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MOLECULAR EPIDEMIOLOGY AND PHENOTYPIC AND GENETIC CHARACTERIZATION OF MYCOBACTERIA ISOLATED IN CENTRAL VIETNAM

Direttore:
Prof. Bruno Masala

Tutor:
Prof. Pietro Cappucinelli

Tesi di dottorato della:
Dott.ssa Ngo Viet Quynh Tram

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ABBREVIATION

16S rDNA PCR:	**16S rRNA gene realtime PCR**
AFB:	**Acid-fast bacillus**
ARNr 16S :	**S16 ribosomal RNA**
BCG:	**Bacille Calmette-Guérin**
CAS:	**Central-Asian**
CDS:	**Coding Sequence**
CR3:	**Complement receptor 3**
CSF:	**Cerebral spinal fluid**
CTAB:	**Cetyltrimethyl Ammonium Bromide**
CTL:	**Cytolytic T lymphocytes**
DC:	**Dendritic cells**
DC-SIGN:	**Dendritic cell-specific intercellular-adhesion molecule-3-grabbing non-integrin**
DIP:	**Deletion Insertion Polymorphism**
DST:	**Drug susceptibility testing**
EAI :	**East African-Indian**
EMB:	**Ethambutol**
ETR-A:	**Extract tandem repeat A**
GM-CSF:	**Granulocyte-macrophage colony-stimulating factor**
IFN:	**Interferon**
IS6110:	**Insertion sequence 6110**
ISC:	**Indian Subcontinent**
***kat*G:**	**catalase-peroxidase**
LAM :	**Latin American and Mediterranean**
LSP:	**Large Sequence Polymorphisms**
MDR:	**Multidrug-resistant**
MDR-TB:	**Multidrug-resistant tuberculosis**
MHC:	**Major histocompatibility complex**
MIRU:	**Mycobacterium Intespersed Repetitive Units**
MMR:	**Macrophage mannose receptor**
MODS:	**Microscopic-Observation Drug-Susceptibility**
MDR-TB:	**Multi-drug resistant tuberculosis**
NaOH-NALC:	**N-acetyl-L-cysteine**
NCBI :	**NationalCenter for Biotechnology Information**
NK:	**Natural killer**
OADC:	**Oxalic acid, albumin, dextrose, and catalase**
ORF:	**Open reading frame**
PANTA:	**Polymyxin, Amphotericin B, Nalidixic Acid, Trimethoprim, and Azlocillin**

PBS:	**Phosphate buffered saline**
PCR:	**Polymerase Chain Reaction**
PE :	**proline-glutamate**
PGRS:	**Polymorphic GC-rich sequences**
PMN :	**Polymorphonuclear neutrophils**
PPE:	**Proline-Proline-Glutamate**
RD:	**Direct Repeat**
RFLP:	**Restriction fragment length polymorphism**
***rpo*B:**	**β subunit of DNA dependant RNA polymerase**
***rRNA* gene:**	**gene coding ribosomal RNA**
SIT:	**Spoligo-international type**
SNP:	**Single-nucleotide polymorphism**
ST:	**shared type**
STR:	**Streptomycin**
TB:	**Tuberculosis**
TDM:	**Trehalose 6, 6'-dimycolate**
TGF:	**Transforming growth factor**
TGF-β:	**Transforming growth factor-β**
TLR:	**Toll-like receptors**
TM cells:	**Memory T cell**
TNF:	**Tumour necrosis factor.**
Treg:	**Regulatory T**
tRNA gene:	**Gene coding transfer RNA**
VDR:	**Vitamin D receptor**
VNTR:	**Variable numbers of tandem repeats**
WHO:	**World Health Organization**
XDR-TB	**Extensively drug- resistant tuberculosis**

1. ABSTRACT

The overall objective of this study is to determine molecular epidemiology and phenotypic and genetic characterization of mycobacteria isolated in central Vietnam.

In this study, the MODS assay was used to isolate mycobacteria and determine the proportion of drug resistant strains in 252 clinical samples. *M.tuberculosis* was detected in153 samples (60.7%) and 46 (30.1%) were antibiotic resistant. One drug resistance was present in 30 strains (19.6%): 18 for RIF, 6 for INH and 3 for STR and EBM. Multidrug resistant *M.tuberculosis* as defined by WHO (resistant to RIF and INH) was observed in 13 strains. There were additional 14 strains showing resistance to two or more drugs.

Identification of *M.tuberculosis* using the 16S rDNA based PCR assay detected 258 positive in 480 clinical samples(53.8%), including 73 smear negative cases. The IS6110 PCR of positive cases could not confirm the positivity in 3 samples, probably due to the absence of the insertion sequence.

Spoligotyping produced a total of 36 different patterns for the 122 *M.tuberculosis* strains examined. The EAI family genotype (65.6% of isolates) dominated in our study: 15.6 % were of the EAI4-VNM genotype and 46.7 % were of EAI5. Beijing genotype was observed in 12.3 % of isolates. There were 4 isolates (3.3%) in which spoligotype pattern did not match with any of the updated international spoligotype database of the *M.tuberculosis* complex - SpolDB4. The remaining 22% were of other genotypes (including U, T, MANU, H and CAS).

Sequence and analysis of the whole genome of MTB_HUE_20 strain - a multidrug resistant strain with unknown spoligotype pattern - showed that it is 4,397,928 bp in length and contains 64.8% of GC. This strain harbors only one copy of IS6110 and has a mutation on *kat*G gene.

2. INTRODUCTION

2.1. Epidemiology of tuberculosis

2.1.1.Global burden of tuberculosis

The consequences of tuberculosis (TB) on human society are immense. Worlwide, one person out of three is infected with *Mycobacterium tuberculosis* – two billion people in total. TB accounts for 2.5% of the global burden of disease and is the commonest cause of death in young women, killing more women than all causes of maternal mortality combined. TB currently holds the seventh place in the global ranking of causes of death.[177,210]

Effective drugs to treat and cure the disease have been available for more than 50 years, yet every 15 seconds, someone in the world dies from TB. Even more alarmingly a person is newly infected with *Mycobacterium tuberculosis* every second of every day. Left untreated, a person with active TB will infect an average of 10 to 15 other people every year.[66]

TB hinders socioeconomic development: 75% of people with TB are within the economically productive age group of 14-54 years. 95% of all cases and 99% of deaths occur in developing countries, with the greatest burden in sub-Saharan Africa and South East Asia. Household costs of TB are substantial.[67]

World Health Organization (WHO) estimated 8.9 million new cases of TB in 2004 (140/100,000 population). About 3.9 million cases (92/100,000 population) were acid fast bacilli (AFB) sputum smear-positive, the most infectious form of the disease. There were 14.6 million prevalent case (229/100,000), of which 6.1 million were AFB sputum smear-positive (95/100,000).[67]

In 2009, there were an estimated 9.4 million incident cases (range, 8.9 million–9.9 million)1 of TB globally (equivalent to 137 cases per 100,000 population) (table1, figure 1). The absolute number of cases continues to increase slightly from year to year, as slow reductions in incidence rates per capita continue to be outweighed by increases in population. Most of the estimated number of cases in 2009 occurred in Asia (55%) and Africa (30%)(Asia here means the WHO regions of South-East Asia and the WesternPacific. Africa means the WHO African Region); smaller proportions of cases occurred in the Eastern Mediterranean Region (7%), the European Region (4%) and the Region of the Americas (3%). The 22 highest burden countries (HBCs) that have received particular attention at the global level since 2000 account for 81% of all estimated cases worldwide (Table 1).

The five countries with the largest number of incident casesin 2009 were India (1.6–2.4 million), China (1.1–1.5 million), South Africa (0.40–0.59 million), Nigeria (0.37–0.55 million) and Indonesia (0.35–0.52 million). India alone accounts for an estimated one fifth (21%) of all TB cases worldwide, and China and India combined account for 35%.[267]

Of the 9.4 million incident cases in 2009, an estimated 1.0–1.2 million (11–13%) were HIV-positive, with a best estimate of 1.1 million (12%) (table 1, figure 2). These numbers are slightly lower than those reported in previous years, reflecting better estimates as well as reductions in HIV prevalence in the general population. Of these HIV-positive TB cases, approximately 80% were in the African Region.[267]

There were an estimated 14 million prevalent cases (range, 12 million–16 million) of TB in 2009, equivalent to 200 cases per 100,000 population.[267]

In 2009, an estimated 1.3 million deaths (range, 1.2 million–1.5 million) occurred among HIV-negative cases of TB (table 1). This is equivalent to 20 deaths per 100,000 population. In addition, there were an estimated 0.4 million deaths (range, 0.32 million – 0.45 million) among incident TB cases that were HIV-positive (data not shown); these deaths are classified as HIV death in the 10th revision of the International Classification of Diseases (ICD-10). Thus in total, approximately 1.7 million people died of TB in 2009. The number of TB deaths per 100,000 population among HIV-negative people plus the estimated number of TB deaths among HIV-positive people equates to abest estimate of 26 deaths per 100,000 population.[267]

The emergence of drug resistance strains of *Mycobacterium tuberculosis* and particularly multidrug-resistant *M.tuberculosis* strains, has become a significant public health problem in a number of countries and an obstacle for an effective control of tuberculosis (TB). More than 400,000 cases of MDR-TB emerge every year as a result of under investments in basic activities, poor management of patients that results in the transmission of the deadly strains. Despite its global magnitude, the problem has not been addressed adequately**.** MDR-TB has been a topic of growing interest. MDR-TB are much more difficult and costly to treat than drug-susceptible TB, since more than 2 year of therapy may be required to treat a patient infected with MDR-TB strains compared with the 6-8 months required to treat those infected with a sensitive strains. As a results of long lasting treatment and the use of more expensive drugs, the cost of the therapy may increase up to 100 folds.[56, 265]

Table1: Estimated epidemiological burden of TB, 2009 (From WHO, 2010)
Number in thousands except where indicated.[a]

