP is also treating XDR TB cases at NIDCH and Chest Disease Hospital, Chittagong.

New drugs and new regimens are required to reduce the duration and adverse events, and improve effectiveness of treatment of DR-TB cases. A clinical trial on Bedaquilline and Delamanid has started at NIDCH in May 2016 under Interactive Research and Development (IRD) project.

Implementing tuberculosis diagnostics

Tuberculosis (TB) laboratories play a critical part in national TB programmes, providing clinicians with invaluable information that is used to diagnose and guide the care of patients. Because of the specialized nature of the different technical procedures needed to diagnose TB, and the need for quality assurance and effective laboratory management, TB control programmes require a tiered network of laboratories in which different tiers use complementary diagnostic tools and mechanisms for referring specimens. Establishing, equipping and maintaining a laboratory network to ensure that there are timely and universal access to quality-assured diagnostics is challenging, complex and expensive, and the following core elements must be addressed simultaneously:

- Planning for the implementation of diagnostic services;
- Developing laboratory infrastructure and plans for maintaining the infrastructure, as well as implementing appropriate biosafety measures;
- Developing schedules for equipment validation and maintenance;
- Establishing mechanisms for specimen collection, transport and referral;
- Establishing systems for managing laboratory commodities and supplies;
- Developing systems for managing the laboratory and the data collected;
- Establishing systems for assessing the quality of each laboratory's services; and
- Ensuring there are appropriate strategies for managing human resources and adequate funding for human resources.

Diagnostic capacity continues to be a major bottleneck in TB control, including scaling up management and control efforts to tackle drug-resistant TB and TB associated with HIV(http:www.stoptb.org/wg/gli). An unprecedented effort to improve and expand the capacity of TB laboratories is under way, coordinated by the World Health Organization's (WHO's) Global TB Programme and with the active involvement of the Global Laboratory Initiative (GLI), a working group of the Stop TB Partnership (for more information, see http://www.stoptb.org/wg/gli).

The targets for laboratory strengthening in The global plan to stop TB 2011–2015 include ensuring:

- There is 1 microscopy centre per 100 000 population (for smear examinations for acid-fast bacilli [AFB]);
- There is 1 laboratory per 5 000 000 population to perform culture testing;
- That 50% of tests for drug resistance for new TB patients and more than 90% of tests for previously treated patients are done using rapid TB diagnostic tests.

However, with the roll-out of the Xpert MTB/RIF assay (Cepheid, Sunnyvale, CA, United States), the number of microscopy centres and facilities offering culture and drug-susceptibility testing (DST) will need to be adjusted depending on the extent of the roll-out in different settings and the epidemiology of TB, multidrug-resistant TB (MDR-TB) and HIV infection. Smear examinations for AFB remain essential for monitoring patients' responses to treatment, as well as for the initial evaluation of patients with suspected pulmonary TB when Xpert MTB/RIF testing is not available. Provided that molecular methods are made available, the priority for using culture and DST would be to monitor response to treatment for MDR-TB, as well as to determine whether there is susceptibility to second-line anti-TB agents.

WHO's global strategy for TB prevention, care and control for 2015–2035 (known as the EndTB Strategy) prioritizes the early diagnosis of TB, which should include the universal availability of DST, and systematic screening of contacts and high-risk groups.2 Therefore, all national TB control programmes should prioritize the development of a robust network of TB laboratories that have adequate biosafety standards, use modern methods of diagnosis, use standard operating procedures (SOPs) and appropriate quality assurance processes, and that have qualified and sufficient human resources; these priorities should be comprehensively addressed in national strategic plans.

Overall, the development landscape for TB diagnostics is promising: many different organizations are developing products, and there is a robust pipeline of technologies. The range of technologies that may replace sputum-smearmicroscopy continues to expand, and smaller, simpler and more robust products are expected to become available in the coming years. Several technologies aim to deliver results in less than 1 hour, including DST results; this should improve the time to treatment, enable point-of-care testing programmes and provide greater access to DST.

Until these new technologies become available, the use of existing WHO-recommended diagnostic techniques must be accelerated and strengthened. This will require ensuring that laboratories have adequate infrastructure and human-resources capacity. Additionally, there must be clear country-level policies on using these recommended tests in the most effective screening and diagnostic algorithms, depending on each country's specific epidemiology and resources.

Understanding the genetic diversity of M. tuberculosis complex (MTBC) as well as TB epidemics by using molecular typing methods is important to carry out supervision of TB

control programs in a country (Banu et al., 2015). IS*6110* restriction fragment length polymorphism is a labor intensive method for large scale genotyping and results vary between laboratories (Banu et al., 2004). Relatively recent epidemiological studies have preferred two state-of-the-art molecular techniques – spacer oligotyping/spoligotyping and mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) typing(Brudey et al., 2006b). Spoligotyping has less discriminatory power than IS*6100* typing, however, this is a rapid and robust method and is particularly suitable for genotyping of *M. tuberculosis* strains having few copies of IS*6110* elements which make this useful for typing of South Asian strains(Das et al., 1995; Goyal et al., 1997)

For initial analysis of genotypic diversity of MTBC strains, spoligotyping is widely used. This polymerase chain reaction (PCR) based technique that can simultaneously detect and type *M. tuberculosis* strains was first introduced by Kamerbeek in 1997. This technique detects presence or absence of forty three unique spacer sequences in the chromosomal direct repeat (DR) locus (Kamerbeek et al., 1997).

The DR region in *Mycobacterium bovis* (BCG) consists of directly repeated 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 BCG strain was determined to be 49. In other *Mycobacterium tuberculosis* complex strains the number of DR elements was found to vary significantly. The vast majority of the MTBC strains contain one or more IS*6110* elements in the DR region. 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 DR's and other spacers. One DR and its neighboring non-repetitive spacer are termed “Direct Variant Repeat” (DVR).

Spoligotyping technique

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 DR's occur (Groenen et al., 1993). The mechanism, by which spacers and copies of DR are generated, is unknown. The presence or absence in the DR region of 43 spacers of known sequence can be detected by hybridization of PCR-amplified spacer DNA to a set of immobilized oligonucleotides, representing each of the unique spacer DNA sequences. This method will be referred to as spoligotyping.

By spoligotyping one can detect the presence or absence of spacers of known sequence. The first step in the method 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 (Figure. 1).

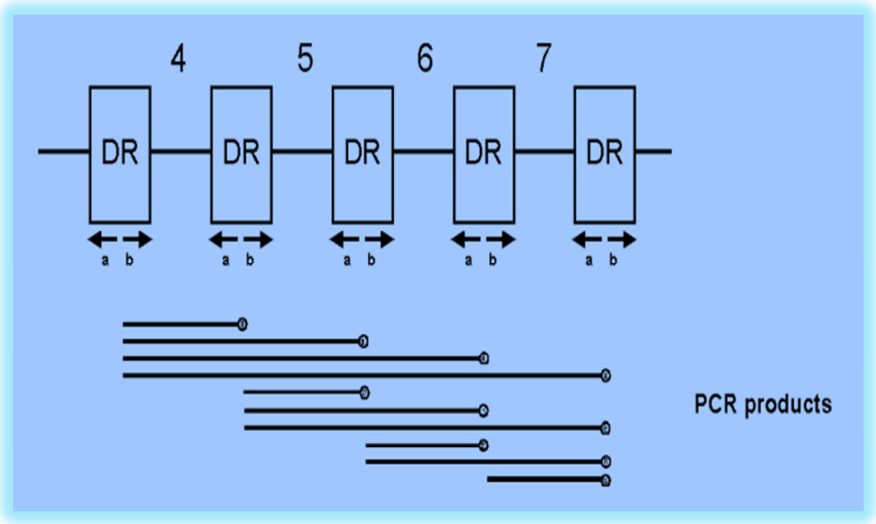


Figure 1.2: Principle of the *M. tuberculosis* amplification of DNA within the DR region of MTBC

The obtained PCR products differ in length because of two reasons. First, the product contains several spacers and the DR's in between if the primers anneal to DR's not next to each other. Second, the product itself can act as a primer, and become elongated with one or more DVRs.

Therefore, the PCR product provides no reliable information about spacer order or total length of the DR region.

Spoligotyping may offer an alternative for typing Southern blotting when rapid results are required. The method is in particular useful to simultaneously detect and type *M. tuberculosis* complex bacteria in clinical samples (suspected nosocomial infections, outbreaks in prisons, etc.). The level of differentiation by spoligotyping is less compared to IS*6110* fingerprinting for strains having five or more IS*6110*copies, but higher for strains with less than five copies.Due to the introduction and development of SITVIT WEB (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/),the largest online database containing genotyping markers for *M. tuberculosis,* assigning MTBC strains in major phylogenetic clades and comparing strains with isolates of other countries have become possible(Demay et al., 2012). This technique is successfully being utilized to assign isolates to different clades named Beijing, CAS, EAI, T, Haarlem etc(Ferdinand et al., 2004; Sebban et al., 2002).

MIRU-VNTR technique

Another useful method for genotyping of MTBC strains is **MIRU-VNTR technique**. Variable independent markers for the analysis of the population structure are desirable for a finer phylogenetic classification of *M. tuberculosis* complex (MTBC). Micro- or minisatellites are powerful tools that have been successfully applied for high resolution population-genetics. For the MTBC a typing system based on Variable Number of Tandem Repeats (VNTR)-typing applying genetic elements called Mycobacterial Interspersed Repetitive Units (MIRU) as genetic markers has been proposed as a suitable tool to analyze the genetic diversity of clinical isolates (Supply et al., 2006). First evaluations already showed that MIRU-VNTR typing can provide unique high-resolution insights into the population structure of the MTBC and provides clear criteria for the identification of the different MTBC lineages and sub-lineages.

Variable Number Tandem Repeat (VNTR)sequences have emerged as valuable markers for genotyping of several bacterial species, especially for genetically homogeneous pathogens such as *Bacillus anthracis*, *Yersinia pestis*(Le Flèche et al., 2001)and the *M. tuberculosis* complex members (see below). VNTR genotyping basically rely on PCR amplification using primers specific for the flanking regions of the VNTRs and on the determination of the sizes of the amplicons, after electrophoretic migration. As the length of the repeat units is known, these sizes reflect the numbers of the amplified VNTR copies. The final result is a numerical code, corresponding to the repeat number in each VNTR locus. Such numerical genotypes are intrinsically portable and are thus particularly convenient for both intra- and inter-laboratory comparative studies. In addition, compared to IS*6110*-RFLP, VNTR typing has the advantages of being faster, and appropriate for virtually all *M. tuberculosis* isolates, including strains that have a few IS*6110* copies(Mazars *et al.,* 2001).Initial VNTR typing systems for *M. tuberculosis* complex strains made use of very limited sets of loci, which turned out to not be sufficiently discriminatory(Kremer *et al.,* 1999). More extensive sets of VNTR loci have been described subsequently, including a system based on 12 loci(Supply et al., 2001), which has been shown to be applicable for reliable genotyping and molecular epidemiology studies of *M. tuberculosis*. These loci contain VNTR of genetic elements named **Mycobacterial Interspersed Repetitive Units (MIRUs)** that are located mainly in intergenic regions dispersed throughout the *M. tuberculosis* genome. As the other VNTRs sequences mentioned above, the lengths of MIRU repeat units are in the range of 50-100 bp, and belong therefore to the "minisatellite" VNTR category (Supply *et al.* 2001).

A MIRU-VNTR-based high-speed genotyping system has been developed, which combines the analysis of multiplex PCRs for the target loci on a fluorescence-based DNA analyzer with computerized automation of the genotyping (Supply *et al.* 2001). Both this system and the

simpler system using electrophoresis with agarose gels are highly reproducible at intra- and inter-laboratory levels (Cowan *et al.,* 2002). A recent population-based study indicated that the use of the 12 locus-based MIRU-VNTR typing as a first-line method, together with spoligotyping, provides adequate discrimination in most cases for large-scale, prospective genotyping of *M. tuberculosis.*

A discriminatory subset of 15 loci with the highest evolutionary rates was then defined, that concentrated 96 % of the total resolution obtained with the full 24-loci set. Its predictive value for evaluating *M. tuberculosis* transmission was found to be equal than to that of IS*6110* RFLP typing, as shown by results from a companion population-based study (Oelemann*et al.* 2007). This 15-loci subset system is thus therefore proposed as the new standard for first-line routine epidemiological discrimination of *M. tuberculosis* isolates, and the 24-loci system as a high-resolution tool for phylogenetical studies in different settings (Supply et al., 2006).

The knowledge of anti-TB drug resistance levels is an essential public health management tool for evaluating and improving the performance and efficiency of tuberculosis control in Bangladesh. This knowledge is also essential for the management of patients with drug-resistant TB. A surveillance system based on routine DST of all TB cases is able to provide continous information on drug resistance patterns among patient groups, and is therefore able to accurately detect trends over time, as well as localized outbreaks4. This is feasible in high resource low TB burden countries. But in low resource and high TB countries llike Bangladesh a minimum, systematic DST should be established among all previously treated TB cases and a periodic survey should be conducted regularly among new cases every five years4.

1.2 Literature Review

1.2.1 Causative agent of tuberculosis

***Mycobacterium tuberculosis* complex**

M. tuberculosis is a member of *M. tuberculosis* complex (MTBC), which is made up of *M. tuberculosis, M. bovis, M. africanum, M. pinnipedii, M .caprae, M. microti*and *M. canetti*. These members are closely related, with 99.9% sequence similarity on the nucleotide level (Sreevatsan et al., 1997). But even with this close relatedness, they still have different phenotypes, they bring about different pathologies and they demonstrate different host specificities. The distinguishing features of tubercle bacilli are given in table 1.1. The precise origin of the evolution of *M. tuberculosis* however, is still unknown, but it has been suggested previously that is derived from *M. bovis* (which is an organism infecting cattle), as a result of cross-species jump (Stead et al., 1995). However, there was new evidence that was later found which suggested that *M. bovis* actually derived from *M. tuberculosis* (Hewinson et al., 2006; Mostowy et al., 2002).

Table 1.1: Distinguishing characteristics of tubercle bacilli

Character	***M.tuberculosis***	***M. bovis***	***M. africanum***	**BCG**
Colonial growth on egg medium	Eugenic	Dysgenic	Dysgenic	Dysgenic
Growth on semi solid agar	Aerobic	Microaerophilic	Microaerophilic	Aerobic
Growth on PNB medium	–	–	–	–
Growth on TCH medium	+	–	–	–
Niacin Production	+	–	+/–	–
Nitrate reduction	+	–	+/–	–
Sensitivity to pyrazinamide	+	–	+	–

(i) Mycobacterium tuberculosis

Mycobacterium tuberculosis (*M. tuberculosis*) is the main cause of TB in humans, and debatably the most successful bacterial pathogen. It was first described by Robert Koch in 1888 and is thus sometimes referred to as Koch's bacillus (Koch, 1882). There have been speculations about *M. tuberculosis* evolving from *M. bovis* by specific adaptation of an animal pathogen to the human

host (Stead et al., 1995). This was proposed before the availability of the whole genome sequence of *M. Tuberculosis* (Cole et al., 1998) and before several variable genomic regions in the members of the MTBC was uncovered by comparative genomics. The degree of relatedness to the last common ancestor of the MTBC was proposed based on the presence or absence of such conserved regions. It showed that the lineages of M. tuberculosis and M. bovis separated before the occurrence of the *M. tuberculosis* deletions TbD1. This analysis therefore, made it clear that it is impossible for *M. bovis* to have been the ancestor of M. tuberculosis, but rather, appears either to be descended from M. tuberculosis or to have emerged independently (Brosch et al., 2002, Cole, 2002, Huard et al., 2003).

(ii) Mycobacterium africanum

Mycobacterium africanum(*M. africanum*) is predominantly isolated in different parts of Africa (David et al., 1978). Up to 60% of isolates obtained from patients with pulmonary TB are represented by *M. africanum* in certain regions (Haas et al. 1997). This species was first isolated from a Senegalese patient suffering from pulmonary TB in 1968 (Castets et a., 1968). It was then discovered that it has characteristics that seem to lie in between *M. tuberculosis* and *M. bovis. M. africanum* species are subdivided into two major subgroups, based on their biochemical properties as well as their geographic origin: Subtype I are closer to M. bovis and are from West Africa; Subtype II are from East Africa and are closer to M. tuberculosis (Niemann et al., 2002). There are differences in the distribution of *M. africanum* in various regions of Africa, the different prevalence of this species were Ivory Coast 5%, Guinea-Bissau 60%, Burkina Faso 18% and Uganda 49-60% (Castets et al 1968; Kallenius et al., 1999, Niemann et al., 2002). The issue of whether *M. africanum* subtype II is a genetically well-defined subspecies of the MTBC has been quite a considerable debate (Mostowy, 2002; Sola et al.; 2003).

(iii) Mycobacterium bovisand Mycobacterium bovisBCG

The host range of *M. bovis* is broad, and it can cause TB in different domestic or wild animals such as goats or cattle, but can also cause Tb in humans (Wayne et al., 1986). Bovine TB remains a significant disease in a number of countries of the world, as it is a cause of high rate of economic losses in certain countries. The risk factors for *M. bovis* infection in both animals and humans have been observed to be close, food hygiene practices, such as the consumption of dairy and meat products that have not been processed, and HIV/ AIDS, and this is more so in African settings where domestic animals are a part of human social life (Cosivi et al., 1998). *M. bovis*BCG (Bacillus Calmette-Guerin) is from a virulent *M. bovis* strain, where 230 in vitro passages of *M. bovis* were performed by Calmette and Guerin until the organism lost its virulence (Calmette, 1927). Even though this strain has been used as an attenuated vaccine, it is possible that it may cause disease, but only after vaccination with BCG.

(iv) Mycobacterium microti

Mycobacterium microti(*M. microti*) had earlier been considered non-pathogenic for humans, and only causes TB mainly in small rodents such as voles (Kremer et al., 1998, Wells et al., 1937). This species was proved to be a member of the MTBC based on the DNA sequence of the 16S rRNA gene and the 16S-to-23S internal transcribed spacer region. *M. microti* is very slow to grow and this has complicated the primary isolation and differentiation of this species using biochemical tests, however, spoligotyping has allowed the simultaneous detection and typing of *M. microti* (van Soolingen et al., 1998). This technique was applied in two studies of *M. microti* infections the Netherlands and England, allowing the identification of cases in Ilamas, cats and ferrets, and in the four humans that were used in the study only one was described as immunocompetent. Another study was done by Niemann et al (2000) and he reported cases of *M. microti* infections that caused pulmonary TB in Germany, and the isolates, according to

spoligotyping patterns, were identified as *M. microti* of the ilama and vole types, which displayed reactions with only two of the 43 spacers (spacers 37 and 38).

(v) Mycobacterium caprae

A test was done in Spain where isolates from the MTC were cultured from caprine pathological tissue samples. These isolates were genetically and biochemically characterized, and the result was that they segregated from the members of the MTB complex. Based on the results of the test as well as the repeated relationship of this microorganism with goats, it was suggested that a new member of the complex had been identified, which does not match any of the already existing classical species. These isolates where then given the proposed name *M. tuberculosis* subsp. Capraesubsp.nov (Aranaz et al., 1999). Strains of this unusual member of the MTC have shown a unique combination of pncA, axyR, katG and gyrA gene polymorphisms, while the analysis of the sequence of the gyrB gene in the same strains revealed nucleotide substitutions that are not found in other members of the MTC that could be used in the differentiation of caprine mycobacterial strains from *M. bovis* and other members of the MTC. Based on this, it was then proposed that *M. tuberculosis*subspcaprae be elevated to species status, as *Mycobacteriumcaprae*comb.nov, sp.nov (Aranaz et al., 1999).

(vi) Mycobacterium pinippedii

In 1993, Tuberculosis was diagnosed in Australia in seals (pinnipeds) (Brosch, 2002). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and Western blotting techniques were once used to determine the similarity of mycobacterium species strains that were isolated from the lungs of 2 seals, the secretory protein MPB70 that is present in *M. bovis* was not detected in the wild seal isolates. When further analysis of protein and DNA fragment profiles was done, it indicated that TB isolates that were found in seals formed a special cluster within the *MTC.* Another test was done where *MTC i*solates from seals in Australia, Argentina,

Uruguay, Great Britain and New Zealand were compared to one another, in an effort to determine their relationships to each other and their taxonomic position within the complex (Cousins et al., 2003), and using biochemical tests, it was confirmed that the seal isolates belonged to the MTB complex. When grown on media it was noted that, in many cases the isolates grew preferentially on media containing sodium pyruvate and there were minor differences that were noted (Asiimwe, 2008). Further analysis was done which showed all isolates contained the sequences IS6110, IS1081, mpb70 and mtp40 however, they failed to produce detectable MPB70 antigen. The pncA gene in *Mycobacterium pinippedii* contained CAC (His) at codon 57 and the oxyR gene showed G at position 285, and this was noted to be similar to *M. tuberculosis, M. microti*as well as *M. africanum*. When spoligotyping was done on the seal isolates, the spoligotypes were noted to from a cluster that differed from those of other MTC members. The proposed name for this novel member of the MTC was *Mycobacterium pinnipedii* sp. nov. (Cousins et al., 2003).

(vii) Mycobacteruim canettii

An unusual mycobacterial strain was once isolated from a 2 year old patient with lymphadenitis in Somali. Van Sooligen et al (1997), in an attempt to characterize the strain, applied various molecular methods. It was noted that the bacillus produces smooth and glossy colonies, this characteristic was exceptional this species. Another study was conducted by Miltgen et al (2002), and they identified the same smooth, glossy bacillus from an unusual strain of mycobacteria from two different patients who had pulmonary TB. Spoligotyping and IS6110 RFLP revealed that it was *M. canettii*. All known cases of TB caused by *M. canettii* are said to have been contracted in the Horn of Africa (Pfyffer et al., 1998). *M. canettii* and M. tuberculosis have some close similarities, and because of that the prevalence of *M. canettii* taxon isolates may be underestimated in the routine microbiology laboratory (Asiimwe, 2008).

1.2.2 Characteristics of M.tuberculosis

(i) Growth characteristics of M.tuberculosis

Mycobacteria are obligate aerobes and derive energy from oxidation of simple carbon compounds. Increased CO_2 tension enhances growth. Biochemical activities are not characteristic, and the growth rate is much slower than that of most bacteria. The doubling time of tubercle bacilli is about 18 hours. Saprophytic forms tend to grow more rapidly at 22-23^{o}C, produce more pigment and less acid-fast than pathogenic forms.

(ii) Cell Wall structure

The cell wall structure of *M. tuberculosis* deserves special attention because it is unique among procaryotes and it is a major determinant of virulence for the bacterium. In addition to peptidoglycan, the cell wall complex contains glycolipids such as arabinogalactan-lipid complex and mycolic acid. Over 60% of the mycobacterial cell wall is lipid. The lipid fraction of the cell wall consists of three major components.

Mycolic acids are unique alpha-branched lipids found in cell walls of *Mycobacterium* spp. They make up 50% of the dry weight of the mycobacterial cell envelope. Mycolic acids are strong hydrophobic molecules that form a lipid shell around the organism and affect permeability properties at the cell surface.

Trehalosedimycolate, also named “cord factor” has been an intensely studied cell envelope componund of *M. tuberculosis*. There is a high correlation between the virulence of stains of tubercle bacilli and their morphologic appearance in culture in the form of serpentine cords consisting of bacilli in close parallel arrangements. Growth in cords can be correlated with the presence of the trehalosedimycolate.

(iii) Genomics of M. tuberculosis

The genome of *M.tuberculosis* contains 4000 genes, distributed fairly evenly between the two strands and accounting for >91% of the potential coding capacity. Genes were classified into 11 broad functional groups and today, precise or putative functions can be attributed to 52%, with the remaining 48% being conserved hypotheticals or unknown (Camus *et al*, 2002). Over 51% of the genes have arisen as a result of gene duplication or domain shuffling events and 3-4% of the genome is composed of insertion sequences (IS) and prophages (phiRvl, phiRv2). These are 56 copies of IS elements belonging to the well-known IS*3*, IS*5*, IS*21*, IS*30*, IS*110*, IS*256* and IS*L3* as well as a new IS family, IS*1535*, that appears to employ a frameshifting mechanism to produce its transposaes (Gordon et al. 1999). IS *6110*, member of the IS*3* family, is the most abundant element and has played an important role in genome plasticity. The complete genome sequence of *M. tuberculosis* H37Rv comprises 4411529 bp and has a mean G+C content of 65.6 mol%.

(iv) Virulence Factors

Studies of *M. tuberculosis* virulence factors have been hindered by the fact that the generation time of *M. tuberculosis* is 18 to 24 h(which means it take about a week to form a visible colony on agar medium). Despite difficulties, methods for extracting clonable DNA and introduction of foreign DNA into *M. tuberculosis* have been developed. In fact, a transposon that integrates randomly enough to be used for transposon mutagenesis has been found. Cultured mammalian cells, especially lines of macrophagelike cells, are now being used to investigate *M.tuberculosis* virulence factors.

(v) Entry and survival in phagocytes

A key virulence property of *M.tuberculosis* is its ability to multiply inside monocytes and macrophages. It also invades PMNs, but macrophages, especially the activated ones, are the key

to development of the infection. *M.tuberculosis* binds directly to macrophage surface protein CR3, the normal receptor for iC3b. The bacteria also bind to other macrophage receptor protein, such as CR4. Binging is followed by internalization of the bacteria in a vesicle. Macrophage that take up *M.tuberculosis* have a reduced ability to carry out phagolysosome fusion, because the bacteria prevent the interior of the vesicle from acidifying and reduced oxidative burst. Another effect of the bacteria on the macrophage is to reduce the production of IL-12, a cytokine that stimulates the Ph response.

The ability of *M.tuberculosis* to bind to more than one surface protein on phagocytic cell may increase the ability of the bacteria to invade a variety of cells. Some of the bacteria escape the phagosome and multiply in the cytoplasm. The infection with *M.tuberculosis* elicits an MHC-I associated cytotoxic T-cell response which is usually elicited by microorganisms growing in the cytoplasm or nucleus of the macrophage. The phenollic glycolipids from mycobacteria can scavenge toxic oxygen radicals in vitro (Salyers and Whitt, 2002).

(vi) Avoidance of the activated macrophage response

M.tuberculosis is killed by activated macrophages. IFN-γ (and possibly other cytokines as well) produced by activated T cells, especially $CD4^+$ cells, is essential for activating macrophages. *M.tuberculosis* produces some compounds that (at least in vitro) interfere with T-cell activation. For example, lipoarabinomannan, a mycobacterial cell wall glycolipid, suppresses T-cell proliferation. It also blocks transcriptional activation of interferon-inducible genes in macrophage cell lines and might thus prevent interferon from trigerring macrophage activation.

A protein complex secreted by *M.tuberculosis*, antigen 85, is now thought to catalyze the mycolation of cell wall polysaccharides. Antigen 85 might serve as a virulence factor in the sense that it is a powerful immunostimulant that elicits an antibody response (Salyers and Whitt, 2002).

(viii) Ability to elicit a destructive inflammatory response

A few proteins have been identified that can evoke a skin test response when administered in purified from (e.g., antigen 85B, 85C and MPB70, a 23-kDa secreted protein of *M.bovis* BCG), but little is known about the antigens most important in provoking the destructive cell-mediated response that causes lung damage. Mycolic acids from the mycobacterial cell wall are toxic when injected into animals, and these compounds could act by stimulating the inflammatory response. Also, muramyl dipeptide, a cell wall component cycobacteria, is well known for its ability to stimulate the immune system and trigger cytokine production.

One popular hypothesis is that realease of TNF-α, provoked by a combination of mycobacterial cell surface components, cause most of the lung damage. Injection of TNF-α into lungs causes damage similar to that seen in tuberculosis, and antibodies to TNF-α reduce lung damage in animals infected by the bacteria without significantly affecting the growth of the bacteria themselves. Thus, bacterial products that elicit TNF-α could be important in the disease process. Release of toxic lysosomal components by macrophages trying to ingest and kill the bacteria by also contribute to lung damage *(Salyers et al., 2002)*

1.2.3 The Disease Tuberculosis

(a) Transmission of tuberculosis

Tuberculosis (TB) is transmitted exclusively by people with active pulmonary or larengeal forms of disease who expectorate bacilli in droplet nuclei as they cough, sneeze or talk. In poorly ventilated, enclosed environments, bacilli can remain air-borne for several hours. *M.tuberculosis* is an intracellular pathogen. Transmission required inhaled bacteria to reach the alveoli in the lung periphery and to be ingested by the alveolar macrophage. Each macrophage then rapidly transports the bacilli via the lymphatic system to the hilar lymph nodes and if replication is not checked infection can reach almost any area of the body, including bones,

joints, liver, spleen, gastrointestinal tract and brain giving rise to miliary tuberculosis which is always fatal.

(b) Clinical presentation

Pulmonary disease is the most common clinical manifestation in 80% cases. The patients with pulmonary Symptoms of TB are as follows: i) cough of more than two weeks duration; ii)weight loss; iii) fatigue; iv) fever; v) night sweat; vi) shortness of breath; vii) chest pain; and viiii) haemopthysis (coughing up blood) in later stages. Tissue destruction can result in scarring and fibrosis and if infection is untreated erosion though blood vessels can occur leading to massive haemoptysis.

(c) Stages of the Disease

TB is a highly contagious disease. Transmission is most likely when there is prolonged close contact between a susceptible person and a person who has an active TB. The word "infect" needs to be defined carefully. A person is considered infected with *M.tuberculosis* if he or she concerts from negative to positive on a TB skin test. Only 1% of infected people develop active TB (Salyers et al., 2002). Disease progression depends on: i) stain of *M. tuberculosis*; ii) prior exposure iii) vaccination; iv) infections dose; and v) immune status of the host

Stage 1: Droplet nuclei are generated by during talking coughing and sneezing of a patient with tuberculosis. The droplet nuclei contain tubercle bacilli. When these droplet nuclei are inhaled, by healthy adult, activated macrophages generally appear early enough to stop the infection before appreciable damage is done the lung. But if such things do not happen, *M.tuberculosis* multiplies virtually unrestricted within inactivated macrophages until the macrophages burst. Other macrophages begin to extravasate from peripheral blood. These macrophages also

phagocytose *M.tuberculosis*., but they are also inactivated and hence cannot destroy *M.tuberculosis.*

Stage 2: Since phagocytic cells are not clearing the infection, new T cells, polymorpho nuclear lymphocytes (PMN) and macrophages continue to be attracted to the area and accumulate around the sites where bacteria are growing. Macrophages in the vicinity of the bacteria fuse to form giant cells and a layer of macrophages and T cells forms around a growing focus of damaged tissue containing the bacteria. The lymphocytes, specifically T-cells, recognize processed and presented MTB antigen in context of MHC molecules. This results in T-cell activation and the liberation of cytokines including gamma interferon (IFN). The liberation of IFN causes in the activation of macrophages. These activated macrophages are now capable of destroying *M.tuberculosis.*

It is at this stage that the individual becomes tuberculin-positive. This positive tuberculin reaction is the result of the host developing a vigorous cell mediated immune (CMI) response. A CMI response must be mounted to control an *M.tuberculosis* infection. An antibody mediated immune (AMI) will not aid in the control of a *M.tuberculosis* infection because *M.tuberculosis* is intracellular and if extracellular, it is resistant to complement killing due to the high lipid concentration in its cell wall. Although a CMI response is necessary to control an *M.tuberculosis* infection, it is also responsible for much of the pathology associated with tuberculosis. Activated macrophages may release lytic enzymes and reactive intermediates that facilitate the development of immune pathology. Activated macrophages and T-cells also secrete cytokines that may also play a role in the development of immune pathology, including Interleukin 1 (IL-1), tumor necrosis factor (TNF) and IFN-γ.

In some cases, although the phagocytes are not able to kill the bacteria, the T cells and macrophages are successful in walling off the growing lesion with a thick fibrin coat. The walled-off lesion is called a tubercle. The center of the tubercle is characterized by "caseation necrosis" meaning semi-solid or "cheesy" consistency. *M.tuberculosis* cannot multiply within these tubercles because of the low pH and anoxic environment but can, however, persist within these tubercles for extended periods.

Stage 3: Although many activated macrophages can be found surrounding the tubercles, many other macrophages present remain inactivated or poorly activated. *M.tuberculosis* uses these macrophages to replicate and hence the tubercle grows.

The growing tubercle may invade a bronchus. If this happens, *M.tuberculosis* infection can spread to other parts of the lung. Similarly the tubercle may invade and artery or other blood supply line. The hematogenous spread of *M.tuberculosis* may result in extrapulmonary tuberculosis otherwise known as miliary tuberculosis. The secondary lesions caused by miliary TB can occur at almost any anatomical location, but usually involve the genitourinary system, bones, joints, lymph nodes, and peritoneum.

Stage 4: As bacteria continue to divide and phagocytes continue to enter the area, the caseous centers of the tubercles liquefy. This liquid is very conducive to MTB growth and hence the organism begins to rapidly multiply extracellular. After time, the large antigen load causes the walls of nearby bronchi to become necrotic and rupture. This results in cavity formation. This also allows MTB to spill into other airways and rapidly spread to other parts of the lung.

When the primary lesion heals, it becomes fibrous and calcifies. When this happens the lesion is referred to as the Ghon complex. Depending on the size and severity, the Ghon complex may never subside. Typically the Ghon complex is readily visible upon chest X-ray.

An unusual feature of *M.tuberculosis* is its ability to survive decades in the lesions Later in life, suppression of the immune system (e.g., by cancer, immunosuppressive drugs or AIDS) may allow the bacteria to break out of the lesion and begin to multiply again. This form of the disease, called reactivation TB, is identical to primary TB in its infectiousness and in the damage it can cause. A person who has an HIV infection has a 5 to 10% per year probability of developing reactivation TB.

(d) Diagnosis of tuberculosis

Early identification of people with symptomatic TB not only allows therapy to be administered before serious lung damage occurs but also helps to prevent the spread of the bacteria to other susceptible people.

(i) Microscopy

Acid-fast staining of sputum (material coughed up from the lungs) has been used as a diagnostic test for years. This test is complicated by the fact that other pathogenic *Mycobacterium* species also are acid-fast, so detecting acid-fast rods does not give a definitive diagnosis of TB. Also, unless the bacteria are present in high numbers, they will not be readily found by microscopic examination. Despite these limitations, acid-fast staining has proved to be a very useful test.

(ii) Light-emitting diodefluorescence microscopy

Light-emitting diodes (LEDs) provide a relatively inexpensive light source for fluorescence microscopy. LED microscopes or attachments require less power than conventional fluorescence microscopes and can run on batteries. Also, the bulbs have a long half-life and do not release potentially toxic products if they are broken.

Evidence shows that the diagnostic accuracy of LED microscopy is comparable to that of conventional fluorescence microscopy and it surpasses that of conventional Ziehl–Neelsen microscopy (by an average of 10%). Therefore, WHO recommends replacing conventional fluorescence microscopy with LED microscopy, and that LED microscopy should be phased in as an alternative to conventional Ziehl–Neelsen light microscopy in all settings, prioritizing high-volume laboratories.

(iii) Culture and species identification

Mycobacteria can be cultured in specific solid or liquid media. Bacterial growth can be identified visually (that is, by identifying specific characteristics) or by automated detection of its metabolism. All positive *mycobacterial* cultures must be tested to confirm the identification of *M. tuberculosis complex* (MTBC).

The *M. tuberculosis* complex comprises eight distinct closely related organisms, the most common and important agent of human disease is *M. tuberculosis*. The complex includes *M. bovis* (the bovine tubercle bacillus—characteristically resistant to pyrazinamide, once an important cause of TB transmitted by unpasteurized milk, and currently the cause of a small percentage of human cases worldwide), *M. caprae* (related to *M. bovis*), *M. africanum* (isolated from cases in West, Central, and East Africa), *M. microti* (the "vole" bacillus, a less virulent and rarely encountered organism), *M. pinnipedii* (a bacillus infecting seals and sea lions in the Southern Hemisphere and recently isolated from humans),

M.mungi (isolated from banded mongooses in southern Africa), *M. orygis* (described recently in oryxes and other Bovidae in Africa and Asia and a potential cause of infection in humans), and *M. canetti* (a rare isolate from East African cases that produces unusual smooth colonies on solid media and is considered closely related to a supposed progenitor type).