		MORTALITY[b]			PREVALENCE			INCIDENCE			HIV PREVALENCE IN INCIDENT TB CASES (%)		
	POPULATION	BEST[c]	LOW	HIGH	BEST	LOW	HIGH	BEST	LOW	HIGH	BEST	LOW	HIGH
Afghanistan	28 150	11	7.1	15	94	43	160	53	43	64	–	–	–
Bangladesh[d]	162 221	83	60	110	690	320	1 100	360	300	440	0.2	0.1	0.3
Brazil	193 734	4.5	2.2	8.4	100	36	180	87	72	100	12	11	12
Cambodia	14 805	10	7.4	14	100	47	170	65	56	76	6.4	4.5	8.3
China	1 345 751	150	100	220	1 800	740	3 000	1 300	1 100	1 500	1.5	0.9	2.2
DR Congo	66 020	50	36	67	430	200	690	250	200	300	8.4	6.4	11
Ethiopia	82 825	54	38	75	480	220	790	300	240	360	12	8.8	15
India	1 198 003	280	170	430	3 000	1 300	5 000	2 000	1 600	2 400	6.4	3.9	9.8
Indonesia	229 965	62	36	95	660	270	1 100	430	350	520	2.8	1.7	4.3
Kenya	39 802	6.2	3.0	12	110	45	190	120	99	150	44	42	46
Mozambique	22 894	8.8	6.3	12	86	43	130	94	76	110	58	58	58
Myanmar*[e]	50 020	29	18	43	300	130	490	200	160	240	11	7.7	14
Nigeria	154 729	110	89	140	830	380	1 400	460	370	550	19	19	19
Pakistan	180 808	60	36	93	640	270	1 100	420	340	500	1.5	1.0	2.2
Philippines	91 983	32	21	45	480	450	510	260	210	310	0.5	0.3	0.8
Russian Federation	140 874	25	17	38	190	65	320	150	120	180	8	7	9
South Africa	50 110	23	10	44	390	160	650	490	400	590	60	54	65
Thailand	67 764	12	7.2	18	130	57	210	93	75	110	17	12	22
Uganda	32 710	9.3	3.9	17	91	39	170	96	78	120	56	39	73
UR Tanzania	43 739	4.0	1.5	9.2	72	27	130	80	75	85	47	33	61
Viet Nam	88 069	32	18	50	290	130	510	180	130	230	4.2	2.9	5.8
Zimbabwe	12 523	10	7.5	14	96	48	150	93	76	110	52	51	52
High-burden countries	**4 297 498**	**1 100**	**930**	**1 200**	**11 000**	**8 900**	**14 000**	**7 600**	**7 100**	**8 100**	**12**	**11**	**13**
AFR	824 401	430	390	470	3 900	3 300	4 600	2 800	2 700	3 000	37	35	39
AMR	929 509	20	16	24	350	280	450	270	260	290	8.5	8.1	8.9
EMR	596 509	99	74	130	1 000	690	1 500	660	590	750	1.6	1.3	2.1
EUR	891 559	62	51	74	560	430	720	420	390	450	5.3	4.9	5.7
SEAR	1 783 587	480	360	630	4 900	3 300	7 100	3 300	2 900	3 700	5.7	4.1	7.8
WPR	1 800 640	240	180	310	2 900	1 900	4 200	1 900	1 700	2 100	1.8	1.4	2.3
Global	**6 826 205**	**1 300**	**1 200**	**1 500**	**14 000**	**12 000**	**16 000**	**9 400**	**8 900**	**9 900**	**12**	**11**	**13**

- Indicates no data reported.

[a] Numbers for mortality, prevalence and incidence shown to two significant figures. Totals for HBCs and global computed prior to rounding using Monte Carlo simulations.

[b] Mortality excludes deaths among HIV-positive case. Deaths among HIV-positive TB cases are classified as HIV deaths according to ICD-10.

[c] Best, low and high indicate best estimates followed by lower and upper bounds. The lower and upper bounds are defined as 2.5^{th} and 97.5^{th} centiles of outcome distributions produced in simulations.

[d] Banladesh completed a survey of the prevalence of TB disease in 2009. A reassessment of the epidemiological burden of TB, using data from the survey combined with an in-depth anlysis of surveillance and programmatic data, will be undertaken in 2011.

[e] Myanmar completed a survey of the prevalence of TB disease in 2010. A reassessment of the epidemiological burden of TB will be undertaken finalization and dissemination of survey results.

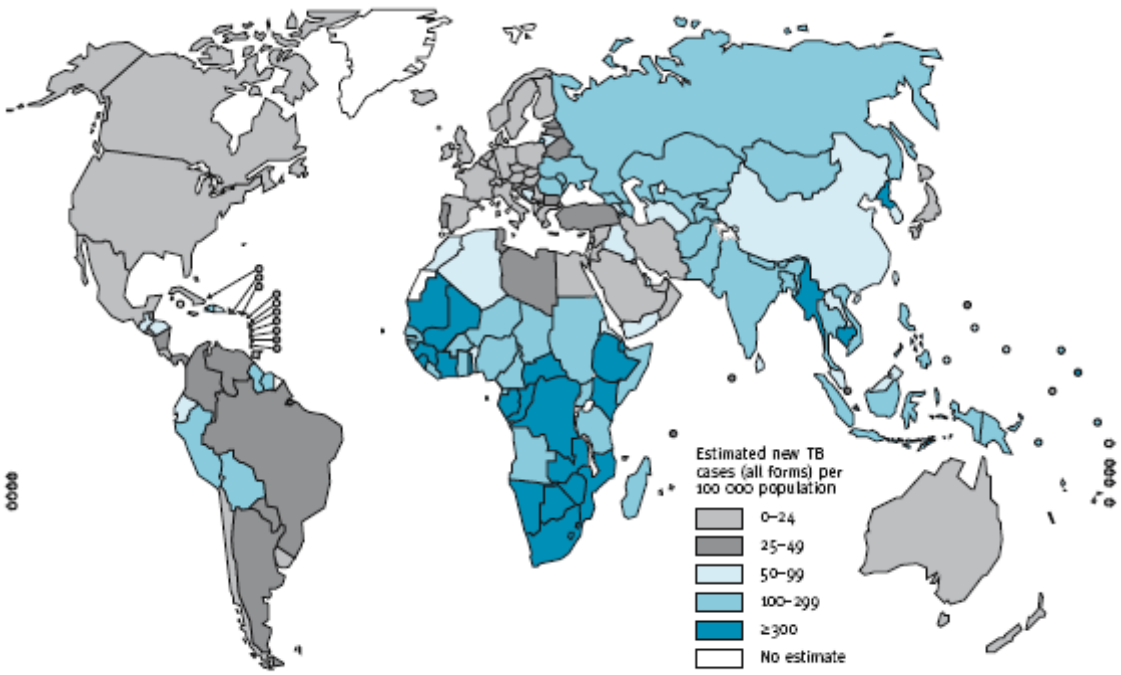


Figure 1: Estimated TB incidence rates, by country, 2009 (FromWHO, 2010)

MDR-TB most commonly develops in the course of TB treatment, and is most commonly due to doctors giving inappropriate treatment, or patients missing doses or failing to complete their treatment. It has been proposed that MDR-TB strains appear to be less fit and less transmissible, and outbreaks tend to occur in people with weakened immune systems (e.g., patients with HIV), although outbreaks among immunocompetent people do occur and the number of cases of TB due to MDR-TB on the rise. A 1997 survey of 35 countries found rates above 2% in about a third of the countries surveyed. The highest rates were in the former USSR, the Baltic states, Argentina, India and China, and was associated with poor or failing national tuberculosis control programmes.[263]

There were an estimated 440,000 cases of multi-drug resistant TB (MDR-TB) in 2008 (range, 390,000–510,000). The 27 countries (15 in the European Region) that account for 86% of all such cases have been termed the 27 high MDR-TB burden countries. The four countries that had the largest number of estimated cases of MDR-TB in absolute terms in 2008 were China (100,000; range, 79,000-120,000), India (99,000; range, 79,000–120,000), the Russian Federation (38,000; range, 30,000–45,000) and South Africa (13,000; range 10,000–16,000). By July 2010, 58 countries and territories had reported at least one case of extensively drug-resistant TB (XDR-TB).

2.1.2. TB burden in Vietnam

The Viet Nam National Tuberculosis Control Programme (NTP) is based on the principles of DOTS, the core control strategy recommended by the World Health Organization (WHO). According to WHO estimates, since 1997 Viet Nam has reached and exceeded the global targets for tuberculosis control[98,252] i.e. to detect >70% of new smear-positive pulmonary tuberculosis cases and cure >85% of these detected cases.[266] If these targets were met, tuberculosis incidence in Viet Nam would predictably have decreased over the period 1997–2004 by 44%.[64,252] Although there was indeed a small decrease in tuberculosis notification rates among women and persons older than 35 years, this was offset by an increase among young men, which led to stabilization in notification rates during this period.[128]

In Vietnam, almost 30,000 people die every year (one death every 18 minutes) from TB. Vietnam ranks 12th among the 22 countries[262] which added together account for 80% of the global TB burden, with a prevalence of smear-positive tuberculosis of 89 per 100,000 population (WHO, unpublished data, 2008). If TB is detected early and properly treated using a combination of medicines for 6 to 9 months, the patients quickly become non-infectious and are eventually cured. Multi-drug resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB), HIV-associated TB and weak health systems are major challenges in Viet Nam. There are an estimated 7,000 new MDR-TB cases and 6,400 new TB/HIV cases every year. Both of these forms of complicated TB carry a high risk of early mortality.[262]

2.2. Bacteriology of *Mycobacterium tuberculosis*

2.2.1. The bacillus

Bacteria of the genus *Mycobacterium* are non-motile and non-sporulated rods. They are grouped in the suprageneric rank of actinomycetes that, unusually, have a high content (61-71 %) of guanine plus cytosine (G+C) in the genomic desoxyribonucleic acid (DNA), and a high lipid content in the wall, probably the highest among all bacteria. *Mycobacterium* and other closely related genera (i.e. *Corynebacterium,Gordona, Tsukamurella, Nocardia, Rhodococcus* and *Dietzia*) have similar cell wall compounds and structure, and hence show some phenotypic resemblance. Several mycolic acids in the envelope structure distinguish the mycobacteria. These quirky lipids may act as carbon and energy reserves. They are also involved in the structure and function of membranes and membranous organelles within the cell. Lipids constitute more than half of the dry weight of the mycobacteria. However, the lipid composition of the tubercle bacillus may vary during the life cycle in culture, depending on the availability of nutrients. The waxy coat confers the idiosyncratic characteristics of the genus: acid fastness, extreme hydrophobicity, resistance to injury, including that of many antibiotics, and distinctive immunological properties. It probably also contributes to the slow

growth rate of some species by restricting the uptake of nutrients.[177]

Comprised within the *M. tuberculosis* complex and generically called the tubercle bacillus, the various etiologic agents of tuberculosis (TB) have distinct hosts, zoonotic potential and reservoirs (table 2). *M. tuberculosis,* and the regional variants or subtypes *Mycobacterium africanum* and *"Mycobacterium canettii*" are primarily pathogenic in humans. *Mycobacterium bovis* and *Mycobacterium microti* are the causative agents of TB in animals, and can be transmitted to humans. Some particular strains isolated from goats and seals have been named *Mycobacterium caprae* and *Mycobacterium pinnipedi,* although sometimes they are identified as *M. bovis* subspecies or variants. It could be expected that the major evolutive shifts involved in adaptation to different hosts would have entailed significant microbiological differentiation.[177] However, the above mentioned agents of TB together with the vaccine bacille Calmette-Guérin (BCG) strains rank close to each other along a phenotypically continuous taxon.[168,217,247,249]

Table 2: Members of *M. tuberculosis* complex with specific names (From Davies et al., 2008)

Species	Principal hosts	Humans as secondary hosts
M. tuberculosis	Humans	–
M. bovis	Cattle, deer, elk, bison, badger, opossum	Yes
M. caprae	Goats	Yes
M. africanum	Humans	–
M. microti	Vole, hyrax, llama	Very rare
'M. canettii'	Humans	–
M. pinnipedii	Seal	Very rare

The microscopic appearance does not allow the differentiation of the pathogenic agents of TB, mainly *M. tuberculosis*, from other mycobacteria although some characteristics may be indicative. In smears stained with carbol fuchsin or auramine and examined under light microscope, the tubercle bacilli typically appear as straight or slightly curved rods. According to growth conditions and age of the culture, bacilli may vary in size and shape from short coccobacilli to long rods.

By microscopic observation, Robert Koch first described the arrangement of bacilli in braided bunches and associated this phenomenon with virulent strains of *M. tuberculosis*. He also detailed the aspect of cultures in blood serum as compact scales which could be easily detached. In general, fresh virulent *M. tuberculosis* bacilli produce rough textured colonies on solid media, expanded gummy veils on the surface of liquid

media and serpentines on microscopic smears. In contrast, non-virulent mycobacteria and tubercle bacilli attenuated by prolonged cultures usually develop smooth colonies on solid media, form discrete mats in liquid media and distribute randomly in loose aggregates when smeared. The recognition of these two peculiarities, cording and crumbly colony formation, provides a reliable and timely clue to the experienced microbiologist for the presumptive distinction of *M. tuberculosis* from other mycobacteria in cultured specimens and even in sputum smears.[177]

When numerous and actively multiplying, the bacilli are strongly acid fast and show an evident and distinctive tendency to form hydrophobic bundles (Figure 2-aand 2-b). Free bacilli can also be seen, though, especially at the border of the swarms. In unlysed host tissue, the bacilli are more numerous within the phagocytic cells.

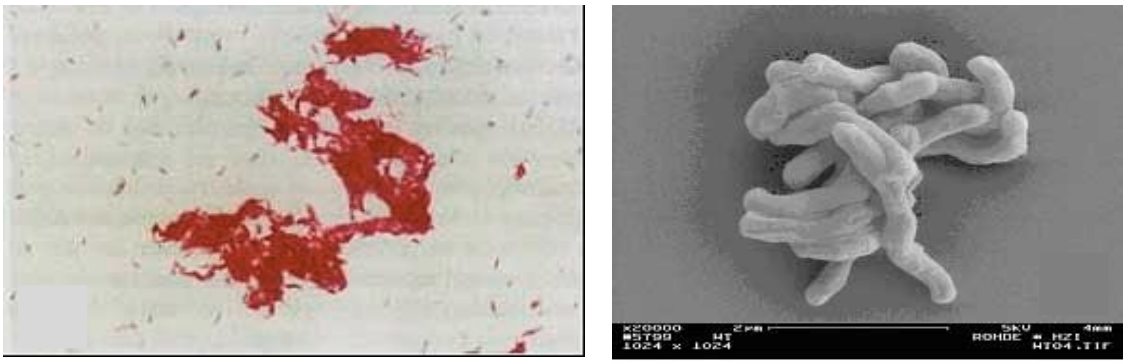


Figure 2: *Mycobacterium tuberculosis* growing in culture (From Palomino et al., 2007)
Ziehl-Neelsen staining at 1000x magnification.
Electron microscopy at 20000x magnification

These distinctive characteristics of the virulent bacilli have been attributed to the trehalose 6, 6'-dimycolate (TDM). This compound, also known as cord factor, was described as an extractable glycolipid consisting of two mycolic acid molecules loosely bound in the outer layer of the cell wall.[170] A myriad of biological activities related to pathogenicity, toxicity, and protection against the host response have been attributed to this molecule. However, it does not seem to be essential for bacterial multiplication *in vitro*.[99] Several models were used to identify the role of the TDM in the microscopic and macroscopic morphology of *M. tuberculosis*. In this way it was demonstrated that beads coated with this substance generate an oriented hydrophobic interaction and aggregate in elongated structures similar to cords.[16]

The tubercle bacillus is prototrophic (i.e. it can build all its components from basic carbon and nitrogen sources) and heterotrophic (i.e. it uses already synthesized organic compounds as a source of carbon and energy). The microorganism macromolecular structure and physiological (metabolic) capabilities result in high adaptation to the specific environment. In turn, the

nutritional quality of the environment determines the bacillus lifestyle and limitations, either in the natural habitat or in culture media, as do various physical conditions such as oxygen availability, temperature, pH and salinity.[177]

As the environment changes, the bacillus is able to bring into play different physiological pathways in order to survive even in harsh conditions. This is a highly resourceful strategy, not only for pathogenicity but also for species persistence. It has been shown that, during the course of infection in mice, *M. tuberculosis* metabolism may shift from an aerobic, carbohydrate-metabolizing mode to one that is more microaerophilic and utilizes lipids. These demonstrations, which were reported a long time ago, were supported in recent times by the completese quencing of the *M. tuberculosis* genome in which an unusually high number of genes putatively involved in fatty acid metabolism were identified. This phenomenon may be related to the ability of the pathogen to grow or persist in host tissues where fatty acids may be the major carbon source.[163]

The tubercle bacillus requires oxygen as a final electron acceptor in aerobic respiration. Molecular oxygen is reduced to water in the last step of the electron transport system. In nature, the bacillus grows most successfully in tissues with high oxygen partial tension, such as the lungs, particularly the well-aerated upper lobes. Carbon dioxide is essential and may be taken from the atmosphere and also from carbonates or bicarbonates. In the laboratory, an atmosphere of 5 to 10 % carbon dioxide favors culture growth, at least during the early stage of incubation. On the other hand, *M. bovis* is microaerophilic, i.e. it grows preferentially at a reduced oxygen tension.[177]

M. tuberculosis is mesophile and neutrophile as its multiplication is restricted to conditions offered by warm-blooded animals: about 37ºC and a neutral pH. The temperature and hydrogen ion concentration ranges, in which the bacillus is able to multiply, are relatively narrow. High saline concentration such as that found inmedia containing 5% sodium chloride, inhibits the growth of the microorganism.[177]

2.2.2. Immune response against tuberculosis

The immune response against tuberculosis plays a fundamental role in the outcome of *M. tuberculosis* infection. It is clear that the immune system reacts efficiently in the vast majority of infections. This is particularly evident in the case of TB, where most people infected by the tubercle bacillus (~ 90 %) do not develop the disease throughout their life times. Nevertheless, the risk of developing the disease increases considerably when TB infection co-exists with an alteration in the immune system, such as co-infection with human immunodeficiency virus (HIV).[177]

The likelihood and intensity of aerogenic transmission of *M. tuberculosis*

from a TB index case to a contact person in a shared air space depends on (i) exposure duration; (ii) intensity of exposure; (iii) cough and sputum-related host factors; and (iv) *M.tuberculosis* strain-related virulence characteristics. Infectious droplet nuclei are deposited in the alveolar spaces where *M. tuberculosis* can be phagocytosed by alveolar macrophages, epithelial cells, dendritic cells (DC) and neutrophils. Alveolar macrophages and DC are then believed to transport *M. tuberculosis* to local lymph nodes where T cells are primed and clonally expanded.[57]

During active TB disease there is an exuberant local pulmonary immune response characterized by an alveolitis of activated a/b T-cell receptor-positive lymphocytes, recently recruited immature macrophages, [193,201] and strongly enhanced *M.tuberculosis* antigen-specific Th1 responses,[95,202,228] with large amounts of locally secreted IFN-γ. Increased numbers of alveolar neutrophils can also be found in subgroups of patients. *M.tuberculosis*-induced immune evasion mechanisms with production of suppressive cytokines and effector molecules may counteract protective immune responses and abrogate bactericidal immune mechanisms. The role of regulatory T (Treg) cells in suppressing local immune responses and probably permitting mycobacterial growth is not yet well understood.[57]

Susceptibility to TB is associated with HIV-1 infection (decreased numbers and function of CD4+ and CD8+ T cells [218,46]), therapeutic tumor necrosis factor-a (TNF-α) blockage,[254] and hereditary IFN-γ and IL-12 receptor abnormalities.[6] These conditions have provided insights into immune requirements for protection against *M. tuberculosis*. However, because the potentially protective Th1responses (IFN-γ and TNF- α) are undermined *in vivo* by suppressive immune mechanisms, clear correlates of protection are still lacking for the assessment of new tuberculous vaccines and adjuvant immune therapies.