(iv) Drug-susceptibility testing

Drug Susceptibility Testing (DST) determines whether a strain is susceptible to particular anti-TB agents: a result indicating that the strain is sensitive to particular agents means that treatment with those agents will most likely be successful; a result indicating that a strain is resistant means that there is a high possibility that treatment with those agents will fail and, therefore, other agents should be used. Thus, using standardized and reliable DST for *M. tuberculosis* provides guidance on treating a patient. Following are the types of different available techniques:

- Phenotypic methods involve culturing *M. tuberculosis* in the presence of anti-TB agents to detect growth (which indicates resistance) or inhibition of growth (which indicates susceptibility).
- Genotypic methods target specific molecular mutations associated with resistance against individual anti-TB agents.

(v) Drug-susceptibility testing for second- line anti-TB agents

Commercial liquid methods and the proportion method used on solid media have been studied; methods for the absolute concentration or resistance ratio methods on solid media for second-line anti-TB agents have not been validated. The recommended gold standard for DST for second-line anti-TB agents is the automated liquid system.36 Routine DST for second-line agents is not recommended unless laboratory infrastructure and capacity have been established, rigorous quality assurance is in place and sustained proficiency has been demonstrated.

Phenotypic DST for second-line injectableagents (kanamycin, amikacin, capreomycin) and fluoroquinolones (ofloxacin, levofloxacin, moxifloxacin, gatifloxacin) is generally reliable and reproducible across various settings. The susceptibility of M. *Tuberculosis* to all

fluoroquinolones used by a national TB programmes should be tested to guide the choice of the most appropriate agent for treatment.

Current molecular methods cannot replace phenotypic DST for second-line agents because there is incomplete cross-resistance among second-line injectable agents. Current molecular methods cannot identify resistance to specific second-line injectable agents; thus, they cannot be used to guide the choice of second-line agents included in individualized MDR-TB regimens.

Routine DST for other second-line agents (such as ethionamide, prothionamide, cycloserine, terizidone, p-aminosalicylic acid, clofazimine, amoxicillin/clavulanic acid, clarithromycin and linezolid) is not recommended because the reliability and reproducibility tests for these anti-TB agents cannot be guaranteed.

(vi) Molecular testing

Genotypic methods have considerable advantages when the programmatic management of drug-resistant TB is being scaled up, in particular with regard to their speed, the standardization of testing, their potentially high throughput and the reduced requirements for biosafety. The ultimate aim should be to use molecular assays – including LPAs, Xpert MTB/RIF, and any other molecular platform that may be recommended by WHO in the future – for rapid first-step identification of RR- TB and MDR-TB.

Line-probe assays

Performing an LPA involves extracting DNA from M. tuberculosis isolates or directly from clinical specimens and using polymerase chain reaction (PCR) to amplify the resistance-determining region of the rpoB gene using biotinylated primers. Subsequently, labelled PCR products are hybridized with specific oligonucleotide probes immobilized on a strip.

Colorimetric development of the captured and labelled hybrids enables the presence of *M. tuberculosis* complex to be detected as well as the presence of wild- type *M. tuberculosis*. It also detects mutations associated with drug resistance. If a mutation is present in one of the target regions, the amplicon will not hybridize with the relevant probe. Therefore, mutations are detected by a lack of binding to wild-type probes as well as by binding to specific probes for the most commonly occurring mutations. The post-hybridization reaction leads to the development of coloured bands on the strip at the site of probe binding, and it can be read by the laboratory technician.40 In 2015, WHO plans to update the policy recommendations on LPA for the detection of rifampicin resistance conferring mutations as well as utility of LPA in detection resistance to fluoroquinolones (FQ) and second-line injectable anti-TB agents.

Xpert MTB/RIF assay

The Xpert MTB/RIF assay is an automated, cartridge-based nucleic acid amplification test (NAAT) that uses the multidisease GeneXpert platform. The Xpert MTB/RIF assay is performed directly on sputum, processed sputum sediment and selected extrapulmonary specimens from adults and children. GeneXpert instruments are modular, and options include systems with the capacity to have 1, 2, 4, 16, 48 or 80 independently functioning modules.

The technology was first recommended by WHO in 2010, and a policy update was issued in 2013 following the meeting of an expert group to assess its use for detecting pulmonary and extrapulmonary TB and rifampicin resistance in adults and children.35, 41 The "how to" Xpert MTB/RIF implementation manual was updated in 2014 by WHO; it describes the operational aspects of and practical considerations associated with introducing and using the system.

(vii) TB skin test

Skin Testing is performed as the tuberculin or Mantoux test PPD (purified protein derivative) is employed as the test antigen in the Mantoux test. PPD is a crude extract containing many mycobacterial proteins. 5 TU (tuberculin units), which equals 0.000lmg of PPD. in a O.l ml volume is injected intradermally. If the person being tested has or previously had tuberculosis, PPD stimulates preprimed $CD4^+$ T-helper cells at the site of the injection to secrete cytokine These cytokines cause PMNs, monocytes and macrophages, to be recruited to the injection site. Leakage of fluid due to migration of phagocytes through the blood vessel wall, together with the local inflammatory effect of cytokines, produces an area of redness and swelling (erythema). Fibrin deposition triggered by the monocytes and macrophages causes the area to harden (induration). The result is a raised, tough red area around the injection site (positive skin test). No reaction is seen in people who have not been sensitized by previous infection (negative skin test). The skin test must be interpreted with care. After initial exposure to *M.tuberculosis*, it takes about 4 weeks before the person converts to skin test positive. Thus, the skin test does not detect very recent infection. People who have been immunized with the anti-TB vaccine *M. bovis* BCG, become skin test positive, so a positive result gives no information about previous infection in such cases AIDS can cause a person to convert form skin test positive to negative due to loss of skin test reactivity (anergy). People infected with other mycobacterial species can become weakly positive on the skin test.

The need for better diagnostic tests to detect *M.tuberculosis* infections has spurred efforts to develop detection methods that do not require growth of these slow growing organisms. Using PCR to amplify 16S rRNA sequences specific to *M.tuberculosis* strains identified to date carry the same insertion sequence IS6110. Detection of IS 6110 sequence by PCR or by DNA hybridization provides a means of demonstrating that *M.tuberculosis* is present in clinical

specimens. PCR is a rapid detection method but the main drawback is the danger of cross-contamination of specimens with DNA.

(viii) Testing for latent TB infection

Persons with latent TB infection (LTBI) do not have active TB disease but may develop it in the near or remote future, a process called TB reactivation. The lifetime risk of TB reactivation for a person with documented LTBI is estimated to be 5–10%, with the majority developing TB disease within the first five years after initial infection.

A direct measurement tool for M. tuberculosis infection in humans is currently unavailable; hence, there is no gold standard for the diagnosis of LTBI. The tuberculin skin test (TST) and Interferon- gamma release assays (IGRAs) indirectly measure TB infection by detecting memory T-cell response signifying the presence of host sensitization to *M. tuberculosis* antigens.

1.2.4 Multidrug resistant Tuberculosis

(a) Terminology of drug resistance

The term multi-drug resistant *M. tuberculosis* (MDR-TB) has been used in the strict sense of the definition referring to isolates resistant to both isoniazid and rifampicin with or without resistance to other drugs. Primary resistance is that which has not resulted from the treatment of the patient with the drug concerned. It includes resistance in wild strain which have never come into contact with the drug (natural resistance) and the resistance occurring as a result of exposure of the drug but in another patient. Initial resistance is the resistance in patients who give a history of never having received chemotherapy in the past. It includes primary resistance and resistance to previous treatment concealed by the patient or of which the patient was unaware (Vareldzis*et al.*, 1994). The term acquired resistance has often been used with the implication that resistance has developed due to exposure of the strain to anti-tuberculosis drugs and the

consequent selecting out of resistant mutant bacilli. However, some of the drug-resistant isolates in previously treated patients may actually represent primary resistance among patients who remain uncured (Friedene*t al.*, 2002). Susceptible strains are those that have not been exposed to the main anti-tuberculosis drugs and respond to these drugs in a uniform manner. Resistant strains differ from the sensitive strains in their capacity to grow in the presence of higher concentration of a drug. Wild strains are those that have never been exposed to anti-tuberculosis drugs. Naturally resistant strains are wild strains resistant to a drug without having been in contact with it. It is species specific and has been used as a taxonomic marker (Vareldzis*et al.*, 1994).

(b) Factors contributing to multi drug resistance

The factors associated with the emergence of multi drug resistant *M. tuberculosis* and their effects on the epidemiology of tuberculosis are complex and multi-faceted. The factors include: i) non adherence to the therapy; ii) inadequate treatment regimens; iii) poor medical management; iv) lack of direct observed treatment; v) limited or interrupted drug supplies; vi) poor drug quality vii) cost of drugs; viii) widespread availability of anti-TB drug without prescription; ix) dissociation between public and private sector; and x) poorly managed national control programmes (Espinal*et al.*, 2001).

(c) Molecular Basis of Antimicrobial Resistance in Tuberculosis

Understanding the genetic events that lead to drug resistance in clinical *M. tuberculosis* isolates is important for the development of genetic assays, elucidation of the mechanisms of action of antimicrobial agents, and the design of novel antibiotics that are active against drug-resistant strains (Piatek et al., 2000).

(d) Resistance to rifampicin (RIF)

Rifampicin is extremely effective against *M. tuberculosis*. Because of its high bactericidal action, RIF, along with isoniazid, forms the backbone of short-course chemotherapy (Kochi *et al.*, 1993)

RNA polymerase, a complex oligomer composed of four different subunits (α, β, β′and ם, encoded by *rpo*A, *rpo*B, *rpo*C and *rpo*D respectively), is highly conserved among bacterial species (Ovchinnikov*et al.*, 1981). RIF specifically interacted with the β subunit of RNA polymerase, thereby hindering transcription, and that mutations in the *rpo*B locus conferred conformational changes leading to defective binding of the drug and consequently resistance (Jin and Gross, 1988). The *rpo*B locus from *M. tuberculosis* was characterized and mutations conferring the resistant trait were identified (Telenti*et al.*, 1993).

Analysis of approximately 500 Rifampicin strains from global sources has found that 96% of Rifampicin resistant clinical isolates of *M. tuberculosis* have mutations in the 81-bp core region of *rpo*B gene, which encodes the *B* subunit of RNA polymerase. (Musser, 1995). These mutations are absent in susceptible organisms. Missense mutations in codons 513, 526, or 531 result in high level Rifampin resistance, whereas amino acid changes at position 514 or 533 usually result in low levels of Rifampicin resistance. It is estimated that 90% of rifampicin-resistant isolates in some areas are also resistant to isoniazid, making rifampicin resistance a useful surrogate marker for multidrug resistance and indicating that second and third line drugs to which these isolates are susceptible are urgently required (Yuen *et al.*, 1999). The consistency of mutations in the *rpo*B locus and the RIF- resistant phenotype (>95%) has marked clinical implications. Because it may act as a surrogate marker for MDRTB, RIF resistance has prompted development of various diagnostic tests to improve the sensitivity of mutation detection. Although automated sequencing has been unambiguously applied to characterize mutations associated with RIF resistance, a number of other techniques such as polymerase

chain reaction-single stand conformational polymorphism (PCR-SSCP) (Kapur*et al.*, 1995), dideoxy fingerprinting (Felmlee et al., 1995), heminested PCR (Whelen*et al.*, 1995), PCR heteroduplex analysis (Williams *et al.*, 1994), and line probe hybridization (Cooksey *et al.*, 1997) have been successfully applied to detecting these mutations.

(e) Resistance to isoniazid (INH)

Isonizid (isonicotinic acid hydrazide, 4-pyridinecarboxylic acid hydrazide), highly active against the MTB complex (*M. tuberculosis, M. bovis, M. africanum,* and *M. microti)* Evidence suggests that INH inhibits the biosynthesis of cell wall mycolic acids (long-chain α-branched β-hydorxylated fatty acids), thereby making the mycobacteria susceptible to reactive oxygen radicals and other environmental factors. Activation of INH to an unstable electrophilic intermediate requires the enzyme catalase-peroxidase (KatG, coded by *katG*) and an electron sink (hydrogen peroxide) (Shoeb*et al.*, 1985), although hydrazine formed after INH spontaneously decomposes may also mediate activation of INH (Maggliozzo and Marcinkeviciene, 1996). Nevertheless, KatG is the only enzyme capable of activating INH, and consequently, KatG mutant *M.tuberculosis* strains are invariably INH resistant. Sequence analysis of *kat*G from INH-resistant strains showed randomly distributed mutations, including point mutations and deletions and insertions of up to 1 to 3 bases (Cockeril*et al,*. 1995). These mutations could disrupt the *kat*G gene, leading to the production of an inactive gene product or a gene product with compromised peroxidative activity. The most common mutation was G>T transversion in codon 463 (Heym*et al,*. 1995). In this G>T change, Leu is substituted for Arg. Four additional missense mutations were identified, including GTG>GCG (f-Met>Ala, position 1), GAC>GCC (Thr-275Pro), AGC>ACC (Ser-315Thr), and CTG>ATG (Leu-587Met). Strains with a deletion of 12 bp resulting in absence of amino acids 120 to 123 and strain with an insertion of 3bp (CAT, IIe) between codons 125 and 126 were identified.

Efforts to determine the factors involved in resistance to INH led to the discovery of the *inh*A locus, which was proposed as the primary target for coresistance to INH and ethionamide. This locus is composed of two open reading frames (ORFs), designated *orf*l and *inh*A, separated by a 21-bp noncoding region. Inh, an enoyl-ACP-reductase, activity is though to use NAD(H) as co-factor. INH susceptibility could result from incorporation of iso-NAD, which is formed as a consequence of the action of KatG on INH, and thus hinders the enzymatic activity of InhA and blocking fatty acid synthesis (Cole, 1994). A T>G transversion, observed in few of the resistant strains, at position 280 in the *inh*A gene, results in the ser94 to ala94 replacement (Banerjee *et al.*, 1994). This replacement, thought to alter the binding affinity of InhA to NAD(H), ultimately results in INH resistance (Dessen*et al.*, 1995). Alternatively, because of mutations in the putative promoter region, hyperexpression of InhA could result in INH resistance.

(f) Resistance to streptomycin (SM)

Streptomycin, one of the oldest drugs known to be active against *M. tuberculosis*, disrupts the decoding of aminoacyl-tRNA and thus inhibits mRNA translation or causes inefficient translation (Davis and Wright, 1997). In *M .tuberculosis*, resistance to SM is attributed, at least partially, to two distinct classes of mutations including point mutations in S12 ribosomal protein, encoded by rpsL gene (Meier *et al.*, 1994), and mutations in the *rrs* operon encoding the 16S rRNA (Meir *et al.*, 1994).

Point mutations in the *rps*L gene result in single amino acid substitutions (Meier *et al.*, 1994) that affect higher order structures of 16S rRNA and thereby confer SM resistance. Mapping of the mutations in the *rps*L gene demonstrated that they primarily affected one of two critical lysine residues at positions 43 and 88 and led to the substitution with either arginine at 88 or arginine and threonine at position 43 (Nair *et al.*, 1993). Mutations in the *rps*L gene accounted for more than two thirds of SM resistant cases.

The genesis of SM resistance in some of the SM-resistant isolates is due to pint mutations in the 16S rRNA(Fig-1.7). Mutations in the *rrs* locus have been mapped to two regions, the 530 loop and the 915 region. Within the 530 loop, C>T transitions at 491, 512 and 516, in addition to the A>C transversion at position 513, are consistent with the SM-resistant phenotype (Meier *et al.*, 1994). Base pairing between residue 524-526 (of the 530 region of the hairpin loop) and residue 504-507 (of the adjacent 510 region bulge loop) (Shaila*et al.*, 1973) results in SM resistance in clinical isolates of *M.tuberculosis* (Meier *et al.*, 1994). Further, G-U wobble base pairing between residues 522-501 stabilizes the pseudoknot formation within 16s rRNA and thereby confers resistance to SM.

(g) Resistance to pyrazinamide (PZA)

Pyrazinamide is a structural analogue of nicotinamide that is used as a first line TB drug. PZA kills semi-dormant tubercle bacilli under acidic conditions. It is believed that in the acidic environment of phagolysosomes the tubercle bacilli produce pyrazinamidase, an enzyme that converts PZA to pyrazinoic acid the active derivative of this compound. To define the molecular mechanism of PZA resistance the *mycobacterium tuberculosis pncA* gene encoding pyrazinamidase has been sequenced. The results have provided evidence that *pncA* mutations conferred PZA resistance. Substitution of Cys 138 with Ser, Gln 141 with Pro, and Asp63with His and deletion G nucleotide at positions 162 and 288 resulted in a defective pyrazinamidase. In contrast, susceptible organisms had wild type sequences. A remarkably wide array of *pncA*mutations resulting in structural changes in the *PncA* has been identified in greater than 70% of drug resistant isolates. It is presumed that these structural changes detrimentally change enzyme function, thereby altering conversion of PZA to its bioactive form (Ramswamy&Musser, 1998).

(h) Resistance to ethambutol (EMB)

Ethambutol [dextro-2,2′-(ethyldiimino)-di-lonol], synthetic compound with profound anti-mycobacterial effects, is a first-line anti-MTB drug. EMB led to rapid cessation of mycolic acid transfer to the cell wall and equally rapid accumulation of trehalose mono and di-mycolates (Kilburn and Takayama, 1981). Mycolic acids attach to the 5′-hydroxyl groups of D-arabinose residues of arabinogalactan and form mycolyl-arabinogalactan-peptidoglucan complex in the cell wall. Disruption of the arabinogalactan synthesis inhibits the formation of this complex and may lead to increased permeability of the cell wall. Subsequently, it was demonstrated that EMB specifically inhibited arabinogalactan synthesis (Takayama and Kilburn, 1989). This agent has been proposed to be an arabinose analog suggesting that arabinosyl transferase was the primary cellular target for EMB.