A role for protective immune response also underlies the persistence of latent TB infection (a state assumed to exist in a third of humans worldwide) during which *M. tuberculosis* growth is suppressed. These non-replicating bacilli are characterized by intracellular lipid bodies[84] and foamy lipid-laden macrophages may be associated with TB-related tissue pathology.[194]

In the parenchymal tissue, *M. tuberculosis* may induce the formation of pathognomonic granulomas (inflammatory reaction characterized by a ball-likecollection of immune cells). Necrotizing granuloma centres[58] provide ideal culture conditions for *M. tuberculosis* that multiply extracellularly to large numbers, gain access to the airways and thus eventually disseminate the disease within the lung and to close contacts.

Innate immunity to *M. tuberculosis*

Upon entry into the host lungs by aerosol inhalation, *M. tuberculosis* interacts with various receptors such as pattern recognition receptors such as toll-like receptors (TLR),[240]complement receptor 3,[200] mannosereceptor,[71] scavenger receptor,[71]DC-specific intercellular-adhesion-molecule-3-grabbing nonintegrin, on the surface of macrophages and DC (Figure 3). These receptors recognize components of *M. tuberculosis* such as lipoprotein, CpG-containingDNA, mannose-capped lipoarabinomannan and phosphatidylinositol mannoside, respectively. Lung surfactant protein D binds *M. tuberculosis* surface lipoarabinomannan and limits the intracellular growth of *M. tuberculosis* by increasing phagosome lysosome fusion.[74] In addition, cytosolic nucleotide-binding and oligomerization domain-like receptors such as NOD2 that recognizes muramyl dipeptide[59]and also C-type lectin dectin-1 that interacts with *M. tuberculosis* cooperate with TLR-2 to activate NF-kBand mediate pro-inflammatory cytokine and antimicrobial responses. Toll NF-kB pathway activation promotes nucleus translocation of NF-kB and activatesvitamin D pathway: (i) the activation of NF-kB results in production and secretion of many proinflammatory mediators including cytokines TNF-a,IL-1, IL-12, IL-18 and chemokines, which attract neutrophils, natural killer (NK) cells, T cells, and more DC and macrophages to the infection site,[116,131] and nitricoxide. It is of interest to note that *M.tuberculosis* secreted protein ESAT-6 could inhibit activation of NF-kB through preventing interaction between MyD88 and downstream kinase IRAK4;[180] (ii) TLR activation also up regulates expression of the vitamin D receptor (VDR) and the vitamin D-1-hydroxylase genes, which converts pro-vitamin D into the active form 1,25(OH)2D3 and leads to induction of the antimicrobial peptides cathelicidin and β-defensin to kill intracellular mycobacteria.[40,146] NADPH oxidase2, which mediates phagocytic killing of ingested pathogens like *M. tuberculosis* via reactive oxygen species, interacts with TLR-2 and affects VDR mediated antimicrobial peptide production. Knockdown of NADPH oxidase 2 inhibited 1,25D(3)-mediated antimicrobial activity against *M. tuberculosis* through the modulation of cathelicidin expression in human macrophages.[269] The importance of the host VDR and TLR in controlling TB is shown in polymorphisms in VDR and TLR-2 that are associated with increased susceptibility to TB infection.[18,236]In contrast, DC-specific intercellular-adhesion molecule-3-grabbing non-integrin signal pathway activation leads to production of IL-10 and transforming growth factor-β (TGF-β), which suppress the immune response.[116] Macrophages are heterogeneous and have different roles during TB infection, that is,IL-23-producing type 1 macrophages that promote Th1 immunity to mycobacteria, and IL-10-producing type 2 macrophages that suppress immunity to mycobacteria.[250] The type 2 macrophages have been shown to induce CD4+ T cells to adopt a Treg CD25+FoxP3+mTGFβ-1+

suppressor phenotype.[198] Interferon-γ, secreted from activated T cells and NK cells have the capability to activate macrophages and promote bacterial killing by permitting phagosomal maturation and production of antimicrobial reactive nitrogen intermediates and reactive oxygen intermediates.[109] Recent research also found that IFN-γ and TLR signalling pathways induce autophagy in macrophages,[91] which enhances the delivery of ubiquitin conjugates to the lysosome and increases the bactericidal capacity of the lysosomal soluble fraction.[5] Th1cytokine IFN-γ facilitates phagosome lysosome fusion (autophagy) through cell signalling pathway IRGm1(LRG-47) and PI3K, whereas Th2 cytokines IL-4 and IL-13 abrogate autophagy and autophagy-mediated killing of *M. tuberculosis* through Akt signalling pathway.[57] Besides IFN-γ, TNF-α also plays an important role in killing intracellular *M. tuberculosis* through reactive nitrogen intermediates together with IFN-γ and is involved in granuloma formation.[81] TNF is needed for controlling LTBI as anti-TNF antibody infliximab increases the risk of activating latent TB47 through direct neutralization of TNF and also depletion of granulysin-expressing CD45RA+ subset of effector memory CD8+ T cells that contributes to the killing of intracellular *M. tuberculosis*.[29]

The CD1d-restricted NKT cells have recently been shown to mediate protection against *M. tuberculosis* in the mouse model.[195] NK cells also contribute to protective immunity through killing CD4+CD25+FoxP3+Treg cells.[116] In addition, invariant NKT have recently been found to inhibit development of Th17 cells,[137] which may impact the pathology mediated by *M. tuberculosis* infection. The role of neutrophils in host defence against *M. tuberculosis* is conflicting. Polymorphonuclear neutrophils (PMN) are the first cells recruited to sites of microbial entry and express a range of receptors and avast arsenal of antimicrobial effector molecules as in macrophages.[116] Most experiments on human PMN suggest that PMN could be activated in response to *M. tuberculosis*, and have the ability to restrict mycobacterial growth *in vitro*. For example, PMN produce human neutrophil peptides 1–3 and cathelicidin LL-37 and lipocalin 2, which have the ability to kill or restrict *M.tuberculosis* growth. Furthermore, PMN could activate macrophages through releasing granule proteins and heat shock protein 72 from apoptotic neutrophils. However, on the other hand, PMN accumulated in *M. tuberculosis*-infected sites seem to have no obvious effect on mycobacterial growth and play a pathological rather than protective role in active disease. The results from different animal models are also conflicting. Some studies suggest neutrophils have a protective role during early stage of infection, while others suggest PMN do not play a role in control of TB. Mast cells seem to play a role in controlling *M. tuberculosis* infection as activation of mast cells via TLR-2 could compensate the defect in controlling *M. tuberculosis* infection in TLR-2 knockout mice.[57]

V-γ-9V-δ-2-encoded T cells are the main γ-δT cells in humans, which recognize non-peptidic phosphoantigens without known requirement for MHC molecules. Mature V-γ-9V-δ-2 T cells are rapidly recruited to the *M. tuberculosis* infection site, displaying a potent NK-like cytotoxic activity, expressing chemokine receptors, secreting a plethora of cytokines including IFN-γ and TNF-α, and killing *M. tuberculosis*-infected cells.[33] Likeγ-δ T cells, activated CD1 molecules-restricted T cells that present mycobacterial glycolipid antigens produce IFN-γ and express cytolytic activity.[109] These antigen-specific γ-δ T cells and CD1-restricted T cells have adaptive immune responses, and can have immune memory and mount rapid, strong recall expansion after *M. tuberculosis* reinfection.[140] Furthermore, parts of γ-δT cells produce IL-17 and express TLR-1 and TLR-2, as well as dectin-1, and could directly interact with certain pathogens to orchestrate an inflammatory response after bacterial invasion.[139]

Collectively, these data indicate that several pathways and cell types interact to mediate innate immunity against *M. tuberculosis*, and provide likely mechanisms for the observation that many individualsfail to display any immunodiagnostic evidence of TB infection despite significant and prolonged exposure to *M. tuberculosis*.[57]

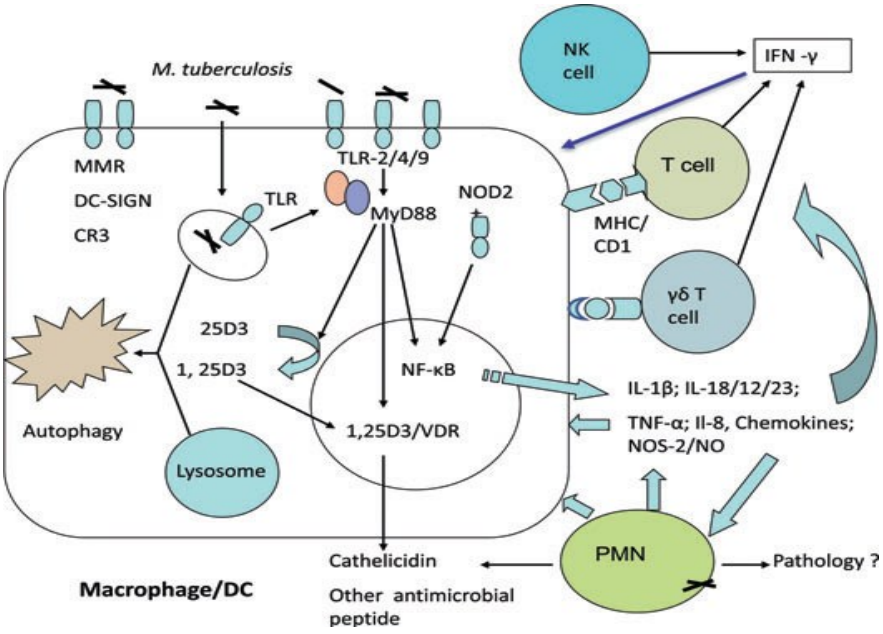


Figure 3: Innate immunty to tuberculosis infection. *M. tuberculosis* is phagocytosed by macrophages and dendritic cells through membrane-bound receptors such as CR3, scavenger receptor, MMR, TLR, NOD2 and DC-SIGN. These lead to activation of macrophage signalling pathways (NF-kB), causing secretion of pro-inflammatory cytokines, chemokines, and antimicrobial molecules, and activation of VDR, which induces the expression of the antimicrobial peptides cathelicidin and β-defensin. In addition, induction of autophagy mediates antimicrobial activity. PMN cells recognize and engulf *M. tuberculosis* and secrete antimicrobial peptides to kill bacteria. NK cells, γδT cells and CD1-restricted T cells are also be activated by specific ligands and cytokines, release cytotoxic factors and

secrete IFN-γ, which activates macrophages. CR3, complement receptor 3; DC-SIGN, dendritic cell-specific intercellular-adhesion molecule-3-grabbing non-integrin; INF, interferon; MMR, macrophage mannose receptor; NK, natural killer; PMN, polymorphonuclear neutrophils; TLR, toll-like receptors; TNF, tumour necrosis factor; VDR, vitamin D receptor. (From Dheda et al., 2010)