Molecular genetics approaches applied to *M. tuberculosis* revealed a highly conserved 14-kb region comprising three homologous ORFs designated embC, embA, and embB preceded by a predicted coding region and by orfx (which encodes a putative protein belonging to the short chain alcohol dehydrogenase family) (Telenti*et al*, 1997). The embCAB proteins are believed to be integral membrane proteins, consistent with their role in the synthesis of various arabinan-linkage motifs of the arabinogalactan and lipoarabinomannan (Belanger *et al.*, 1996; Telenti*et al.,* 1997). The great majority (98%) of strains had mutations in codon 306; however, mutations were also identified in 3 additional codon 285, 330 and 630. EmbB mutations are associated with Ethambutol resistance in roughly 70% of Ethambutol isolates of *M. tuberculosis.*

(i) Resistance to fluoroquinolones (FQ)

DNA gyrase (Gyr), a member of the type II DNA topoisomerases is the primary target for fluoroquinolones. Gyr introduces negative supercoils in closed circular DNA molecules and is a heterotetramer (A_2B_2), coded by *gyr*A and *gyr*B respectively.

FQs, synthetic derivatives of nalidixic acid, act by inhibiting DNA supercoiling and relaxation activity of Gyr without affecting the ATPase activity (Kirkegaard and Wand, 1981) and enhance the rate of DNA cleavage by Gyr. Consequently, by binding to the single-stranded DNA, the quinolones may inhibit relegation, thereby imposing an effective transcriptional blick (Lewis *et al.*, 1996), culminating in cellular death. However, question about the specific interaction of quinolones and the Gyr/DNA complexes remain unsolved (Takiff*et al.*, 1994).

Mutations were found to be clustered in a small region in GyrA that is close, approximately 40 residues amino-terminal, in the linear amino acid sequence to the active site tyrosine, Tyr122 (*E. coli* numbering) (Revel *et al.*, 1994). Other single amino substitutions, for residues 88 to 94, were also identified in ciprofloxacin-resistant MTB isolates. Because polymorphism encountered at codon 95 (Ser95>Thr95) occurred in both resistant and susceptible isolates, it may not be involved in acquiring the FQ-resistant phenotype. Alternative mechanisms to *gyr*A mutations, including changes in cell wall permeability and active quinolone efflux pumping, have also been proposed and could account for the low-level resistance among MTB isolated.

(j) Resistance to ethionamide

Ethionamide is a second line anti tuberculosis drug that is thought to inhibit mycolic acid biosynthesis in *M. tuberculosis*. Studies have shown that for certain strains, low level of INH resistance is correlated with co-acquisition of Ethionamide resistance, suggesting that INH and Ethionamide share a common molecular target and most likely the *mab-inhA* genes (Bannerjee*et al.*, 1994).

1.2.5 Molecular epidemiology

One of the aspects of TB research is the epidemiology of M. tuberculosis, with the aim to document the dynamics of the disease in different groups of individuals (Coggon et al., 1997).

This makes it possible for epidemiologist to assume the reason for the disease occurring in a particular setting (Falmer, 2008). In recent years, molecular epidemiological methods have been used comprehensively in transmission studies of M. tuberculosis. The release of the M. tuberculosis genome has greatly increased the knowledge of researchers about the organism significantly, so much that focus can now be directed towards its evolution and genetics (Falmer, 2008).

In the past years, molecular typing approaches have made a great enhancement in the understanding of TB epidemiology by indicating possible epidemiological links between TB patients and also by detecting suspected outbreaks (WHO, 1998). A number of cases of multi drug resistance (MDR) and lack of adherence to therapy were reported in the last few years (Ereqat et al., 2011a; Ereqat et al., 2011b). This recognized that the deterioration in the economical, nutritional and health situations caused by regional conflict increases the likelihood of an increase in misdiagnose patients and the rise of MDR TB case (Ereqat et al., 2012). Early diagnosis, effective treatment as well as identification of factors influencing disease dynamics are therefore considered as major factors in the control of TB.

Just about a decade ago, the only molecular markers available for the epidemiology study of TB were drug susceptibility profiles and phage types (Falmer, 2008). However, there were some limitations to these methods and in recent years, quite a number of genotyping methods have been developed that are based on DNA polymorphisms in the genome of the organism M. tuberculosis. The discovery of polymorphic regions within the genome has led to a whole new era of epidemiology based on molecular methods using these polymorphic regions as markers (Falmer, 2008). Generally, these polymorphisms are located in non-coding regions with different frequencies shown between different strain families. As a consequence, molecular epidemiology of *M. tuberculosis* uses specific genetic markers within the *M. tuberculosis*

genome to study the distribution of *M. tuberculosis* strains as well as how the strain distribution changes over a period of time. Compared to traditional methods, molecular methods give more information and because molecular methods are rapid, they are suitable for use in the diagnosis of TB (Falmer, 2008). It is imperative when conducting epidemiological studies that the marker chosen for a specific method must be suitable for the study setting.

(a) Molecular characterization of multidrug resistant M. tuberculosis

The genome of *M. tuberculosis* has been shown to contain several polymorphic repetitive DNA elements that can be used to discriminate between isolates. Repetitive DNA elements which have been used in molecular typing studies include insertion sequences (IS), such as IS6110, the direct repeat elements (DR), the major polymorphic tandem repeat sequences (PGRS), the polymorphic GC-rich tandem repeat sequences (MPTR), (GTG)5, and exact tandem repeat (ETR) sequences (Frothingham& Meeker O'Connell, 1998). Studies have shown that combinations of molecular typing methods utilizing different repetitive elements may improve discrimination between *M. tuberculosis* isolates (Kremer et al., 1999).

Spoligotyping*:* The direct variable repeat (DVR) spacer oligonucleotide typing technique (spoligotyping) detects DNA polymorphism within the direct repeat (DR) locus of *M. tuberculosis* complex organisms. The DR locus contains multiple DVRs that consist of well-conserved 36-bp DRs interspersed with spacer sequences (spacers) 34 to 41 bp long (Hermans et al., 1991). The order of the DVRs is strongly conserved in the various isolates. Polymorphism in this region appears to comprise mainly the presence or absence of single, discrete DVRs or stretches of contiguous DVRs (van Embden et al., 2000). Differentiation of strains is based on the presence or absence of the spacers in a hybridization pattern (spoligotype), and this has been exploited for the study of the epidemiology of tuberculosis. Although its level of discriminations is much lower than that obtained with the restriction fragment length polymorphism associated

with IS*6110* in most strains (strains carrying five or more IS*6110* copies), the performance of the technique regarding the degree of differentiation and reproducibility is good Furthermore, the polymorphism obtained by spoligotyping of the IS*6110* low-copy-number *M. tuberculosis* complex isolates proved to be superior to the polymorphism obtained by IS*6110*-associated restriction fragment length polymorphism (Goyal *et al.,* 1997). This technique has gained widespread acceptance because it is a simple, rapid, and robust method. Results can be expressed in a simple digital format and are easier to compare and store in comparison with those of other available techniques. Thus, it has been extensively applied, alone or in conjunction with other techniques, for tracking epidemics; for the description of highly prevalent families such as the Beijing family, multidrug-resistant strain W-Beijing (Bifani*et al.,* 1999), and Westem Cape F11 (Victor *et al., 2004*); and to study global epidemiology.

IS6110-restriction fragment length polymorphism (RFLP) is the current gold standard method for *M. tuberculosis* typing (Kremer *et al.*, 1999) and is extensively used for epidemiological and population-based studies. Different sites within the genome of *M. tuberculosis* have been reported as hot spots for the integration of IS*6110.* These include the DR locus, the *ipl*locus, the DKI locus, and the *dnaA-dnaN*region (Small *et al.,* 1994). This suggests that the integration of IS*6110* is not a truly random event and the frequency of transposition is influenced by the site of insertion within the mycobacterial genome. The identification of IS*6110* insertion hot spots may complicate the interpretation of IS*6110*-RFLP data.

Despite the widespread use of IS*6110I*-RFLP, this method is both technically demanding and time consuming. This method is labor intensive and requires culturing of the slow growing mycobacteria. The comparison of large numbers of RFLP fingerprints, even with the introduction of computerized gel documentation systems, can still be problematic. Perhaps the most important limitation of this method is that the data obtained in independent studies are

difficult to compare because of the lack of reproducibility (Kremer *et al.,* 1999) and probability of the results between different laboratories. For strains containing low copy numbers of IS*6110*, integration hotspots may produce "false" clusters which must be subdivided by a second typing method independent of IS*6110* (Kremer *et al.*, 1999)

Variable number tandem repeat (VNTR) analysis

Variable number tandem repeat (VNTR) typing is an invaluable tool for genotyping in higher eukaryotes and provides data in a simple and nonabiguous format based on the number of repetitive sequences in so-called polymorphic micro- or minisatellite regions,. Frothingham and Meeker-O'Connell (Frothingham and Meeker-O'Connell, 1998) have indentified five VNTR loci in *M. tuberculosis* that have recently been tested for epidemiological purposes. However, the polymorphism of these loci proved to be much too low to accurately discriminate different clones. A type of VNTR sequences called mycobacterial interspersed repetitive units (MIRUs), are scattered throughout the *M. tuberculosis* complex genomes (Supply *et al.*, 1997). A recent genome scan revealed that 12 of the 41 MIRU loci present in the *M. tuberculosis* H37Rv genome correspond to human minisatellite-like VNTR regions among nonrelated isolates of different geographic origins (Supply *et al.,* 2000). Since VNTR analysis detects polymorphisms in five independent genetic loci, it would be useful method for subdividing isolates with low copy numbers of IS*6110*, which are poorly discriminated by IS*6110*-RELP.

DNA sequencing

Principle of Sanger method

This technique utilizes 2, 3-dideoxynucleotide triphosphates (ddNTPs), molecules that differ from deoxynucleotides by the having a hydrogen atom attached to the 3' carbon rather than OH group. These molecules terminate DNA chain elongation because they cannot form a phosphodiester bond with the next deoxynucleotide.

In order to perform the sequencing, the double stranded DNA must be converted into single stranded DNA. Sanger reaction consists of the following: a strand to be sequenced (One of the single strands which was denatured using NaOH), DNA primers (short pieces of DNA that are both complementary to the strand which is to be sequenced and radioactively labeled at the 5'end), a mixture of a particular ddNTP(ddATP, ddGTP, ddTTP) and normal dNTP (dATP, dGTP, dCTP, dTTP). The concentration of ddNTP should be 1% of the dNTP. The logic behind this ratio is that after DNA polymerase is added, the polymerization will take place and will terminate whenever a ddNTP is incorporated into the growing strand. If the ddNTP is only 1% of the total concentration of dNTP, a whole series of labeled strands will be produced. The length of these strands are dependent on the location of the base relative to the 5' end.

This reaction is performed four times using a different ddNTP for each reaction. When these reactions are completed, a polyacrylamide gel electrophoresis (PAGE) is performed. One reaction is loaded into one lane for a total of four lanes. The gel is transferred to a nitrocellulose filter and autoradiography is performed so that only the bands with the radioactive label on the 5' end will appear. In PAGE, the shortest fragments will migrate the farthest, the bottom-most band indicates that its particular dideoxynucleotide was added first to the labeled primer. The band that migrated the farthest, was in the ddATP reaction mixture. Therefore, ddATP must been the base present on the 3' end of the sequenced strand. The reading can be continued in this fashion. If the bases are read from the bottom up, the 5' to 3' sequence of the strand complementary to the strand sequenced is read.

Automated DNA sequencing

In automated DNA sequencing a cycle sequencing kit is used (e.g Big Dye^{TM} terminator cycle sequencing ready reaction kit). The Kit contained AmpliTaq DNA polymerase and both dNTP

and dye labeled ddNTP, and uses dye terminator cycle sequenceing strategies. In this method only one stand of the desired DNA are copied with one primer and results in the liner increase of the number of copies of one strand of the gene. The growing chains are terminated when dye labeled terminators (ddATP, ddGTP, ddCTP, ddTTP) are incorporated as dideoxynucleotides has no hydroxyl group at the 3' end which forms the phosphodiester bond with the next nucleotide. After cycle sequences unincorporated chain terminators are removed from the reaction mix with alcohol precipitation and the DNA fragments of different length, which ended on a fluorescent-labeled dNTP, are separated according to their length through capillary electrophoresis. Finally the nucleotide bases are detected in ABI Prism-310 Genetic Analyzer machine bye the laser detection system.

DNA sequence databases

EMBL

EMBL, the nucleotide sequence database from the European Bioinformatics Institute (EBI), includes sequences both from direct author submissions and genome sequencing groups and from the scientific literature and patent applications (Stoesser*et al.*, 1998). The database is produced in collaboration with DDBJ and GenBank. Information can be retrived from EMBL using the Sequence Retrieval System (SRS) (Etzold*et al.*, 1996); this links the principal DNA and protein sequence databases with motif, structure, mapping and other specialist databases.

DDBJ

DDBJ is the DNA Data Bank of Japan, Which began in 1986 as a collaboration with EMBL and GanBank (Tateno et al., 1998). The database is produced, maintained and distributed at the National Institute of Genetics; sequences may be submitted to it from all corners of the world by means of a Web-based data-submission tool. The Web is also used to provide standard search tools, such as FastA and BLAST.

GenBank

GenBank, the DNA database from the National Center for Biotechnology Information (NCBI), incorporates sequences from publicly available sources, primarily from direct author submissions and large scale sequencing projects.

1.3 Aims and objectives

Early diagnosis of susceptible TB and prevent drug-resistant TB in Bangladesh

General objective of the study was to improve our overall understanding of genotpypic diversity of MTBC strains circulating in Bangladesh.

The specific objectives of the research were:

1. To characterize and classify the multi-drug resistant M. tuberculosis strains collected from different government tuberculosis detection and control centers of Bangladesh
2. To investigate the molecular epidemiology of the multi-drug resistant find out the genotypic mutations in the rpoBandkatGgenes, which might be responsible for the drug resistance M. tuberculosis strains

Chapter 2

MATERIALS AND METHODS

2. Materials and Methods

The study settings were selected purposively and conducted in National TB Reference Laboratory (NTRL), National Institute of Diseases of Chest and Hospital (NIDCH), Mohakhali and Department of Microbiology, University of Dhaka, Dhaka, Bangladesh

Total 371 sputum samples from NTRL were collected. These sputum samples were received from all over Bangladesh (Appendix III).

The materials and methods of the study can be shortly described through the mentioned flow chart (Figure 2.1).

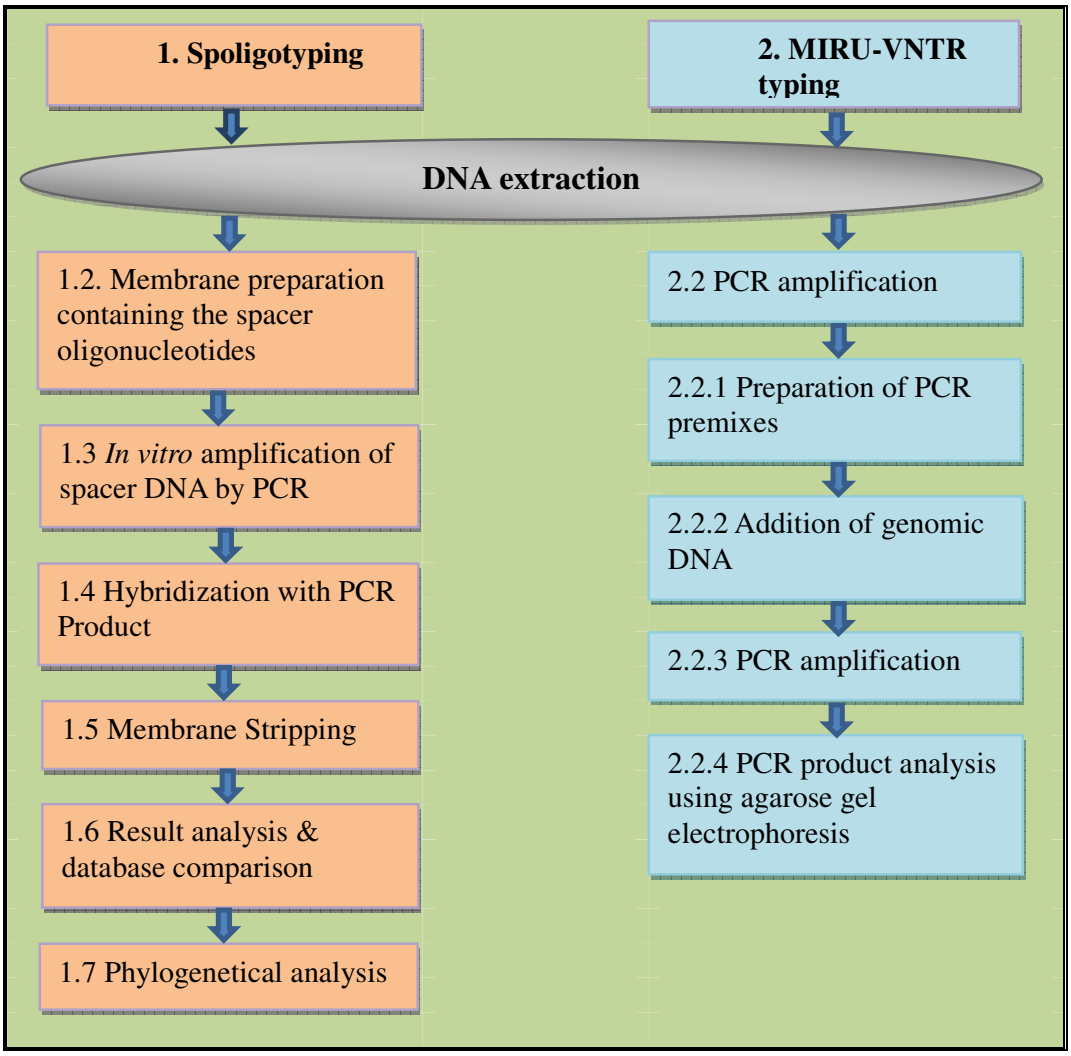


Figure 2.1: Methodological Matrix

2.1 Spoligotyping

2.1.1DNA extraction

DNA extractions from isolates were performed using InstaGene™ Matrix (BioRad). DNA was extracted from *M. tuberculosis* cells gown on solid culture media by using InstaGene matrix kit (Bio-Rad, USA). The growth from a culture was scraped with a loop and bacterial suspension was made in 1 mL of sterile distilled water in a microcentrifuge tube. The bacterial cell suspension was centrifuged for 1 minute at 10,000–12,000 rpm and supernatant was removed. 100 µL of InstaGene matrix (InstaGene matrix was being mixed at moderate speed on a magnetic stirrer to maintain the matrix in suspension) was added to the pellet and incubated at 56°C for 30 minutes. After vortexing the tube at high speed for 10 seconds, it was placed in a 99°C heat block for 10 minutes. Then the microcentrifuge tube was again vortexed at high speed for 10 seconds and then was centrifuged at 13,000 rpm for 2-3 minutes. The resulting supernatant was transferred to another microcentrifuge tube and was stored at -20°C.

2.1.2 Preparation of the membrane containing the spacer oligonucleotides

The spacer oligonucleotide sequences are derived from DNA sequences of the DR region in the strains *Mycobacterium tuberculosis* H37Rv and *M. bovis* BCG P3. All spacer-oligonucleotides are synthesized with a 5' terminal amino group, by which they can be covalently linked to an activated negatively charged Biodyne C membrane.

The spacer oligonucleotides were diluted to the optimized concentrations (Table – 2.1) in 150 µl 500 mM NaHCO3, pH 8.4. The Biodyne C membrane was activated by 10 min incubation in 10 ml freshly prepared 16% (w/v) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) in demineralized water, in a rolling bottle at room temperature. The membrane was then placed in a plastic container and shaken with demineralised water for 2 min and placed on a support cushion in a clean miniblotter system. The screws were turned to hand-tight.

Table 2.1: Oligonucleotide names, sequences and optimum concentration of each of them

Oligo number	Oligo name	Sequence	Concentration pmol/150 ul
1	SP-F310A	ATAGAGGGTCGCCGGTTCTGGATCA[1]	25
2	SP-F320A	CCTCATAATTGGGCGACAGCTTTTG[2]	700
3	SP-F330A	CCGTGCTTCCAGTGATCGCCTTCTA	20
4	SP-F340A	ACGTCATACGCCGACCAATCATCAG	40
5	SP-F350A	TTTTCTGACCACTTGTGCGGGATTA	35
6	SP-F360A	CGTCGTCATTTCCGGCTTCAATTTC	25
7	SP-F370A	GAGGAGAGCGAGTACTCGGGGCTGC	50
8	SP-F380A	CGTGAAACCGCCCCCAGCCTCGCCG	300
9	SP-F390A	ACTCGGAATCCCATGTGCTGACAGC	25
10	SP-F400A	TCGACACCCGCTCTAGTTGACTTCC	20
11	SP-F410A	GTGAGCAACGGCGGCGGCAACCTGG	40
12	SP-F420A	ATATCTGCTGCCCGCCCGGGGAGAT	150
13	SP-F430A	GACCATCATTGCCATTCCCTCTCCC	20
14	SP-F440A	GGTGTGATGCGGATGGTCGGCTCGG	35
15	SP-F450A	CTTGAATAACGCGCAGTGAATTTCG	400
16	SP-F455A	CGAGTTCCCGTCAGCGTCGTAAATC	25
17	SP-F457A	GCGCCGGCCCGCGCGGATGACTCCG	100
18	SP-F460A	CATGGACCCGGGCGAGCTGCAGATG	20
19	SP-F470A	TAACTGGCTTGGCGCTGATCCTGGT	25
20	SP-F480A	TTGACCTCGCCAGGAGAGAAGATCA	25
21	SP-F490A	TCGATGTCGATGTCCCAATCGTCGA	400
22	SP-F500A	ACCGCAGACGGCACGATTGAGACAA	50
23	SP-F510A	AGCATCGCTGATGCGGTCCAGCTCG	75
24	SP-F520A	CCGCCTGCTGGGTGAGACGTGCTCG	75
25	SP-F530A	GATCAGCGACCACCGCACCCTGTCA	35
26	SP-F540A	CTTCAGCACCACCATCATCCGGCGC	35
27	SP-F550A	GGATTCGTGATCTCTTCCCGCGGAT	75
28	SP-F560A	TGCCCCGGCGTTTAGCGATCACAAC	45
29	SP-F570A	AAATACAGGCTCCACGACACGACCA	35
30	SP-F580A	GGTTGCCCCGCGCCCTTTTCCAGCC	20
31	SP-F590A	TCAGACAGGTTCGCGTCGATCAAGT	30
32	SP-F600A	GACCAAATAGGTATCGGCGTGTTCA	200
33	SP-F610A	GACATGACGGCGGTGCCGCACTTGA	500
34	SP-F620A	AAGTCACCTCGCCCACACCGTCGAA	80
35	SP-F630A	TCCGTACGCTCGAAACGCTTCCAAC	40
36	SP-F640A	CGAAATCCAGCACCACATCCGCAGC	20
37	SP-F650A	CGCGAACTCGTCCACAGTCCCCCTT	25
38	SP-F660A	CGTGGATGGCGGATGCGTTGTGCGC	60
39	SP-F670A	GACGATGGCCAGTAAATCGGCGTGG	75
40	SP-F680A	CGCCATCTGTGCCTCATACAGGTCC	40
41	SP-F690A	GGAGCTTTCCGGCTTCTATCAGGTA	30
42	SP-F700A	ATGGTGGGACATGGACGAGCGCGAC	50
43	SP-F710A	CGCAGAATCGCACCGGGTGCGGGAG	150

[1]The actual sequence in H37Rv of the underlined nucleotide is C
[2] The actual sequence in H37Rv of the underlined nucleotide is GC

The slots of the miniblotter were filled with 150 µl of the diluted oligonucleotide solutions except the first and the last slot. The first and the last slots were filled with drawing pen ink (diluted 1:100 in 2xSSPE buffer) to mark the edges of the membrane. After adding all samples to the wells, the membrane was incubated for 2 min at room temperature.

The oligonucleotide solutions were then removed by aspiration in the same order as in which they were applied to the slots. The membrane was removed from the miniblotter using forceps and inactivated by incubating in 250 ml freshly prepared 100 mMNaOH for 10 min (maximum) in a plastic container, while shaking. Then the membrane was rinsed with demineralized water followed by washing the membrane in a plastic container by gentle shaking in 250 ml 2xSSPE/0.1% SDS for 5 min at 60^0C.

After that the membrane wash washed in a plastic container by gentle shaking in 100 ml 20 mM EDTA pH 8 for 15 min at room temperature. Then then membrane was sealed in plastic wrap with at least 30 mL of 20 mM EDTA pH 8. Then the membrane was stored at 4^0C until use.

2.1.3*In vitro* amplification of spacer DNA by PCR

Amplification of the spacers is accomplished by using the primers DRa and DRb, which enable to amplify the whole DR region. The PCR products are labeled with biotin, because one of the primers is biotinylated. The primers for the PCR are based on the DR sequence:

DRa :5'-GGT TTT GGG TCT GAC GAC-3', biotinylated at 5' end

DRb : 5'-CCG AGA GGG GAC GGA AAC-3'

Firstly, a master mix, including all the reagents for PCR reaction except the sample, was prepared (Table 2.2; 2.3) and was aliquoted into PCR tubes before adding different extracted DNA templates. After a brief spin, The PCR tubes were placed in a thermal cycler (Bio-Rad, USA).

Table 2.2: Components of PCR reaction mixture for amplification of IS*6110* sequence

Reagents	Component Volume in 25 µL Reaction Mixture	Final Concentration
PCR grade water	10.875 µL	
10× PCR reaction buffer	2.5 µL	1×
$MgCl_2$ (25 mM)	2.5 µL	2.5 mM
dNTP mixture (2.5 mM each)	2.0 µL	200 µM
DRa (40 pmol/µL)	1.0 µL	40 pmol/µL
DRb (40 pmol/µL)	1.0 µL	40 pmol/µL
Taq polymerase (5 U/µL)	0.125 µL	0.025 U/µL
Template DNA	5 µL	

Table 2.3: The PCR reaction was performed according to the following program

95 °C	5 minutes	Initial denaturation
95 °C	1 minutes	
55 °C	1 minutes	35 cycles
72 °C	30 seconds	
72°C	7 minutes	Final extension

After this, PCR tubes were stored at -20^{0}C until further analysis. Each PCR tube was then tested for positive amplification by agarose gel electrophoresis. In case of positive amplifications, multiple bands or smears were present in the gel (Figure. 2.2).

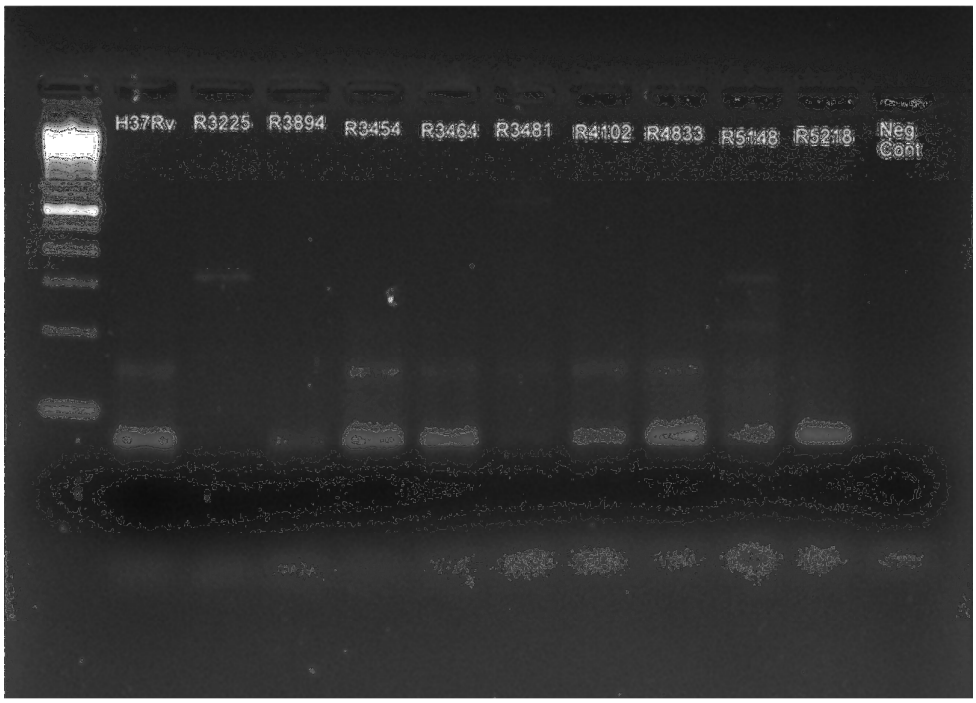


FIGURE 2.2: GEL PICTURE SHOWING POSITIVE AMPLIFICATION OF SPACER DNA BY PCR

2.1.4. Hybridization with PCR Product

The miniblotter for hybridization was thoroughly washed with soap and a dedicated brush before use. All buffers for hybridization purpose was prepared beforehand and was pre-warmed just before use.

The following buffers were prepared prior to carrying out hybridization: 100 ml of SDS with a dilution factor of 10, 250 ml of SSPE/0.1% SDS with a dilution factor of 2 (at 55^0C), 1 L of SSPE/0.5% SDS with a dilution factor of 2 (at 60^0C and 42 ^{0}C) and 500 ml of SSPE with a dilution factor of 2 (at room temperature). All these buffers were pre-warmed before commencement of usage, overnight at 42 ^{0}C except 500 ml of 2 times diluted SSPE buffer.

The hybridizer and water bath were set at 60^0C and 42^0C, respectively. Prior to hybridization, the buffers were warmed according to their recommended temperatures and the membrane was washed for 5 min at 60^0C in 250 ml 2xSSPE/0.1% SDS. The hybridizer was then set at 55^0C and

subsequently the membrane and a support cushion were placed into the miniblotter, making sure that the slots were perpendicular to the line pattern of the applied oligonucleotides. The residual fluids from the slots of the miniblotter were removed by aspiration. Dilution of the PCR products was performed by adding 20-25 µl of each of the products to 150µl 2xSSPE/0.1% SDS. Following heat-denaturation of the PCR products (at 99C for 10 minutes and then cooling immediately on ice), the products were placed in the miniblotter and were hybridized at 55^0C for 1 hour and 15 minutes, on a horizontal surface, avoiding any shaking. Precautions were taken to evade contamination of the neighboring slots. Empty slots were filled with 2xSSPE/0.1% SDS buffer before hybridization.

2xSSPE/0.5% SDS buffer was then warmed in the hybridizer at 60^0C. Samples were removed from the miniblotter by carrying out aspiration for 10 to 20 seconds. Then using forceps, the membrane was removed from the miniblotter and was washed twice in 250 ml 2xSSPE/0.5% SDS for 10 min at 60^0C. In the meantime the hybridizer was set at 42^0C.

After washing, the membrane was placed in a flat bottom container (the shape and size of the container complimented that of the membrane) and was allowed to cool down to prevent inactivation of the peroxidase in the next step. 2ml of streptavidin-peroxidase conjugate (Abcam-Ready to use) (for 500 U/ml stock, 2.5µl is sufficient for 10ml) was added to 30ml of 2xSSPE/0.5% SDS and incubation of the membrane was performed in this solution for 45 to 60 minutes at 42^0C in the container, maintaining moderate shaking. Succeeding incubation, the membrane was washed twice in 250ml of 2xSSPE/0.5% SDS for 10 min at 42^0C and rinsed twice as well with 250ml of 2xSSPE (RT) for 5 min at room temperature. Extra buffer from the membrane was removed by placing the membrane on facial tissue for 30 seconds.

For chemiluminiscence detection of hybridizing DNA, the membrane was incubated precisely for 1 min in 20ml ECL detection liquid. Removal of extra ECL detection liquid from the membrane was executed by touching one corner with facial tissue.

Avoiding any excessive pressure on the membrane, the membrane with the DNA side was placed facing up on a sheet of Saran-wrap and another sheet was placed on top of the membrane (bubbles were carefully removed).

The wrapped membrane was then placed on a photographic cassette, with the DNA side up. Later the cassette was taken to a dark room with only available exposure to red light. The developer, stop bath (water), fixer and final wash (water) were kept on the table and a light sensitive film was positioned on top of the membrane. For the ease of identification of the orientation of the membrane after the developing stage, a random corner of the film was cut. The cassette was then closed and the film was left for exposure for about 5-20 minutes.

After 5-20 min of exposure, the cassette lid was opened and the film was carefully lifted by holding on to one corner. Without any shaking the film was immersed in 1 L of developer solution for 1 min and 30 seconds (~28^0C).

As part of the developing procedure, the film was then lifted from the developer solution just 5 seconds before the preciously mentioned time and was immersed instantly in a container containing water for 30 seconds, with persistent shaking. Following the previous step, the film was placed in fixer solution for 2 to 2.5 minutes (5 seconds of shaking was executed after each 30 seconds). Finally, the film was washed in a separate water container for about 5 minutes and dried by hanging. At this stage the film was ready for examining the pattern of hybridization(Figure 2.3).

Depending on the signal intensity, the membrane could be used again directly, to expose another film for a shorter or longer period.

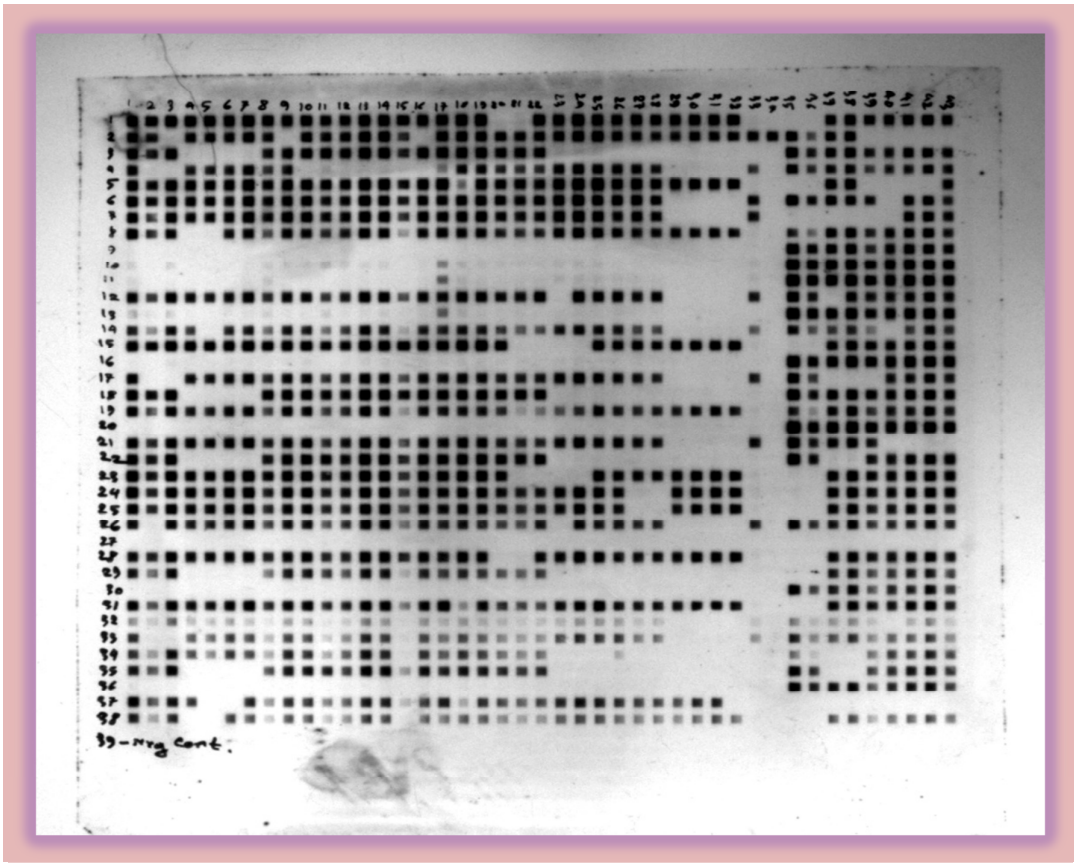


FIGURE 2.3: PATTERN OF HYBRIDIZATION OBTAINED ON LIGHT SENSITIVE FILM

2.1.5. Membrane Stripping

The membrane was washed twice in 250 ml of 1% SDS for 30 minutes while shaking in high speed. Then it was washed with 100ml of 20 mM EDTA solution for 15 minutes. The stripped membrane was sealed in a plastic paper with at least 30 ml 20 mM EDTA solution to avoid drying of the membrane. The membrane was then finally stored at 4^0C for further usage.