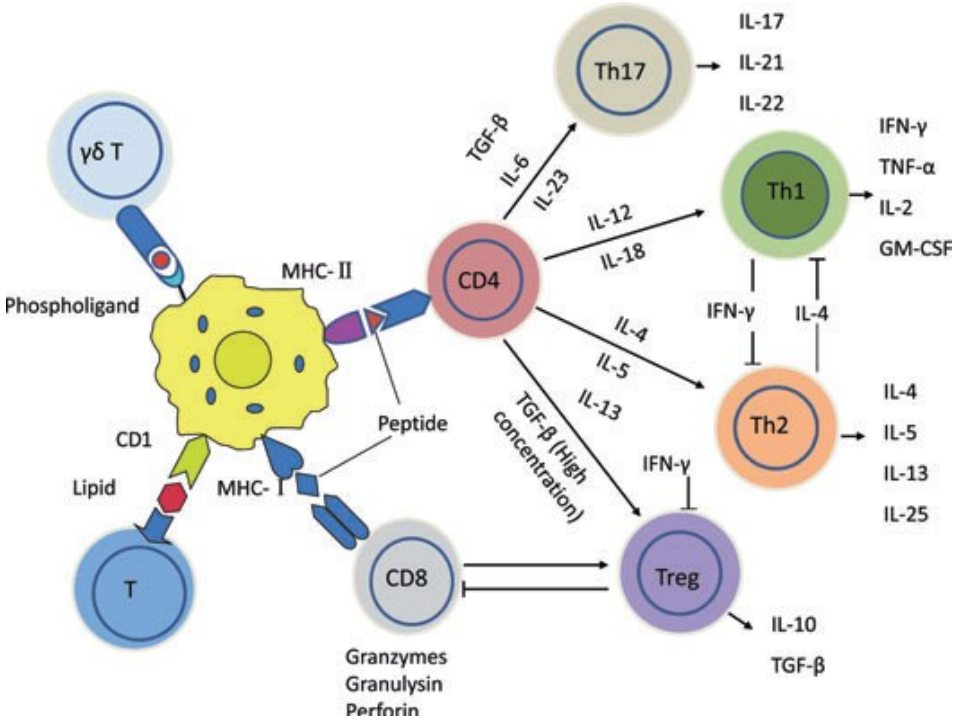


Figure 4: Adaptive immunity to tuberculosis infection. The infected macrophages and dendritic cells secrete cytokines that include IL-12, IL-23, IL-7, IL-15 and TNF-α, and present antigens to several T-cell populations including CD4+ T cells (MHC class II), CD8+ T cells (MHC class I), CD1-restricted T cells (glycolipid antigens) and γδ T cells (phospholigands). These T cells produce the effector cytokine IFN-γ, which activates macrophages in conjunction with TNF-α to effect killing of intracellular mycobacteria through reactive oxygen and nitrogen intermediates. In addition, CD8+ cytotoxic T cells can kill intracellular mycobacteria through granulysin and perforin-mediated pathways. However, CD4+ Th2 cells produce immunosuppressive cytokines such as IL-4, and CD4+CD25+FoxP3+ regulatory T (Treg) cells produce IL-10 and TGF-β that may suppress mycobactericidal effector mechanisms.[190] A new subset of T helper cells called Th17 cells that are produced in the presence of IL-23, and are characterized by production of IL-17, is important modulator of inflammation and recall memory responses. Th17 cells can recruit neutrophils and monocytes, and IFN-γ-producing CD4+ T cells, and stimulate chemokine expression. However, IFN-γ in turn can suppress the IL-17 producing Th17 cells. Thus, there appears to be a more complex cross-regulation of Th1, Th2, Th17 and Treg cell responses, than previously recognized and the precise role of individual responses in protective immunity remain controversial. GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; TGF, transforming growth factor; TNF, tumour necrosis factor. (From Dheda et al., 2010).

Adaptive immunity to Mycobacterium tuberculosis

Mycobacteria-infected macrophages and dendritic cells (DC) of the innate immunity present antigens to T cells and B cells that belong to adaptive immunity. CytokineIL-12p40 plays a fundamental role in the pathogen induced activation of pulmonary DC.[112] Macrophage apoptosis that releases apoptotic vesicles to carry mycobacterial antigens to uninfected DC can lead tomore effective antigen presentation. Inhibition of macrophage apoptosis could reduce transfer of antigens to bystander cells and affect activation of CD8 T cells.[199]

MHC class CD1-restricted CD4 T cells and MHC class CD1-restricted CD8 T cells that recognize peptide antigens and the γ-δ T lymphocytes as well as the CD1-restricted specific T lymphocytes produce IFN-γ and constitute the protective immunity (Figure 4). The CD4+ Th1 cells mount a much stronger IFN-γ response than CD8+ T cells after mycobacterial infection[164] and are thought to play a prominent role in protection.[108] The lack of CD4 T cells may result in delayed distribution of activated CD8 T cells from draining lymph nodes to distant organs and consequently cause a delayed acquisition of immune protection.[255] The CD8 cytolytic T lymphocytes (CTL) secrete granulysin, granzymes and perforins to kill mycobacteria-infected cells and are capable of immune protection against secondary mycobacterial challenge in the absence of CD4+ T cells.[164]

The CD4 T helper cells can be differentiated into Th1, Th2, Th17 and Treg cells. The Th1 cells produce cytokines, notably IFN-γ, TNF-α, IL-2, lymphotoxin and granulocyte-macrophage colony-stimulating factor (GM-CSF), which prompt stimulation of Th1 cells, CTL, and maturation and activation of macrophages as well as granulocytes. The Th2 cells produce B cell-stimulation factors such as IL-4, IL-5,IL-10 and IL-13, which promote antibody production but suppress the Th1 type immune response. The Th17 cells, a distinct subset of helper T cells, produce unique cytokines of IL-17, IL-17F, IL-21 and IL-22, which stimulate defensin production and recruit neutrophils and monocytes to the site of inflammation, and are involved in the early phase of host defence. The Th1, Th2 and Th17 subsets may be modulated by Treg cells, of which there are several types and the list is growing. The CD4+CD25+FoxP3+ natural Treg cells are characterized by TGF-β and IL-10 production,[60,110] while the Treg cells also co-produce IFN-γ.[241] In addition to CD4+ Treg cell subsets, CD8+ Treg cells are also described, which could inhibit T-cell proliferation and cytokine production.[102] The FoxP3-expressing Treg cells are expanded during TB infection,[203] and inhibit human memory γ-δ T cells to produce IFN-γ in response to *M. tuberculosis* antigens.[127] However, phosphoantigen-activated Vγ2Vδ2T cells could antagonize IL-2-induced CD4+CD25+Foxp3+ Treg cells in mycobacterial infection.[86] CD4+CD25+FoxP3+ Treg cells also produce TGF-β to down regulate CD4+ T-cellresponse,[121] and suppress the effector-immune response and induce

bacillary dissemination and disease manifestation.[187,206] High per cent of Treg cells characterized as CD4(+)CD25(high)CD39(+) was also identified in active TB patients.[39] Attenuation of Treg cells has a positive impact on the protective capacity of vaccine against *M. tuberculosis* infection.[14,101] Nevertheless, given the extensive immunopathology and lung damage that characterize human TB, the precise role of Treg cell (deleterious, beneficial or bystander) in the immunopathogenesis of TB remains unclear.

Different cytokines can decide the differentiation of T-cell sets (Figure 4). IL-12, IL-18 and IFN-γ promote Th1 cell development, while IL-4, IL-5 and IL-13 induce Th2 cell development. IL-23, Il-6, IL-21 and low concentration of TGF-β could induce Th17 differentiation, while IL-2 and high concentration of TGF-β could induce Treg differentiation.[110,60] IL-6 inhibits the generation of Treg cells induced by TGF-β, but together with TGF-β induces the differentiation of Th17 cells.[20]

Traditionally, B cells are not generally thought to play a significant role in protection against TB. However, recently it has been demonstrated that B cells[2] are required for optimal protection in *M. tuberculosis*-infected mice through interactions with the cellular immune response and activation of complement.[132] *M. bovis Bacille Calmette Guérin* (BCG) directly activates the classical, lectin and alternative pathways, resulting in fixation of C3b onto macromolecules of the mycobacterial surface,[32] which will contribute to mycobacterial killing.

Memory T (TM) cells form after *M. tuberculosis* exposure or infection. TM cells can be developed into effector ($CCR7^{lo}$, $CD62L^{lo}$, $CD69^{hi}$ in humans) and central TM cells ($CCR7^{hi}$, $CD62L^{hi}$, $CD69^{lo}$ in humans).[108] TM cells proliferate promptly after encounter with antigens, and produce multiple cytokines such as IFN-γ, IL-2, TNF-α, lymphotoxin and/or GM-CSF.[110] Nevertheless, the identification of specific biomarker signature of protective immunity remains elusive. Multifunctional CD4+ T-cell responses, like those raised to the TB subunit vaccine, Ag85B-ESAT-6/CAF01-induced, may be a better marker of protective immunity.[57]

2.2.3. Genomic organization and genes of *M. tuberculosis*

TB research made huge progress with the availability of the genome sequence of the strain *M. tuberculosis* H37Rv.[42] Expectations were generated on the elucidation of some unique characteristics of the biology of the tubercle bacillus, such as its characteristic slow growth, the nature of its complex cell wall, certain genes related to its virulence and persistence, and the apparent stability of its genome. This first available genome sequence of a pathogenic *M. tuberculosis* strain helped to answer some of these questions and, what is even more stimulating, to open many more.[177]

M. tuberculosis H37Rv[42] was revealed to possess a sequence of 4,411,529 bp (Figure 5), the second largest microbial genome sequenced at

that time. The characteristically high guanine plus cytosine (G+C content; 65.5 %) was found to be uniform along most of the genome, confirming the hypothesis that horizontal gene transfer events are virtually absent in modern *M. tuberculosis* .[220.] Only a few regions showed a skew in this G+C content. A conspicuous group of genes with a very high G+C content (> 80 %) appear to be unique in mycobacteria and belong to the family of PE or PPE proteins. In turn, the few genes with particularly low (< 50 %) G+C content are those coding for transmembrane proteins or polyketide synthases. This deviation to low G+C content is believed tobe a consequence of the required hydrophobic amino acids, essential in any transmembrane domain, that are coded by low G+C content codons.[177]

Fifty genes were found to code for functional RNAs. As previously described,[111] there was only one ribosomal RNA operon (*rrn*). This operon was found to be located at 1.5 Mbp from the origin of replication (*oriC* locus). Most eubacteria have more than one rrn operon located much closer to the *oriC* locus to exploit the gene-dosage effect during replication.[43] The possession of a single *rrn* operon in a position relatively distant from *oriC* has been postulated to be a factor contributing to the slow growth phenotype of the tubercle bacillus.[24]

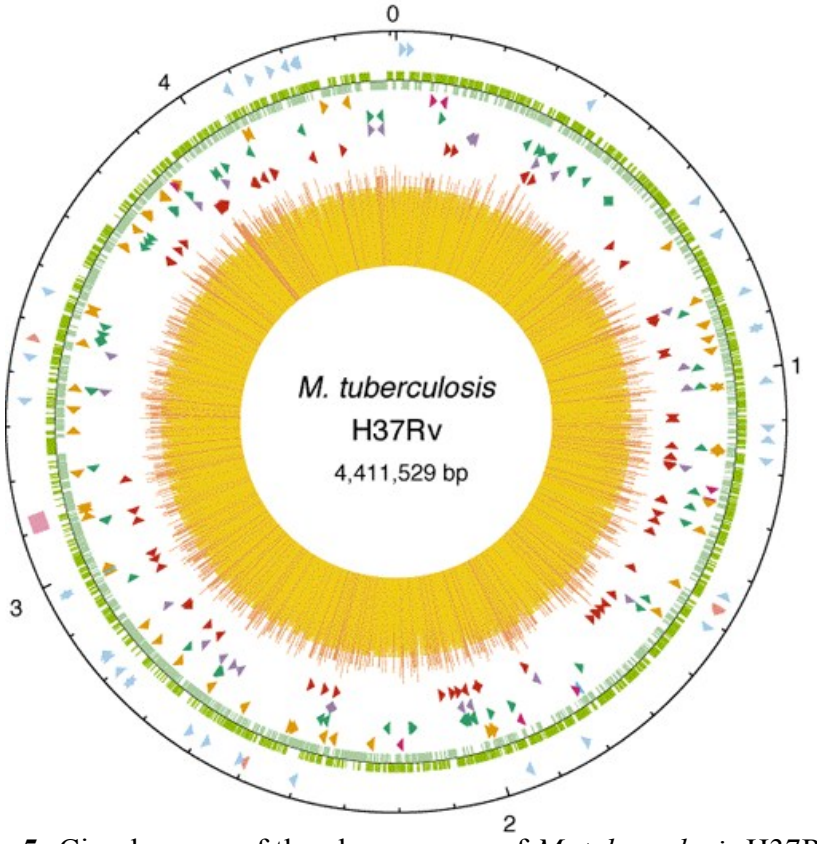


Figure 5: Circular map of the chromosome of *M. tuberculosis* H37Rv. The outer circle shows the scale in Mb, with 0 representing the origin of replication. The first ring from the exterior denotes the positions of stable RNA genes (tRNAs are blue, others are pink) and the direct repeat region (pink cube); the second ring inwards shows the coding

sequence by strand (clockwise, dark green; anticlockwise, light green); the third ring depicts repetitive DNA (insertion sequences, orange; 13E12 REP family, dark pink; prophage, blue); the fourth ring shows the positions of the PPE family members (green); the fifth ring shows the PE family members (purple, excluding PGRS); and the sixth ring shows the positions of the PGRS sequences (dark red). The histogram (centre) represents G + C content, with < 65% G + C in yellow, and > 65% G + C in red. The figure was generated with software from DNASTAR. (From Cole et al., 1998)

One of the most thoroughly studied characteristic of *M. tuberculosis* is the presence and distribution of insertion sequences (IS). Of particular interest is IS*6110*, a sequenceof the IS3 family that has been widely used for strain typing and molecular epidemiology due to its variation in insertion site and copy number.[243] Sixteen copies of IS*6110* were identified in the genome of *M. tuberculosis* H37Rv; some IS*6110* insertion sites were clustered in sites named insertional hot-spots. The same strain was found to harbor six copies of the more stable IS*1081*, an insertion sequence that yields almost identical profiles in most strains when analyzed by Restriction Fragment Length Polymorphism (RFLP).[106,215] Another 32 different insertion sequences were found, of which seven belonged to the 13E12 family of repetitive sequences; the other insertion sequences had not been described in other organisms.[45] Virtually all the ISs found in *M. tuberculosis* so far belong to previously described IS families.[49] The only exception is IS*1556*, which does not fit into any known IS family.[44]

Two prophages were detected in the genome sequence; both are similar in length and also similarly organized. One is the prophage PhiRv1, which in the *M. tuberculosis* H37Rv genome interrupts a repetitive sequence of the family 13E12. This prophage is deleted or rearranged in other *M. tuberculosis* strains.[80] The genome of *M. tuberculosis* possesses seven potential *att* sites for PhiRv1 insertion, which explains the variability of its position between strains.[44] The second prophage, PhiRv2 has proven to be much more stable, with less variability among strains.[44]

Regarding protein coding genes, it was determined that *M. tuberculosis* H37Rv codes for 3,924 ORFs accounting for 91 % of the coding capacity of the genome.[42] The alternative initiation codon GTG is used in 35 % of cases comparedto 14 % or 9 % in *Bacillus subtilis* or *Escherichia coli* respectively. This contributes to the high G+C bias in the codon usage of mycobacteria.[177]

A bias in the overall orientation of genes with respect to the direction of replication was also found. On average, bacteria such as *B. subtilis* have 75 % of their genes inthe same orientation as that of the replication fork, while *M. tuberculosis* only has 59 %. This finding has led to the hypothesis that such a bias could also be part of the slow growing phenotype of the tubercle bacillus.[44] This conjecture, however, does not take into account the fact that *E. coli*, a bacterium that grows much faster than *M. tuberculosis,* has

only 55% of its genes in the same directionas the replication origin.[126]

From the predicted ORFs, all proteins have been classified in 11 broad functional groups, more precisely classified into COG functional categories (http://www.ncbi.nlm.nih.gov/sutils/coxik.cgi?gi=135) according to the National Center for Biotechnology Information (NCBI) of the United States (US). The analysis of the codon usage showed a preference for G+C-rich codons. It was also found that the number of genes that arose by duplication is similar to the number seen in *E. coli* or *B. subtilis*, but the degree of conservation of duplicated genes is higher in *M. tuberculosis*. The lack of divergence of duplicated genes is consistent with the hypothesis of a recent evolutionary descent or a recent bottleneck in mycobacterial evolution.[23]

From the genome sequence it is clear that *M. tuberculosis* has the potential to switch from one metabolic route to another including aerobic (e.g. oxidative phosphorylation) and anaerobic respiration (e.g. nitrate reduction). This flexibility is useful for survival in the changing environments within the human host that range from high oxygen tension in the lung alveolus to microaerophilic/anaerobic conditions within the tuberculous granuloma. Another characteristic of the *M. tuberculosis* genome is the presence of genes for synthesis and degradation of almost all kinds of lipids from simple fatty acids to complex molecules such as mycolic acids. In total, there are genes encoding for 250 distinct enzymes involved in fatty acid metabolism, compared to only 50 in the genome of *E. coli.*[44]

Concerning transcriptional regulation, *M. tuberculosis* codifies for 13 putative sigma factors and more than 100 regulatory proteins. Among the most interesting protein gene families found in *M. tuberculosis* are the PE and PPE multigene families, which account for almost 10% of the genome capacity. The names PE and PPE derive from the motifs of Pro-Glu (PE) and Pro-Pro-Glu (PPE) found near the protein N-terminus in most cases. These proteins are believed to play an important role in survival and multiplication of mycobacteria indifferent environments.[136] There are about 100 members of the PE family, which is further divided into three sub-families, the most important of which is the polymorphic GC-rich sequences (PGRS) class that contains 61 members. Proteins in this class contain multiple tandem repetitions of the motif Gly-Gly-Ala, hence, their glycine concentration is superior to 50 %. The PE_PGRS proteins resemble the Epstein-Barr virus nuclear antigens (EBNA), which are known to inhibit antigen presentation through the histocompatibility complex (MHC) class I.[44]

Interestingly, the analysis of the desoxyribonucleic acid (DNA) metabolic system of *M. tuberculosis* indicates a very efficient DNA repair system, in other words, replication machinery of exceptionally high fidelity. The genome of *M. tuberculosis* lacks the MutS-based mismatch repair system. However, this absence is overcome by the presence of nearly 45 genes related to DNA repair mechanisms,[149] including three copies of the *mutT* gene. This

gene encodes the enzyme in charge of removing oxidized guanines whose incorporation during replication causes base-pair mismatching.[44,149]

As more information was generated, databases grew bigger, more experimental information became available, and better and more accurate algorithms for gene identification and prediction were released. The initial genome annotation in *M. tuberculosis* H37Rv strain soon became out of date. For this reason, a re-annotation of that genome sequence was published in 2002. This re-annotation incorporated 82 additional genes. The gene nomenclature was not altered; the new genes have the name of the preceding gene followed by A, B or D, for example, two new ORFs were described between *Rv3724* and *Rv3725*, hence, they were named *Rv3724A* and *Rv3724B*. The letter C was not included since it usually stands for "complementary", which means that the gene is located in the complementary strand. As expected, the classes that exhibited the greatest numbers of changes were the unknown category and the conserved hypothetical category. The reannotation of the genome sequence allowed the identification of four sequencing errors making the current sequence size change from 4,411,529 to 4,411,532 bp.[31]

In Table 3, the information obtained from a single sequenced genomeis enormous. The advances made on the analysis of such information have accelerated TB research.