2.1.6. Result analysis and database comparison

The patterns obtained for all 43 oligos were taken in binary format – 1 for black spot, 0 for absence of spot. The binary results were then compared by using the publicly released SITVITWEB database (Demay et al., 2012); available online at *http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/*. In this database, Spoligotype International Type (SIT) designates an identical pattern shared by two or more patient isolates, whereas "orphan" designates patterns reported for a single isolate that does not correspond to any of the strains recorded in the database repository.

2.1.7. Phylogenetical analysis

Major phylogenetic clades were assigned according to signatures provided in SITVITWEB for definition of variants within 62 existing lineages/sub-lineages. These include specific signatures for various MTBC members, as well as rules defining major lineages/sub-lineages for *M. tuberculosis* i.e., the Beijing clade, the Central Asian (CAS) clade and two sublineages, the East African-Indian (EAI) clade and nine sublineages, the Haarlem (H) clade and three sublineages, the Latin American-Mediterranean (LAM) clade and 12 sublineages, the "Manu" family and three sublineages, the S clade, the IS6110 – low-banding × clade and four sublineages, and an ill-defined T clade with five sublineages.

Mercat (Molecular Epidemiological Researcher's Collection of Analytical Tools) used to build minimum spanning trees (MST) based on spoligotypingdata.MERCAT expands and improves on an earlier online utility called spolTools and available online at http://mercat.emi.unsw.edu.au/.MST is an undirected network in which all of the isolates are linked together with the fewest possible linkages between nearest neighbors, represented by

branches. The numbers on the edges show weighted genetic distance; the lower the number, the closer the relationship.

2.2 MIRU-VNTR typing

2. 2.1. DNA extraction and dilution

DNA extraction was performed following the protocol described in section 1.1. DNA stored at -20^{0}C was used as template for PCR amplification.

2.2.2 PCR amplification

PCR amplification of different VNTR regions was performed using primers specific for the flanking regions of each VNTR region. PCR was performed on 20 different genotypes using 15 sets of primers that are specific for 15 standard MIRU-VNTR loci (Table 2.4). H37Rv genotype was used as the positive control and a negative control was set up using deionized water. Thus, separate amplification for each locus was performed and further analysis was performed using gel electrophoresis.

Table 2.4: Primers used for amplifying the 15 standard MIRU-VNTR loci are shown in the table along with their corresponding sequences

Multiplex	Locus	Alias	Repeat unit length, bp	PCR primer pairs (5' to 3', with labeling indicated)
Mix 1	580	MIRU 4	77	GCGCGAGAGCCCGAACTGC (FAM) GCGCAGCAGAAACGCCAGC
	2996	MIRU 26	51	TAGGTCTACCGTCGAAATCTGTGAC CATAGGCGACCAGGCGAATAG (VIC)
	802	MIRU 40	54	GGGTTGCTGGATGACAACGTGT (NED) GGGTGAIGTCGGCGAAATCAGATA
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	1644	MIRU 16	53	TCGGTGATCGGGTTCCAGTCCAAGTA CCCGTCGTGCAGCCCTGGTAC (VIC)
	3192	MIRU 31	53	ACTGATTGGCTTCATACGGCTTTA GTGCCGACGTGGTCTTGAT (NED)
Mix 3	424	42	51	GTGGGCGGGGATGAAGGGCATTATT GGCAGCAGAGCCCGGGATTCTTC (FAM)
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	2165	ETR A	75	AAATCGGTCCCATCACCTTCTTAT (NED) CGAAGCCTGGGGTGCCCGCGATT
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	3690	52	98	CGGTTGGAGGCCATGAAGGTCTTC (VIC) TAGAGCGGCACGGGGGAAAGCTTAG
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Mix 5	2163b	QUB-11b	69	CGTAAGGGGGATGCGGGAAATAGG CGAAGTGAATGGTGGCAT (FAM)
	1955		57	AGATCCCAGTTGTCGTCGTC (VIC) CAACATCGCCTGGTTCTGTA
	4052	QUB-26	111	AACGCTCAGCTGTCGGAT (NED) CGGCCGTGCCGGCCAGGTCCTTCCCGAT

2.2.2.1. Preparation of PCR premixes

PCR premixes can be prepared extemporaneously just before the addition of genomic DNA, or stock solutions can be prepared, aliquoted and stored at -20°C until further use. The final concentration of $MgCl_2$ varied from 1.5 mM (default concentration using the 10 X buffer) to 3 mM (by including additional $MgCl_2$), according to the MIRU locus. The final volume per reaction (after addition of DNA) was 25 µl.

In a DNA-free area, the PCR reaction premixes were prepared according to Table 2.5. The void volumes were taken into account and hence 5-10 % proportion in excess of the volume was prepared. After preparing the PCR mixes, 20 µl of the PCR premix were dispensed into each PCR tubes.

Table 2.5: Volumes (µl) for the PCR premixes

Mix	**1**	**2**	**3**	**4**	**5**
Loci	**4-26-40**	**10-16-31**	**0424-0577-2165**	**2401-3690-4156**	**2163b-1955-4052**
$MgCl_2$ final concentration	3mM	2mM	2mM	3mM	3mM
H_20	12.375	12.875	12.875	12.375	12.375
Buffer 10X	2.5	2.5	2.5	2.5	2.5
$MgCl_2$ (50mM)	1.5	1	1	1.5	1.5
dNTP(5mM)	2	2	2	2	2
Primers (each)*	0.75	0.75	0.75	0.75	0.75
Taq. DNA pol (5U/ul)	0.125	0.125	0.125	0.125	0.125
Total Premix	20	20	20	20	20
DNA template	5	5	5	5	5

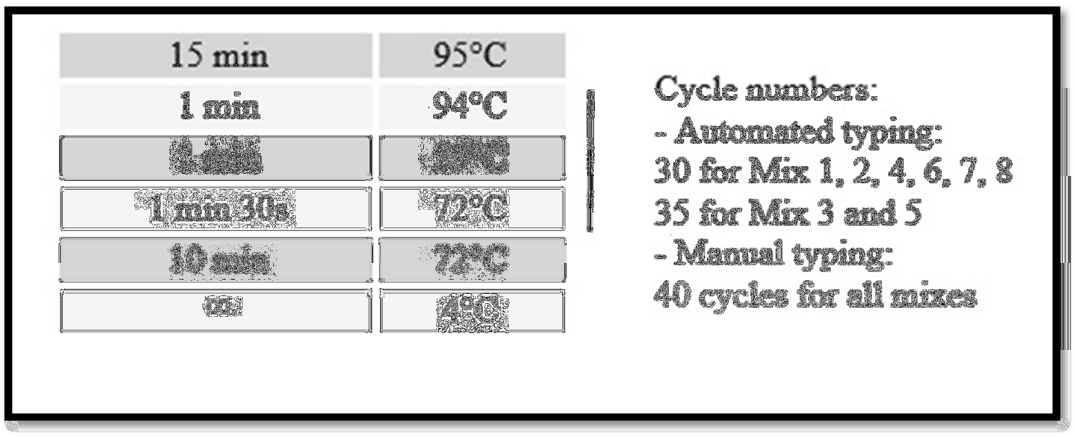
2.2.2.2 Addition of genomic DNA

In another PCR product-free area, 5 µl of extracted DNA was dispensed into the PCR tubes for each set of primers designated to a specific loci. H37Rv DNA and sterile water were used as positive and negative controls, respectively.

2.2.2.3. PCR amplification

The targeted loci were amplified using the PCR conditions indicated in Table 2.6. The number of cycles were adjusted depending on the DNA concentrations routinely obtained in the laboratory, i.e. amplification from weakly or highly concentrated mycobacterial cultures might require 40 cycles or only 25 (Mix 1, 2, 4, 6, 7, 8) to 30 (Mix 3, 5) cycles, respectively. After PCR amplification the products were stored at 4°C or -20°C until further use.

Table 2.6: PCR cycling conditions



2.2.2.4. PCR product analysis using agarose gel electrophoresis

When each locus was amplified separately (i.e. by simplex PCR), the amplified fragments were analysed by electrophoresis using agarose gels. 3% suspension of agarose gel in 1 x TBE solution was prepared and chilled at 4°C to facilitate clump collapse. The agarose was melted

using a microwave oven and agitated periodically until complete dissolution. When the temperature was endurable to the touch, a 25-cm gel was casted using a shark tooth comb. After solidification, the gel was placed into an electrophoresis tank containing 1 X TBE buffer. 2 µl of a 100-bp ladder size standard marker was loaded in both external wells and in the central well of the gel. For each genotype, each well was loaded with 5 µl of PCR amplicon. But prior to loading the PCR products were mixed with approximately 1 µl of loading buffer. The amplicons were run at 100-120 V for a couple of hours. Finally the gel was stained in sterile water containing 0.7 µg/µl of ethidium bromide for 15 to 30 min. At last the gel was exposed to UV light and picture was taken for data analysis (Figure 2.4 and 2.5).

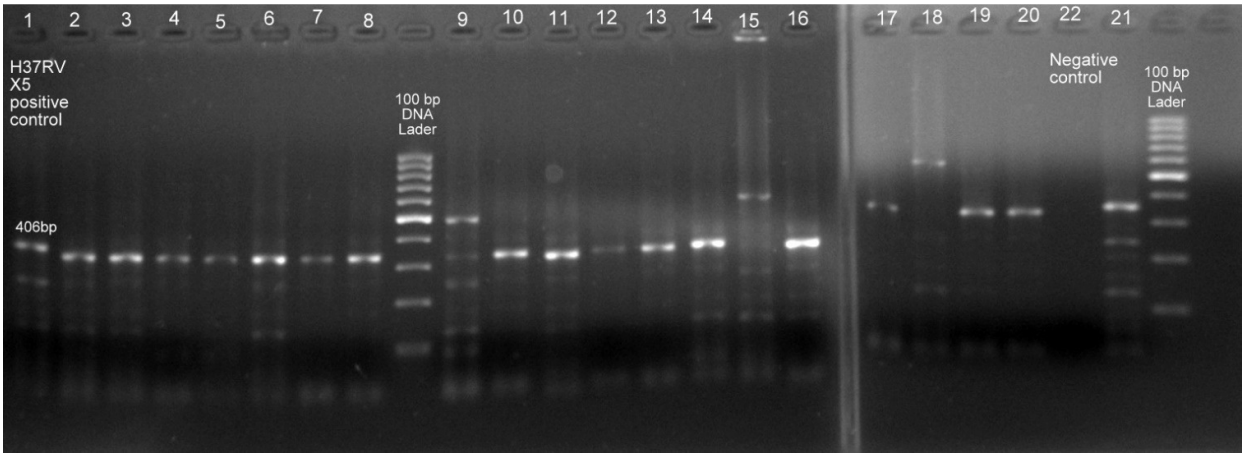


FIGURE 2.4: GEL ELECTROPHORESIS PICTURE OF PCR PRODUCTS TARGETING LOCUS 580

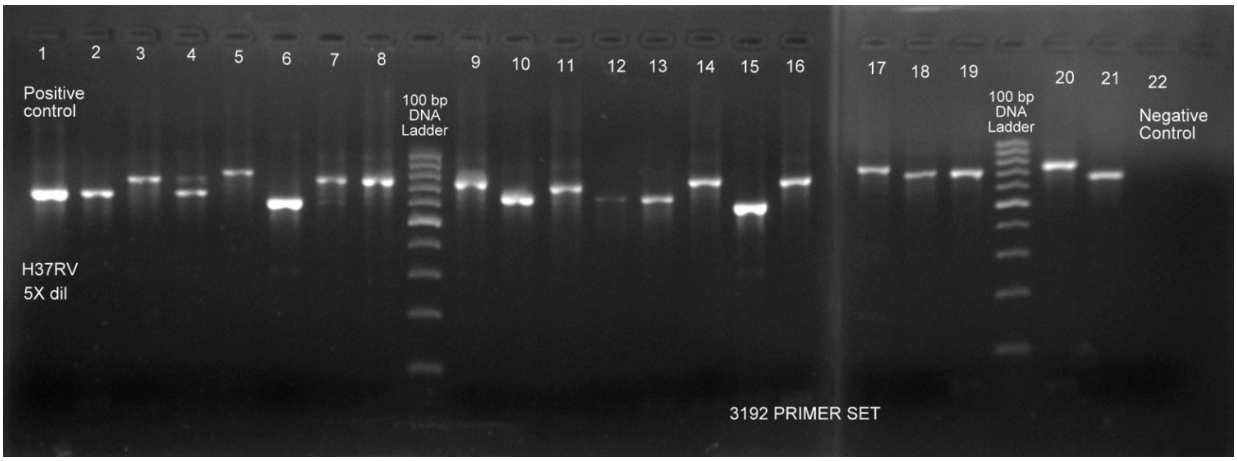


FIGURE 2.5: GEL ELECTROPHORESIS PICTURE OF PCR PRODUCTS TARGETING LOCUS 3192

Chapter 3

RESULTS

3. Results

Altogether 371 samples were collected from NTRL, NIDCH. Spoligotyping revealed 90 different patterns: 55 corresponded to orphan patterns not yet reported in the SITVIT2 database, while 35 patterns containing 286 isolates were assigned a Spoligo International Type (SIT) designation in the database. The seven most common SITs are grouped into 235 isolates (Table 3.1):

Table 3.1: Seven most common sits along with their respective Clades and percentage in the study

SIT	Clade	Percentage
1	Beijing	23.78%
53	T1	13.51%
941	Beijing	9.19%
123	T1	4.86%
42	LAM9	4.59%
358	T1	4.05%
26	CAS1-Delhi	3.51%

A total of 19/35 SITs containing 270 isolates were clustered within this study (2 to 88 isolates per cluster) while 16/35 SITs containing 16 strains were unique. 18 out of 35 SITs have been previously reported in Bangladesh.

If the study sample was classified by clades (and not SITs), the predominant *M. tuberculosis* clades belonged to the Beijing (33.15%) followed by T clade (28.30%), EAI (14.56%) CAS (14.01%), LAM (5.92%) and Haarlem (1.62%). (Tablesw-3.2; 3.3 and 3.4).

Table 3.2: Predominant clades and their respective percentage in the study

Clade	No. of isolates	Percentage (out of a total of 371)
Beijing	123	33.15%
T	105	28.30%
CAS	52	14.01%
EAI	54	14.56%
LAM	22	5.92%
Haarlem	6	1.62%

Table 3.3: Description of clusters containing >3% isolates in this study and their worldwide distribution in the SITVIT WEB database

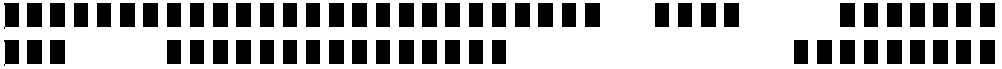
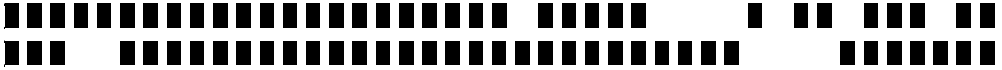
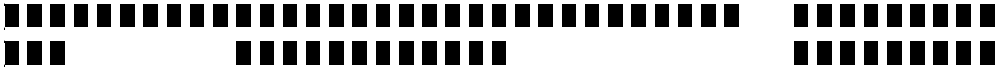
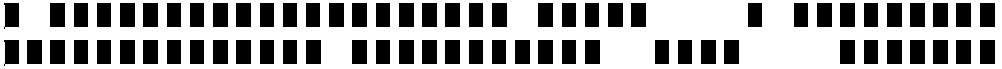
SIT	Clade	No. in study(%)	Distribution in Regions with ≥2% of a given SIT[a]	Distribution in countries with ≥2% of a given SIT[b]
SIT1	Beijing	88(23.78)	AFRI-E 3.8, ARFI-N 3.82, AFRI-D 19.35, AFRI-W 2.48, AMER-N 14.15, ASIA-C 69.09, ASIA-E 60.57, ASIA-N 45.44, ASIA-S 12.35, ASIA-SE 41.93, ASIA-W 8.81	BGD 2.91, JPN 6.21, IDN 2.52, MYS 4.1, ZAF 11.95, VNM 6.67, TWN 2.97
SIT26	CAS1-Delhi	13(3.51)	AFRI-E 1.34, AFRI-N 1.19, AMER-N 1.58, ASIA-S 17.35, ASIA-W 2.58	USA 23.66, IND 23.44, PAK 16.07, BGD 8.04, SAU 7.81, NLD 4.8
SIT42	LAM9	17 (4.59)	AFRI-E 3.97, AFRI-N 9.07, AFRI-S 2.6, AFRI-W 4.54, AMER-C 6.36, AMER-N 2.9, AMER-S 8.8, ASIA-C 7.27, ASIA-N 4.76, ASIA-W 2.58	USA 19.98, BRA 11.99, ITA 8.5, VEN 4.87, ZAF 4.76, BEL 3.33, AEG 3.23, RUS 2.51, FXX 2.31, PRT 2.25, ESP 2.25, NLD 2.15, BGD 2.1, PRY 2.05
SIT53	T1	50(13.51)	AFRI-E 8.38, AFRI-M 3.53, AFRI-N 9.67, AFRI-S 6.73, AFRI-W 10.87, AMER-C 13.74, AMER-N 6.16, AMER-S 7.21, ASIA-C 3.64, ASIA-E 2.83, ASIA-N 6.5, ASIA-S 3.8, ASIA-SE 2.55, ASIA-W 11.54	USA 21.3, ZAF 6.14, ITA 5.9, AUT 5.64, BRA 5.12, TUR 4.38, FXX 3.93, SAU 3.41, NLD 2.54, BEL 2.54
SIT123	T1	18(4.86)		AUT 34.78, TUR 17.39, BGD 17.39, BGR 8.7, USA 4.35, GLP 4.35, FXX 4.35, GRC 4.35, NLD 4.35
SIT358	T1	15(4.05)		BGD 50, TUR 18.75, GBR 12.5, MTQ 6.25, ITA 6.25, FIN 6.25
SIT941	Beijing	34(9.19)		USA 33.33, TWN 16.67, THA 16.67, FXX 8.33, CHN 8.33, MEX 8.33, GBR 8.33

[a]According to the SITVIT WEB database. The definition of macro-geographical regions and sub-regions is according to the United Nations (http://unstats.un.org/unsd/methods/m49/m49regin.htm); Regions: AFRI (Africa), AMER (Americas), ASIA (Asia), EURO (Europe), and OCE (Oceania), subdivided in: E (Eastern), M (Middle), C (Central), N (Northern), S (Southern), SE (South-Eastern), and W (Western).