Table 3: Functional classification of *M. tuberculosis* H37Rv and re-annotation

Group[a]	Function	Number of genes (1998)[b]	Number of genes (2002)[b]	Number of genes (2008)[c]
0	Virulence, detoxification, adaptation	91	99	212
1	Lipid metabolism	255	233	237
2	Information pathways	207	229	232
3	Cell wall and cell processes	516	708	751
4	Stable RNAs	50	50	50
5	Insertion sequences and phages	137	149	147
6	PE and PPE proteins	167	170	168
7	Intermediary metabolism and respiration	877	294	898
8	Protein of Unknown function	606	272	15
9	Regulatory proteins	188	189	194
10	Conserved hypotheticals	910	1051	895
16	Conserved hypotheticals with an orthologue in *M. bovis*	NA	NA	262

a. The functional groups number were taken from the Tuberculist database, publically available at http://genolist.pasteur.fr/TubercuList/.

b. Data taken from Fleischman 2002[80]; *c. Data taken from Målen 2010*[133]

2.2.4. Molecular mechanism ofdrug resistance to rifampicin, izoniazid, ethambutol and streptomycin of *M.tuberculosis*

Tuberculosis is a major threat to global public health that demands new approaches to disease diagnosis and treatment. Understanding at the genomes of different strains, we can know the mechanism of drug resistance of *M. tuberculosis* that may help develop better treatments.

M. tuberculosis becomes resistant to antibiotics by chromosomal mutation, which confers resistance to a TB drug. The accumulation of these mutations leads to multi-drug resistance. Unlike horizontal transfers, which involve the acquisition of DNA fragments with different origins, mutations that confer resistance to TB drugs affect isolated portions of bacterial chromosome. Thus by detecting them, the strain's resistance can be predicted. This is particularly easy in the case of antibiotics to which resistance is caused by the alteration of an essential gene matching the antibiotic target. Very few mutations could cause a loss of interaction between the target and the antibiotic without affecting the gene's function. Thus, only a few permissive mutations of the antibiotic's target will be observed. In the case of resistances to rifampicin (RIF) or fluoroquinolones (FQ), permissive mutations primarily affect a small fragment of *rpo*B (coding for RNA polymerase) and *gyr*A (coding for DNA gyrase) genes respectively. This is also the case for aminoglycoside resistance, which is caused by mutations in the translation machinery genes (*rrs and rps*L) and can only becaused by permissive mutations. These mutations, which affect vital genes, lead to a loss of affinity for the antibiotic without destroying the gene's function. However, when antibiotic resistance is caused by the loss of function of a non-essential gene, a broad spectrum of mutations will be observed, which makes detecting them difficult. This will involve any mutation that inactivates the gene. In order to detect these mutations, the complete sequence of the mutated genes will need to be known. In addition, mutation will not necessarily mean the loss of gene function, and therefore antibiotic resistance. This is the case for example, of the *pnc*A gene, which is inactivated by mutation in the event of resistance to pyrazinamide (PZA), and the genes involved in isoniazid (INH) resistance. The *pnc*A gene is a small gene that can be easily sequenced to find mutations. However, a large number of mutations have been observed in strains susceptible to PZA. These mutations do not affect gene function. High-level resistance to INH is often due (approximately 75% of cases) to the same point mutation in *katG*. Thus isolating it for a large number of strains is easy and can be done to identify the resistance. Resistance can only be linked to a mutation on one gene or be the result of single or multiple complex mutations on different genes as in the case for INH resistance for example.[182]

Rifampicin Resistance

In use since 1966, Rifampicin (RIF) is an important component in treating tuberculosis. It is a broad spectrum rifamycin derivative that interferes with the synthesis of mRNA synthesis by binding to the ß subunit of RNA polymerase (RpoB) in bacterial cells. The RIF binding site is a pocket in the upper wall of the main channel for double-stranded DNA entry just upstream of the polymerase catalytic center. The various RIF-resistant mutations are clustered around this pocket.[273] Thus, RIF resistance is associated with a hotspot (codon 507 to 533) core region called RRDR, for "rifampicine resistance determining region" (81 bp) of the *rpoB* gene.[231,232] More than 95% of RIF resistant *M. tuberculosis* has a mutation in this specific zone.[272]

Resistance occurs at a frequency of 1 out of 107 to 108 bacterial cells. Most RIF-resistant strains show one mutation in the gene. Two to four mutations are rarely reported.[134,205] The most prevalent mutations (81%) affect codons 531 and 526 and usually lead to a high level of phenotypical resistance (MIC > 64 µg/ml) as well as cross resistance to other rifamycins. Mutations 511, 516, 518 and 522 result in a low-level resistance to RIF and rifapentine; and some susceptibility to rifabutin.[272] At the same time, mutations in this hotspot region seem to confer low phenotypical resistance (deletion of codon 508-509, mutation at 515)[229] or variable resistance (L533P),[114] which could lead to an overly hasty interpretation of resistance. The latest observations of Asian strains suggest a geographic variability that can influence the accuracy of genotypic tests.[192]

Silent mutations (Leu511 and Leu521) have been reported in resistant strains. Interestingly, the L511L mutation is always associated with other mutations that confer resistance.[209] In rare cases, double mutations appear to have an additive effect on the degree of resistance. The role of mutations, combined with those known to confer resistance, is uncertain, as in the case of S509R described with H526R.[205]

Finally, less than 5% of resistant strains do not show a mutation in the *rpoB* resistance region.[134,192] Rare loci found outside the hotspot region of *rpoB* are associated with resistance without associated mutation known for conferring resistance. The CTG Leu 533 Pro CCG mutation has been shown on two strains of low-level resistance (MIC 12.5 µg/ml) and on a strain that is susceptible according to phenotypic tests, yet clinically resistant.[192] Other studies describe some sensitive strains with mutations as Ser 450 Leu,[205] Leu 511 Arg or Ser 512 Thr,[150] or Gln CAA 513 Gln CAG.[114] Genotypic detections would therefore be more sensitive in certain circumstances. Resistance to rifampicin, which usually develops with resistance to INH, thus defines the strain as multi-resistant (MDR). However, there are rare isolated mono resistances to rifampicin.[191]

The first mutation detection studies were based on PCR sequencing or PCR-SSCP (single strand conformational polymorphism analysis (SSCP). Now, resistance is frequently screened by one of the methods on the market (INNOLIPARif.TB or GenoType MTBDRplus, HAIN).[96] In these tests, a decontaminated sample is amplified with biotin labeled amplicons. This amplification product is hybridized on a test stripcontaining oligonucleotides. The reaction identifies the signals as parallel marks on the test strip. Thus the presence or absence of a mutation is easily detected in a few hours. To identify rifampicin resistance, the Hain MTBDRplus test strip has 8 probes that recognize wild mutations and four that correspond to the most frequently observed mutations (S531L, H526Y, H526D, and D516V of *rpoB*, mutations observed in 75% of RIF resistances). The test is rapid and easy, and can be applied to samples that are positive upon direct exam or to advanced cultures. There is strong correlation with results obtained through phenotypic susceptibility measurements,[55] automatic sequencing,[25,36,96] or RNA-RNA mismatch assay.[151]

In summary, the RIF binding site to polymerase is a pocket around which the mutations observed on resistant strains are clustered. These mutations are found in nearly all of the strains (>95%). Thus, analyzing this core sequence makes it easy to detect RIF resistant strains and predict their resistance level. It also provides strong concordance among results from different geographic regions. Furthermore, the frequency of mutations among resistant strains is very high: 100% (n=28) in Japan,[205] 100% (n=93) or 98%(n=44) in two Indian studies.[209,134] Only silent mutations are observed. Diagnostic difficulty could be caused by rare inconsistencies where mutated strains are phenotypically susceptible. One of them may even be clinically resistant, which could suggest greater genotypic test sensibility.[182]

Commercial amplification/reverse hybridization kits provide rapid, effective detection of the mutations most commonly responsible for rifampicin resistance.

Based upon *rpoB* core region analysis, the Cepheid gene Xpert System'shands-free sputum processing and real-time PCR system (with rapid ondemand,near-patient technology), simultaneously detects *M. tuberculosis* and RIF resistance within 2 hours from smear-negative and smear-positive clinical sputum samples. The self-contained cartridge fluidics of the XpertMTB/RIF assay make it possible to design a hemi-nested PCR assay with asensitivity that approaches culture-based diagnostics. An internal control target detects false-negative results. The GenoType MTBDR*plus* test (Hain) is able to identify resistance to RIF by detection of mutation in the core region of the *rpoB* gene. Mutations are identified by an amplification method and reverse hybridization on test strips.[182]

The MTBDR*plus* strip contains 17 probes, including 5 amplification and hybridization controls to verify the test procedures. For the detection of

RIF resistance, five *rpoB* wild-type probes (probes WT1 to WT5) encompass the region of the *rpoB* gene encoding amino acids 509 to 534. Four probes(probes *rpoB* MUT D516V, *rpo*B MUT H526Y, *rpoB* MUT H526D, and *rpo*B MUTS531L) specifically target the most common mutations. The Hillemann evaluation[96] detected an association with *rpoB* mutations in 96.8 to 98.7% of RIF resistant strains.

Isoniazid Resistance

Since 1952, Isoniazid (INH) has been one of the most effective and specific agents for treating *M. tuberculosis* infections. This is an essential TB drug that, along with RIF and PZA, forms the base for treating susceptible tuberculosis and is also widely prescribed for treating latent infections. INH is very active (MIC 0.02 to 0.2 μg/ml) on *M. tuberculosis*. It is a simple structure made up of a pyridine ring and a hydrazide group, two essential components to its powerful activity against increasing *M. tuberculosis*.[272] It has one of the most complex modes of action among all antibiotics. INH is a prodrug that requires activation of the catalase-peroxidase enzyme (KatG) coded by the *katG* gene.[274] Once activated, INH seems to destroy the synthesis of mycolic acids of the cell wall by inhibiting NADH-dependent enoyl-ACP reductase coded by the *inhA* gene. It encourages the bacterial cell to produce toxic free radicals. Resistant strains appeared shortly after the use of INH in clinical treatment, many of whom had lost their catalase activity. The increasing observation of INH-resistant strains, up to 20-30% of strains in some regions,[41] which is the first step towards multiresistance, have cast doubts on its use as a first-line treatment of tuberculosis.