[b]The 3 letter country codes are according to http://en.wikipedia.org/wiki/ISO_3166-1_alpha-3

* SITs 123, 358 and 941 could not be distributed in any regions as the percentage of each SIT was less than 1% of all the isolates reported. Moreover, for these SITs, country wise distribution was estimated byusuing a few number of reported cases.

Table 3.4: Spoligotypes and corresponding global lineage, clade, SIT and node (for minimum spanning tree)

ID	Spoligotype (Binary)	Number (%) in Study	Global Lineage	Clade	SIT	Node
1		88 (23.78)	East Asian (Beijing)	Beijing	1	3
2		34 (9.20)	East Asian (Beijing)	Beijing	941	2
3		50 (13.51)	Euro-American	T1	53	86
4		17 (4.59)	Euro-American	LAM9	42	67
5		18 (4.86)	Euro-American	T1	123	76
6		13 (3.51)	East-African-Indian	CAS1-Delhi	26	35
7		2 (0.54)	Indo-Oceanic	CAS	Orphan	30
8		5 (1.35)	Euro-American	T1	244	83
9		2 (0.54)	Indo-Oceanic	CAS	357	32
10		2 (0.54)	Indo-Oceanic	EAI1-SOM	48	81
11		4 (1.08)	Indo-Oceanic	CAS2	Orphan	19
12		3 (0.81)	Indo-Oceanic	EAI6-BGD1	Orphan	73
13		15 (4.05)	Euro-American	T1	358	36
14		11 (3.0)	East-African-Indian	CAS1-Delhi	Orphan	29
15		8 (2.2)	Euro-American	T1	Orphan	84
16		2 (0.54)	Indo-Oceanic	EAI5	138	79
17		3 (0.81)	Indo-Oceanic	EAI5	517	80
18		3 (0.81)	Indo-Oceanic	CAS	Orphan	27
19		3 (0.81)	Indo-Oceanic	Manu2	54	87
20		2 (0.54)	Indo-Oceanic	CAS2	288	20
21		2 (0.54)	Indo-Oceanic	EAI5	Orphan	41
22		2 (0.54)	Indo-Oceanic	EAI5	2152	68
23		6 (1.62)	Indo-Oceanic	EAI6-BGD1	292	74
24		4 (1.08)	Indo-Oceanic	EAI3-IND	11	10
25		3 (0.81)	Indo-Oceanic	CAS	Orphan	21
26		2 (0.54)	Indo-Oceanic	EAI6-BGD1	882	17
27		2 (0.54)	Euro-American	T1	Orphan	62
28		2 (0.54)	Euro-American	T1	Orphan	64
29		2 (0.54)	East-African-Indian	CAS1-Delhi	25	33
30		1 (0.27)	Indo-Oceanic	EAI5	Orphan	75

ID	Spoligotype (Binary)	Number (%) in Study	Global Lineage	Clade	SIT	Node
31		1 (0.27)	Euro-American	T3	Orphan	13
32		1 (0.27)	Indo-Oceanic	EAI5	Orphan	49
33		1 (0.27)	Indo-Oceanic	EAI6-BGD1	Orphan	16
34		1 (0.27)	East-African-Indian	CAS1-Delhi	Orphan	24
35		1 (0.27)	East-African-Indian	CAS1-Delhi	Orphan	25
36		1 (0.27)	Indo-Oceanic	EAI5	Orphan	63
37		1 (0.27)	Indo-Oceanic	EAI5	Orphan	60
38		1 (0.27)	Euro-American	H1	Orphan	53
39		1 (0.27)	Indo-Oceanic	EAI5	Orphan	52
40		1 (0.27)	Euro-American	S	Orphan	39
41		1 (0.27)	Euro-American	T5	Orphan	38
42		1 (0.27)	Indo-Oceanic	CAS	Orphan	23
43		1 (0.27)	Indo-Oceanic	EAI5	Orphan	56
44		1 (0.27)	Indo-Oceanic	CAS	Orphan	4
45		1 (0.27)	Indo-Oceanic	EAI6-BGD1	Orphan	59
46		1 (0.27)	Indo-Oceanic	CAS	Orphan	12
47		1 (0.27)	Euro-American	LAM9	1536	48
48		1 (0.27)	Euro-American	T5-RUS1	254	58
49		1 (0.27)	Indo-Oceanic	EAI4	Orphan	54
50		1 (0.27)	East-African-Indian	CAS1-Delhi	2733	34
51		1 (0.27)	Indo-Oceanic	EAI4	Orphan	65
52		1 (0.27)	Euro-American	T2	52	85
53		1 (0.27)	Indo-Oceanic	EAI5	Orphan	55
54		1 (0.27)	East-African-Indian	CAS1-Delhi	Orphan	28
55		1 (0.27)	Indo-Oceanic	EAI6-BGD1	Orphan	70
56		1 (0.27)	East Asian (Beijing)	Beijing	541	1
57		1 (0.27)	Euro-American	Haarlem1	Orphan	14
58		1 (0.27)	Indo-Oceanic	EAI5	Orphan	78
59		1 (0.27)	Indo-Oceanic	CAS2	Orphan	18
60		1 (0.27)	Euro-American	LAM9	1528	46

ID	Spoligotype (Binary)	Number (%) in Study	Global Lineage	Clade	SIT	Node
61		1 (0.27)	Indo-Oceanic	EAI5	Orphan	72
62		1 (0.27)	Indo-Oceanic	EAI3-IND	Orphan	9
63		1 (0.27)	Indo-Oceanic	EAI5	Orphan	51
64		1 (0.27)	Indo-Oceanic	CAS	Orphan	22
65		1 (0.27)	Euro-American	LAM6	64	66
66		1 (0.27)	Indo-Oceanic	Family 33	Orphan	57
67		1 (0.27)	Indo-Oceanic	EAI5	Orphan	11
68		1 (0.27)	Indo-Oceanic	CAS	Orphan	26
69		1 (0.27)	Indo-Oceanic	EAI8-MDG	109	8
70		1 (0.27)	Indo-Oceanic	EAI1-SOM	1389	5
71		1 (0.27)	Indo-Oceanic	EAI	Orphan	50
72		1 (0.27)	Indo-Oceanic	EAI1-SOM	806	44
73		1 (0.27)	Indo-Oceanic	EAI5	1875	6
74		1 (0.27)	Indo-Oceanic	EAI5	Orphan	77
75		1 (0.27)	Euro-American	Manu2	Orphan	37
76		1 (0.27)	Indo-Oceanic	EAI5	Orphan	43
77		1 (0.27)	Indo-Oceanic	EAI5	Orphan	61
78		1 (0.27)	Euro-American	T2	Orphan	42
79		1 (0.27)	Euro-American	S	Orphan	40
80		1 (0.27)	East-African-Indian	CAS1-Delhi	2692	31
81		1 (0.27)	Euro-American	LAM8	Orphan	45
82		1 (0.27)	Euro-American	H3	512	69
83		1 (0.27)	Indo-Oceanic	EAI5	Orphan	71
84		1 (0.27)	Indo-Oceanic	EAI1	Orphan	15
85		1 (0.27)	Euro-American	LAM7	106	47
86		1 (0.27)	Indo-Oceanic	EAI3-IND	Orphan	7
87		1 (0.27)	Euro-American	H3	50	82
88		1 (0.27)	Euro-American	H3	Orphan	
89		1 (0.27)	Euro-American	X3	92	
90		1 (0.27)	Euro-American	H1	Orphan	

Phylogenetic relationships between MTBC strains by minimum spanning tree (MST) is shown below

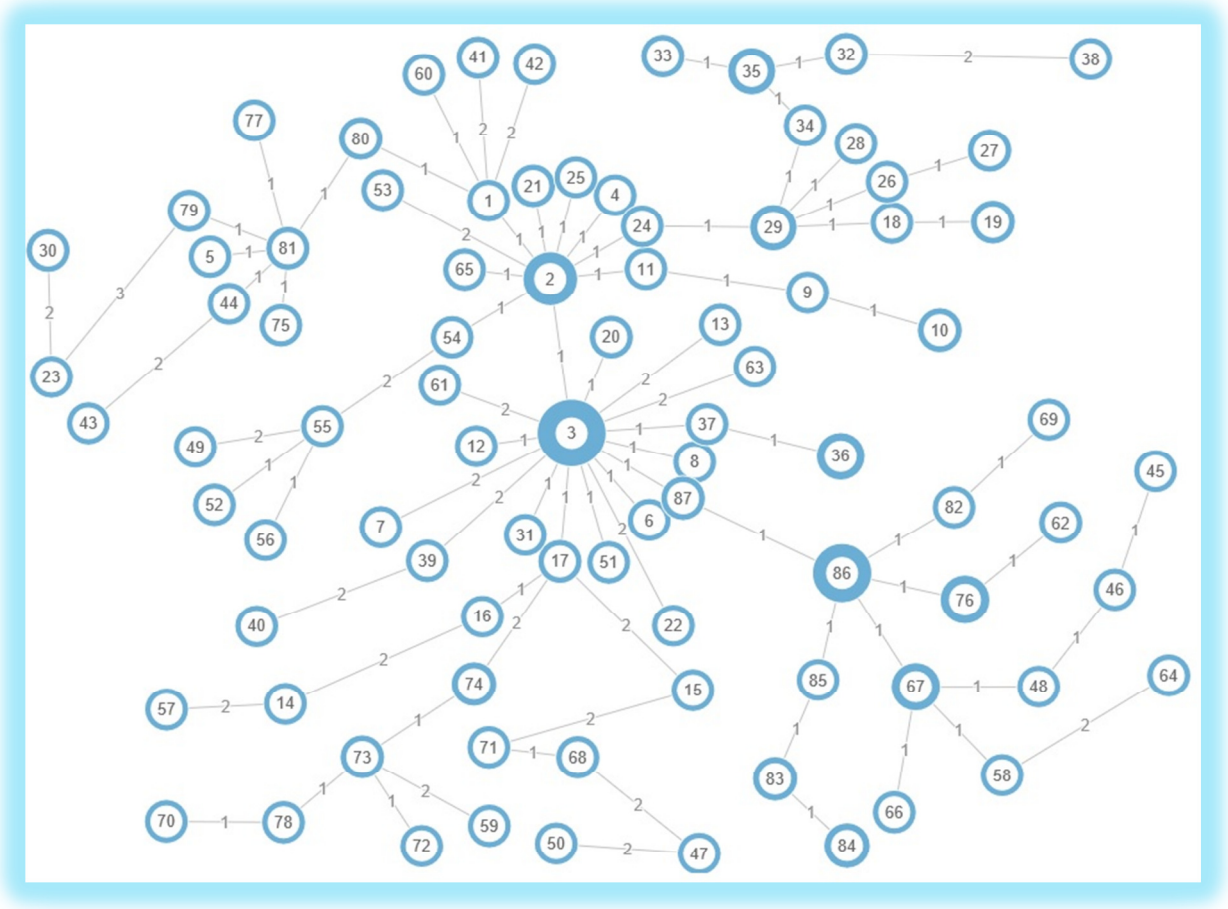


Figure 3.1: A possible evolutionary relationships between different genotypes of M. tuberculosis isolates.

Each ring (Node) represents a single genotype of tuberculosis; the circle size depends on the number of patients with that genotype. The weighted genetic distance s between genotypes are shown by the numbers on the edge. The closer the relationship, the lower the number. The numbers inside the rings represent the different nodes (Figure-3.1).

In addition to genotyping MTBC strains using spoligotyping method, we used another molecular typing technique called MIRU-VNTR (Mycobacterial Interspersed Repetitive Unit – Variable Number of Tandem Repeat) typing. Although there were 15 standard MIRU-VNTR loci, due to low MTB DNA concentration in template and low efficiency of some primer sets, we could only produce partial typing data based on 8 standard MIRU-VNTR loci. (Table-3.5)

Table 3.5: Locus convention allele for each of the 19 samples corresponding to the 8 standard MIRU-VNTR loci. Sample 1 shows results from H37RV DNA template which is used as the positive control

MIRU-VNTR ID	Spoligotype ID	MIRU04 580	MIRU26 2996	MIRU10 960	MIRU31 3192	ETRC 577	Mtub30 2401	QUB2163b 2163	Mtub21 1955
H37Rv	--	3	3	3	3	4	2	5	2
1	--	2	--	--	3	4	5	2	--
2	1	2	--	--	5	4	5	5	--
3	76	2	--	--	3	4	2	--	3
4	3	2	2	4	6	2	2	--	--
5	5	2	3	3	2	4	2	2	3
6	1	2	2	--	5	4	--	--	2
7	1	2	--	3	5	4	4	--	5
8	75	4	--	5	5	2	1	4	3
9	13	2	--	3	3	4	1	3	3
10	2	2	--	3	4	4	--	4	5
11	13	2	--	3	3	4	1	3	3
12	6	2	--	5	5	2	1	2	--
13	74	5	2	4	2	2	1	2	--
14	1	2	--	3	5	4	4	5	--
15	--	2	--	2	5	4	4	6	--
16	73	5	2	4	4	4	4	3	--
17	29	2	--	5	4	2	--	2	4
18	1	2	--	3	5	4	2	5	2
19	3	2	--	--	4	3	4	4	--

Chapter 4

DISCUSSION

4. Discussion

Multidrug resistance, i.e. resistance to at least rifampin and isoniazid, in *Mycobacterium tuberculosis* is caused by the sequential accumulation of mutations in the genes encoding the targets of rifampin and isoniazid. Extensively drug-resistant (XDR) tuberculosis (TB) is defined as multidrug resistance (resistance to isoniazid and rifampin), plus resistance to a fluoroquinolone and any of the injectable second-line drugs (amikacin, capreomycin, or kanamycin). The emergence and spread of multidrug-resistant (MDR) and XDR TB is hampering efforts to control and manage TB. Since the emergence of MDR strains in the 1990s, the prevalence of MDR TB has slowly but constantly increased. More worrisome is the emergence of XDR TB, a nearly untreatable form of the disease with an estimated rate of 15% among the MDR strains.

Genotyping of *Mycobacterium tuberculosis* has been extensively used for investigating epidemics of multidrug-resistant strains of *M. tuberculosis*, in order to identify the factors involved in the transmission of such strains and determine effective control programmes to limit their expansion at both the individual and population levels. Here, we review the methods currently used to study the molecular epidemiology of multidrug-resistant *M. tuberculosis* strains, and the insights provided by these techniques regarding global trends and the transmission dynamics of multidrug-resistant tuberculosis at a world scale.

The aim of this study was to perform genotypic characterization of *M. tuberculosis* strains circulating in Bangladesh. As sputum samples were collected from the only tuberculosis reference laboratory in Bangladesh, it can be assumed that these referred patients came from

different parts of the country, thus not confining this study to Dhaka city only. A total of 371 isolates were studied, producing the largest amount of spoligotyping data on isolates from Bangladesh to date.

Because of these limitations, several PCR-based methods have been developed for MTBC strain typing that require little DNA. These include spoligotyping and Mycobacterial Interspersed Repeat Units (MIRUs) typing, which together have been recently defined as the new gold standard for molecular epidemiological investigation of TB. Spoligotyping patterns are defined based on the presence or absence of 43 unique regions intercalated between direct repeats in the Clustered Regularly Interspaced Short Palindromic Repeats region (CRISPRs) of the MTBC genome. MIRU profiles classify MTBC strains by the number of repeats at different Variable Number of Tandem Repeats (VNTRs) loci. Spoligo-types and MIRU-types can be compared using the SITVITWEB database that includes thousands of spoligotyping patterns and MIRU-VNTR. However, the use of spoligotyping and MIRUs is limited for phylogenetics and strain classification because of the propensity of the corresponding molecular markers for convergent evolution; i.e. because these markers change rapidly, the same or similar patterns can emerge by chance in strains that are phylogenetically unrelated.

According to our findings, the most prevalent tuberculosis clade in Bangladesh is the Beijing clade and constitutes about 33.15% (95% CI 28.36% to 37.94%). This result has close association with worldwide prevalence of Beijing strains which is estimated to be 33.2% (95% CI 31.4–35.2). Continent wise the prevalence of strains of Beijing clade is - Asia 44.7% (39.5–

49.8), Europe 27.9% (25.6 – 30.1), Africa 12.5% (8.9–16.2), and America 8.9% (6.9–10.9) (Ramazanzadeh and Sayhemiri, 2014).

In our study, we found two different spoligotypes of Beijing clade. The less prevalent SIT 941 (9.19%) has one more spacer (Number 41) missing than the more prevalent SIT 1 (23.78%). In previous reports from Bangladesh (Banu et al., 2004; Rahim et al., 2007), only SIT 1 was reported. Previously, SIT 941 has only been isolated from China, France, Mexico, Taiwan, Thailand, UK and USA. This means that, SIT 941 has been introduced in our country in recent years and has spread rapidly to become the 3rd highest SIT of our study. The reason behind this rapid spread might be the hyper virulent nature of Beijing strains(Manca et al., 2001). Moreover, prevalence of Beijing strains has been high in BCG vaccination coverage area. It has been suggested that BCG induced immunity favors the selection of Beijing strains (Kremer et al., 2009). Thus, the extensive coverage of BCG vaccination in Bangladesh might be the reason of rapid dissemination of SIT 941 in Bangladeshi population.

A recent study conducted in Assam, India which is close to Bangladesh has shown the predominance of Beijing clade among clinical isolates collected from designated microscopy centers of Assam (Devi et al., 2015).Although Central-Asian (CAS) and the East-African-Indian (EAI) clade are the predominant strains in India, a recent hospital based study performed in the western part of India show predominance of Beijing strains (23%) with high levels of association with MDR-TB (Arora et al., 2009). However, A study based on isolates from non-hospitalized TB patients in Bangladesh showed predominance of EAI clade (44.2%), making Beijing clade the second most prevalent one (15.2%)(Rahim et al., 2007). In our study, however, EAI clade

constituted 14.56% (3rd most prevalent clade) of all the isolates. The apparent discrepancy in results between this study and our study might be due to the patient type. In our study, the majority of samples came from admitted patients with higher probability of MDR-TB. In Bangladesh, increase in MDR-TB with Beijing strain has been reported previously (Banu et al., 2004), therefore, we can say that predominance of Beijing strain in our study is due to the type of patients from whom samples were collected.

The second most dominant clade was T clade pushing EIA to the 3rd spot. "T" group families comprise the modern Tb strains and hence are ill defined (Brudey et al., 2006a). Among five sublineages (T1 to T5), the sublineage T1 was prevalent (Table 6). We also identified sublineages T2, T3 and T5 in our study. While Rahim et al.(2007) reported 9.4% prevalence of T clade, in our study we found surprisingly high prevalence (28.3%) of this relatively modern TB clade.

From the MST diagram we can observe the evolutionary relationship between different genotypes of our study. The increased circle size of node 3 (Beijing),2 (Beijing) and 86 (T1 SIT 53) indicate that more patients carry these genotypes. The number 1 between node 3 and 2 suggests that the genetic distance between two variants of Beijing strains are relatively low. Node 1 (Beijing SIT 541), 4 (CAS), 11 (EAI5), 21 (CAS), 24 (CAS1-Delhi), 25 (CAS1-Delhi), 54 (EAI4), 65 (EAI4) is closely related to node 2 (Beijing SIT 941). The more prevalent node 3 (Beijing strain SIT 1) shows the close relationship to node 6 (EAI 5), 8 (EAI8-MDG), 12 (CAS), 13 (T3), 17 (EAI6-BGD1), 20 (CAS 2), 31 (CAS1-Delhi), 37 (Manu 2), 51 (EAI5), 61 (EAI 5), 63 (EAI 5) and 87 (Manu 2). Another major cluster can be observed around node 86 (T1). This node 86 is related to node 3 (Beijing SIT 1) via node 87 (Manu 2). Moreover, this node 86 is

closely related to node 67 (LAM 9), 76 (T1 SIT 123), 82 (H3) and 85 (T2). More short clusters can be observed around node 1, 29,55 and 81. This minimum spanning tree diagram shows that Beijing strains (Node 2 and 3) are linked with many different nodes (genotypes). This might be due to the fact that Beijing strains lack all the spacers from 1 to 36. Because of this a single major deletion event from many other genotypes can directly lead to Beijing strains.

To impart higher discriminatory power between lineages and sub-lineages, we tried to genotype our study samples using another high resolution technique called MIRU-VNTR. This MIRU-VNTR technique can be performed manually using agarose gel electrophoresis or can be automated using fluorescence-based DNA analyzer with computerized automation of the genotyping (Supply et al. 2001). The technique with fluorescence-based DNA analyzer is far better than manual visual observation of agarose gel technique as the PCR sensitivity of fluorescence based technique is much higher (Supply et al., 2006). As we didn't have settings for fluorescence based technique, we opted for manual agarose gel based technique. But, as this manual technique lacks proper sensitivity, several primers could not produce any visible band in the agarose gel. That is why results could be obtained for 8 out of 15 targeted loci. As results of only 8 loci was obtained, we could not identify the lineage and sub-lineage of different genotypes using the tool MIRU-VNTRplus web application (available: *http://www.miru-vntrplus.org/MIRU/index.faces*). However, the results of 8 loci showed that different genotypes show different patterns. Moreover, two different types of MIRU-VNTR patterns were observed for the same spoligotype. This is possible as discriminatory power of MIRU-VNTR technique is more than spoligotyping technique (Allix-Béguec et al., 2008). Although, MIRU_VNTR results

could not be imparted with spoligotyping data, the spoligotyping technique alone was sufficient to identify predominant clades like Beijing, T1 and EAI in Bangladeshi population.

In conclusion, our study successfully genotyped 371 MTBC isolates and found predominance of Beijing strains conforming to other recent studies conducted in nearby countries. Moreover, we have reported a surprising increase in T clade strains in this region.

Chapter 5

REFERENCES

5. References

Allix-Béguec, C., Fauville-Dufaux, M., Supply, P., 2008. Three-Year Population-Based Evaluation of Standardized Mycobacterial Interspersed Repetitive-Unit-Variable-Number Tandem-Repeat Typing of *Mycobacterium tuberculosis*. J. Clin. Microbiol. 46, 1398–1406. doi:10.1128/JCM.02089-07

Arora, J., Singh, U.B., Suresh, N., Rana, T., Porwal, C., Kaushik, A., Pande, J.N., 2009. Characterization of predominant *Mycobacterium tuberculosis* strains from different subpopulations of India. Infect. Genet. Evol. J. Mol. Epidemiol. Evol. Genet. Infect. Dis. 9, 832–839. doi:10.1016/j.meegid.2009.05.008

Banu, S., Gordon, S.V., Palmer, S., Islam, R., Ahmed, S., Alam, K.M., Cole, S.T., Brosch, R., 2004. Genotypic Analysis of *Mycobacterium tuberculosis* in Bangladesh and Prevalence of the Beijing Strain. J. Clin. Microbiol. 42, 674–682. doi:10.1128/JCM.42.2.674-682.2004

Banu, S., Rahman, M.T., Uddin, M.K.M., Khatun, R., Khan, M.S.R., Rahman, M.M., Uddin, S.I., Ahmed, T., Heffelfinger, J.D., 2015. Effect of Active Case Finding on Prevalence and Transmission of Pulmonary Tuberculosis in Dhaka Central Jail, Bangladesh. PLOS ONE 10, e0124976. doi:10.1371/journal.pone.0124976

Brudey, K., Driscoll, J.R., Rigouts, L., Prodinger, W.M., Gori, A., Al-Hajoj, S.A., Allix, C., Aristimuño, L., Arora, J., Baumanis, V., Binder, L., Cafrune, P., Cataldi, A., Cheong, S., Diel, R., Ellermeier, C., Evans, J.T., Fauville-Dufaux, M., Ferdinand, S., Garcia de Viedma, D., Garzelli, C., Gazzola, L., Gomes, H.M., Guttierez, M.C., Hawkey, P.M., van Helden, P.D., Kadival, G.V., Kreiswirth, B.N., Kremer, K., Kubin, M., Kulkarni, S.P., Liens, B., Lillebaek, T., Ho, M.L., Martin, C., Martin, C., Mokrousov, I., Narvskaïa, O., Ngeow, Y.F., Naumann, L., Niemann, S., Parwati, I., Rahim, Z., Rasolofo-Razanamparany, V., Rasolonavalona, T., Rossetti, M.L., Rüsch-Gerdes, S., Sajduda, A., Samper, S., Shemyakin, I.G., Singh, U.B., Somoskovi, A., Skuce, R.A., van Soolingen, D., Streicher, E.M., Suffys, P.N., Tortoli, E.,

Tracevska, T., Vincent, V., Victor, T.C., Warren, R.M., Yap, S.F., Zaman, K., Portaels, F., Rastogi, N., Sola, C., 2006a. Mycobacterium tuberculosis complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbiol. 6, 23. doi:10.1186/1471-2180-6-23

Brudey, K., Filliol, I., Ferdinand, S., Guernier, V., Duval, P., Maubert, B., Sola, C., Rastogi, N., 2006b. Long-Term Population-Based Genotyping Study of Mycobacterium tuberculosis Complex Isolates in the French Departments of the Americas. J. Clin. Microbiol. 44, 183–191. doi:10.1128/JCM.44.1.183-191.2006

Cowan, L.S., Mosher, L., Diem, L., Massey, J.P., Crawford, J.T., 2002. Variable-Number Tandem Repeat Typing of Mycobacterium tuberculosis Isolates with Low Copy Numbers of IS6110 by Using Mycobacterial Interspersed Repetitive Units. J. Clin. Microbiol. 40, 1592–1602. doi:10.1128/JCM.40.5.1592-1602.2002

Das, S., Paramasivan, C.N., Lowrie, D.B., Prabhakar, R., Narayanan, P.R., 1995. IS6110 restriction fragment length polymorphism typing of clinical isolates of Mycobacterium tuberculosis from patients with pulmonary tuberculosis in Madras, south India. Tuber. Lung Dis. Off. J. Int. Union Tuberc. Lung Dis. 76, 550–554.

Demay, C., Liens, B., Burguière, T., Hill, V., Couvin, D., Millet, J., Mokrousov, I., Sola, C., Zozio, T., Rastogi, N., 2012. SITVITWEB--a publicly available international multimarker database for studying *Mycobacterium tuberculosis* genetic diversity and molecular epidemiology. Infect. Genet. Evol. J. Mol. Epidemiol. Evol. Genet. Infect. Dis. 12, 755–766. doi:10.1016/j.meegid.2012.02.004

Devi, K.R., Bhutia, R., Bhowmick, S., Mukherjee, K., Mahanta, J., Narain, K., 2015. Genetic Diversity of *Mycobacterium tuberculosis*Isolates from Assam, India: Dominance of Beijing Family and Discovery of Two New Clades Related to CAS1_Delhi and EAI Family Based on Spoligotyping and MIRU-VNTR Typing. PLOS ONE 10, e0145860. doi:10.1371/journal.pone.0145860

Ferdinand, S., Valétudie, G., Sola, C., Rastogi, N., 2004. Data mining of *Mycobacterium tuberculosis* complex genotyping results using mycobacterial interspersed repetitive units validates the clonal structure of spoligotyping-defined families. Res. Microbiol. 155, 647–654. doi:10.1016/j.resmic.2004.04.013

Goyal, M., Saunders, N.A., van Embden, J.D., Young, D.B., Shaw, R.J., 1997. Differentiation of *Mycobacterium tuberculosis* isolates by spoligotyping and IS6110 restriction fragment length polymorphism. J. Clin. Microbiol. 35, 647–651.

Groenen, P.M., Bunschoten, A.E., van Soolingen, D., van Embden, J.D., 1993. Nature of DNA polymorphism in the direct repeat cluster of *Mycobacterium tuberculosis*; application for strain differentiation by a novel typing method. Mol. Microbiol. 10, 1057–1065.

Kamerbeek, J., Schouls, L., Kolk, A., van Agterveld, M., van Soolingen, D., Kuijper, S., Bunschoten, A., Molhuizen, H., Shaw, R., Goyal, M., van Embden, J., 1997. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J. Clin. Microbiol. 35, 907–914.

Kremer, K., van der Werf, M.J., Au, B.K.Y., Anh, D.D., Kam, K.M., Rogier van Doorn, H., Borgdorff, M.W., van Soolingen, D., 2009. Vaccine-induced Immunity Circumvented by Typical *Mycobacterium tuberculosis* Beijing Strains. Emerg. Infect. Dis. 15, 335–339. doi:10.3201/eid1502.080795

Kremer, K., van Soolingen, D., Frothingham, R., Haas, W.H., Hermans, P.W.M., Martín, C., Palittapongarnpim, P., Plikaytis, B.B., Riley, L.W., Yakrus, M.A., Musser, J.M., van Embden, J.D.A., 1999. Comparison of Methods Based on Different Molecular Epidemiological Markers for Typing of *Mycobacterium tuberculosis* Complex Strains: Interlaboratory Study of Discriminatory Power and Reproducibility. J. Clin. Microbiol. 37, 2607–2618.

Le Flèche, P., Hauck, Y., Onteniente, L., Prieur, A., Denoeud, F., Ramisse, V., Sylvestre, P., Benson, G., Ramisse, F., Vergnaud, G., 2001. A tandem repeats database for bacterial genomes: application to the genotyping of Yersinia pestis and Bacillus anthracis. BMC Microbiol. 1, 2. doi:10.1186/1471-2180-1-2

Manca, C., Tsenova, L., Bergtold, A., Freeman, S., Tovey, M., Musser, J.M., Barry, C.E., Freedman, V.H., Kaplan, G., 2001. Virulence of a *Mycobacterium tuberculosis* clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-α/β. Proc. Natl. Acad. Sci. 98, 5752–5757. doi:10.1073/pnas.091096998

Mazars, E., Lesjean, S., Banuls, A.L., Gilbert, M., Vincent, V., Gicquel, B., Tibayrenc, M., Locht, C., Supply, P., 2001. High-resolution minisatellite-based typing as a portable approach to global analysis of *Mycobacterium tuberculosis* molecular epidemiology. Proc. Natl. Acad. Sci. U. S. A. 98, 1901–1906. doi:10.1073/pnas.98.4.1901

Rahim, Z., Zaman, K., Zanden, A.G.M. van der, Möllers, M.J., Soolingen, D. van, Raqib, R., Zaman, K., Begum, V., Rigouts, L., Portaels, F., Rastogi, N., Sola, C., 2007. Assessment of Population Structure and Major Circulating Phylogeographical Clades of *Mycobacterium tuberculosis* Complex in Bangladesh Suggests a High Prevalence of a Specific Subclade of Ancient *M. tuberculosis* Genotypes. J. Clin. Microbiol. 45, 3791–3794. doi:10.1128/JCM.01247-07

Ramazanzadeh, R., Sayhemiri, K., 2014. Prevalence of Beijing family in *Mycobacterium tuberculosis* in world population: Systematic Review and Meta-Analysis. Int. J. Mycobacteriology 3, 41–45. doi:10.1016/j.ijmyco.2014.01.001

Sebban, M., Mokrousov, I., Rastogi, N., Sola, C., 2002. A data-mining approach to spacer oligonucleotide typing of *Mycobacterium tuberculosis*. Bioinforma. Oxf. Engl. 18, 235–243.

Supply, P., Allix, C., Lesjean, S., Cardoso-Oelemann, M., Rüsch-Gerdes, S., Willery, E., Savine, E., de Haas, P., van Deutekom, H., Roring, S., Bifani, P., Kurepina, N., Kreiswirth, B.,

Sola, C., Rastogi, N., Vatin, V., Gutierrez, M.C., Fauville, M., Niemann, S., Skuce, R., Kremer, K., Locht, C., van Soolingen, D., 2006. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. J. Clin. Microbiol. 44, 4498–4510. doi:10.1128/JCM.01392-06

Supply, P., Lesjean, S., Savine, E., Kremer, K., van Soolingen, D., Locht, C., 2001. Automated high-throughput genotyping for study of global epidemiology of *Mycobacterium tuberculosis* based on mycobacterial interspersed repetitive units. J. Clin. Microbiol. 39, 3563–3571. doi:10.1128/JCM.39.10.3563-3571.2001

WHO | Global tuberculosis report 2015 [WWW Document], n.d. . WHO. URL http://www.who.int/tb/publications/global_report/en/ (accessed 5.28.16).

Appendix-I

Media Composition

Unless otherwise mentioned, all media were sterilized by autoclaving at 121°C for 15 minutes at 15 lbs pressure. Distilled water was used for preparation of all media. The media used in this thesis has shown below:

1. Lowenstein Jensen Medium

Ingredients	**Amount (gm/L)**
Malachite Green	0.4gm
Magnesium Sulphate	0.24gm
Glycerol	12ml
Potato Flour (Potato starch)	30gm
L-Asparagine	3.6gm
Monopotassium Phosphate	2.4gm
Magnesium Citrate	0.6gm
Egg Suspension	100ml
Distilled Water	600ml

2. DMEM (Dulbecco Modified Egales) Media

Ingredients	**Amount**
DMEM	6.73 gm
$NaHCO_3$	1.85 gm
HEPES	2.38 gm
Glutamine	0.58 gm
Deionized Water (18Ω)	Up to 500 ml
P^H	7.2-7.4

***Media was not autoclaved. It was filtered through Millipore membrane of 0.20µm average pore diameter.**

3. Dubos broth (Approximate formula for 900 ml purified water)

Ingredients	**Amount**
Pancreatic Digest of Casein	0.5 gm
L-Asparagine	2.0 gm
Monopotassium Phosphate	1.0 gm
Disodium Phosphate	2.5 gm
Ferric Ammonium Citrate	0.05 gm
Magnesium Sulphate	0.01 gm
Polysorbate 80	0.2 gm
Calsium Chloride	0.5 gm
Zinc Sulphate	0.1 gm
Copper Sulphate	0.1 gm
FBS	100.0 ml

Appendix- II

Apparatus used

Autoclave	HL-42AE, Hirayama Corp, Japan
Balance	Adventurer AR1140, Mettler Toledo EL202
Centrifuge	Eppendorf Centrifuge 5804, Germany
Class II Biosafety Cabinet	Lab Cai VX05re, USA
CO2 Incubator	Shel Lab
Electrophoresis Unit	Sigma E0638, Consort N.V
Freezer (-30°C)	LiebherrConfort, Germany, Siemens
Gel Documentation System	VilberLourmat Doc Print VX05
Heater/Magnetic Stirrer	Spinot
Incubator	Japan
Inverted Microscope	KYOW Optilab TR-T
Laminar Air Flow	HF-48 Flow Laboratories, Japan
Microcentrifuge	Hettich, TarsonsSpinwin MC-022
Microwave Oven	Butterfly, China
pH Meter	Hanna HI2211
Power Pack	Biometra Standard Power Pack
Refrigerator	Royal Frestech, Vestfrost
SDS-PAGE Unit	BioRad Mini-Protean II Cell
Horizontal Shaker	Electro Plus, Schuttelmaschine LS10
Spectrophotometer	Genesys 5
Thermomixer	Eppendorf
Thermocycler	Biorad C 1000, Biorad MJ Mini, USA
Vortex Mixer	IKA MS3 Basic
Vacuum Pump	Sartorius GMBH
Water Bath	Grant SUB6, England

Appendix- III

New bacteriologically confirmed TB case notification rate by district, Bangladesh 2015