Resistance develops with a high mutation frequency (1 for 106), higher than that of rifampicin (1 for 107 to 108). There are many molecular mechanisms causing INH resistance, but only some of them have been characterized. A recent review explains them by their molecular analysis.[251]

Mutations in the *katG* gene, which hinder activation of the INH prodrug, are among the most frequent in INH-resistant strains: 20 to 95% INH-resistant strains have at least one mutation in the *katG* gene; its position varies according to their geographical origin.[92,102,188,192,272,274] The S315T mutation is the most frequent (75 to 90%) even up to 93.6%.[153] It lowers catalase peroxidase activity by 50% and leads to a relatively high level of resistance (MIC from 5 to 10 μg/ml), without cross-resistance to ethionamide. It alters INH activity by preventing bond formation between INH and KatG. Strains with acomplete deletion of the *katG* gene may have an MIC equal to at least 50μg/ml.[182]

R463L substitution, found in *M. bovis*, *M. africanum* and *M. canettii*, is not considered to be a source of resistance in *M. tuberculosis*, where it is found in15 to 30% of cases, regardless of whether the strain is susceptible or resistant.[113] The Siddiqi study in 2002[209] analyzed 24 INH-resistant strains

and reported other insertions, deletions, and substitutions on the *katG* gene: an AC deletion at position 30 on six strains, resulting in the synthesis of a small polypeptide; a G deletion at position 109 on two strains, resulting in a truncated polypeptide; an A inserted at 98 and a C at 185 on four and three strains respectively, causing premature 20 polypeptide chain terminations. Only a few strains had new substitutions: Ala61Thr, Thr12Pro, Thr11Ala, Asp73Asn and Asn35Asp. One strain had a partial gene deletion. All of the isolates in this study had the Arg463Leu mutation, which was not related to resistance. Loss of catalase activity is not the only cause of resistance. Resistance can also develop through alterations or an overexpression of the *InhA*[12] target, which codes for NADH-dependent enoyl-ACP reductase. 0 to 5% of INH-resistant strains have a mutation in the structural gene while 8 to 20% have a mutation in the *InhA*, *MabA* promoter.[13,159,188,274] 20 to 34% of INH-resistant strains have at least one mutation in *MabA* promoter, which is either alone or combined with a mutation in the*InhA* gene. Hazbon[92] identifies 51 mutations in *inhA* in 3/608(0.5%) of INH susceptible strains and 48/403 (12%) of INH resistant strains. All of the promoter mutations (except G17T) are observed in INH resistant strains, which are usually associated with the C-15-T mutation. All of the structural gene mutations (except I47T and I194T) were only detected on INH resistant strains. The *inh*A S94A mutations, which seem to alter the INH-NADH bond with InhA, are restricted to INH resistant strains.[125,251] Most of the strains that have one mutation in *inhA*, with no mutation in *katG*, have a relatively low level of resistance > 0.2 μg/ml and < or equal to 1 μg/ml. In 1998, mutations of the *kasA* gene, which codes for ß-ketoacyl ACP synthetase, were reported in INH resistant strains, with genetic complementation showing no proof that mutations were involved in resistance.[144] These mutations were found in INH resistant strains as well as INH susceptible strains. In Hazbon's study,[92] *kasA* mutations were identified at 66, 269 and 312 in 100/608 (16%) of INH resistant strains and 44/403 (11%) of susceptible strains. The *kasA* G312S mutation is unrelated to resistance in thestudy of 98 strains.[225] These results prove that none of the mutant *KasA* alleles are on INH resistant strains alone. In three *M. tuberculosis* strains, overexpression of *KasA* did not increase resistance to INH or EMB, in contrast to InhA.[122] The role of KasA in resistance is still undetermined.

Mutations were found in the *ahpC* gene, which codes for an alkylhydroperoxide reductase, for 10% to 13% of resistant strains [92,192] and also in susceptible strains.[92] Those that are located at position -46 (the most frequent), -9 (G at A) or D73H are not related to resistance. The only mutation found exclusively on INH-resistant strains is T51. These mutations are always related to mutations in *katG*, but rarely to those of codon 315.[192] Mutations in the *ndh* gene, which codes for NADH dehydrogenase, were found in a small number of clinical strains (3% of INH resistant and INH

susceptible strains for one study and 9% of INH resistant strains in another study).[92,123] These mutations confer resistance to INH and to ethionamide. The most frequent is V18A, which was observed independently from susceptibility. Only one mutation in *ndh*, R268H, is found exclusively on clinical strains of INH-resistant *M. tuberculosis*.[92,123,251] Other genes could be involved in resistance.

The *NAT* gene, coded for an arylamine N-acetyl transferase. The NAT protein of *M. tuberculosis* acetylates the INH nitrogen group, thus preventing the *KatG* gene from activating it. *NAT* mutations observed in clinical strains of INH resistant were always associated with mutations in *katG*, but also found in INH susceptible strains.[188,251]

Studies on clinical INH resistant strains identified mutations on other genes such as *furA*, *Rv 0340-0343*, *Rv1772*, *fadE24*, *efpA*,[251]and so on. These mutations were related for the most part to mutations in *katG* and/or in the ***inhA*** promoter, which makes it difficult to determine their role in INH resistance.[188,272]

Several studies have focused on the interactions between genes. The recognition of simultaneous mutations in two genes, *KatG* and *InhA*, is associated with resistance in 75% to 85% of cases.[90,102] Finally, many of the strains (n=1,011) analyzed in Hazbon's study[92] were able to demonstrate a strong individual association between mutations of the *katG*, *inhA*, and *ahpC* genes and resistance to INH (P = 0.0001), while *kasA* mutations were associated with susceptibility. Remarkably, there were more mutations (particularly *katG*315) among MDR strains than among strains that were only resistant to INH. In this last case, mutations of the *inhA* gene were more frequent. Moreover, significant associations were described between mutations of the *katG*, *inhA*, and *ahpC* genes and RIF resistance, while reverse associations exist between *katG*315 and *inhA* or *ahpC* and between *kasA* and *ahpC*.[182]

The phenotype for INH tolerance in *M. tuberculosis* strains is described as being genetically susceptible, but remaining active after INH treatment. The *ini*A gene of *M. tuberculosis* is associated with this phenomenon. Its overexpression confers a cross-resistance to INH and ethambutol.[182]

In summary,there is high frequency of INH resistant mutations (10^{-6}). INH resistant strains most often show at least one mutation in the *katG* gene. This gene is located in a hypervariable region of the genome, containing repeated elements, and which as a result could contribute to the high frequency of mutations of this gene in resistant strains. There could be a boost in KatG mutations at position 315 (the most frequent) because the mutations in this location seem to decrease INH activation without stopping catalaseperoxidase activity, which is a virulence factor in strains. *M. tuberculosis* can also offset mutations in the *katG* gene by overexpressing the

ahpC gene.[182]

The description of *inhA*S94A mutations, which seem to alter the bond between the INH-NADH compound and InhA, corresponds to known interactions between NH and InhA. This suggests that *inhA* mutations play a role in resistance.[92] The role of the *KasA* gene in INH resistance remains undetermined. The outcomes of the mutations observed in the *ahpC* promoter or *ndh* gene suggest that these genes have very little or no effect on resistance.[182]

Isoniazid resistance is thus complex. The genes involved are either non essential, or extragenic mutations can compensate for their absence. Multiresistance development could be influenced by the interactions between these genes. The complexity of detecting isoniazid resistance was highlighted in 2003 by the description of *katG* mutations, associated with resistance and identified on INH susceptible strains. In addition, approximately 9 to 25% of resistant strains contain no mutations in targets known to confer resistance.[90,115,181,188,231] The decline in the effect of INH may be caused by the persistence of INH-tolerant bacilli.[253] This could explain the high rate of clinically-observed resistance.[182]

The recently marketed GenoType MTBDRplus test (Hain) is able to detect resistance to isoniazid by mutation in the *katG* and *inhA* genes. Mutations are identified by the amplification method and reverse hybridization on test strips. One probe covers the wild-type S315 region of *katG*, while two others (probes *katG* MUT T1 and MUT T2) are designed to assess the AGC-to-ACC (S315T) and the AGC-to-ACA (S315T) mutations. For the promoter region of the *inhA* gene, an *inhA* WT1 probe covers the wild-type region from -9 to -22 and a second *inhA* WT2 probe covers the positions from -1 to-12. Four mutations (-15C/T,-16A/G, -8T/C, and -8T/A) can be targeted with the *inhA* MUT1, MUT2, MUT3A, and MUT3B probes. The absence of hybridization with a wild-type probe indicates resistance. This test can identify mutations in a few hours. Mutations in *katG* resulted in a high level of isoniazid resistance, and mutations in *inhA* resulted in low levels of resistance. The Hillemann evaluation detected an association with *katG*315 or *InhA* mutations is 92% of INH resistant strains.[96]

Ethambutol resistance

Ethambutol (EMB) is very specific. It has been used since 1966 in combination with INH in treating tuberculosis. It is used as an accessory drug in short-course therapy, particularly when resistance to at least one TB drug reaches 4%.[192] EMB inhibits the formation of mycobacterial membrane.[159] Three homologues of arabinosyl transferases, EmbC, EmbA, and EmbB [233] have been proposed as the targets of EMB. These enzymes are involved in the synthesis of arabinogalactan, one of the basic components of a mycobacterial cell wall.

Resistance to EMB is frequent among MDR strains.[175] Phenotypic resistance is difficult to standardize in part due to the instability of EMB in both solid and liquid culture mediums. The overexpression of genes, leading to low intracellular rates of EMB, or mutation of the codon at position 306 of the *embB* gene can cause higher resistance.[233] Some reports have suggested that the *EmbB*306 mutation is not involved in EMB resistance but is instead associated with the development of other drug resistance including MDR-TB. A Russian study found mutations of *embB*306 in 48.3% of resistant strains, as well as in 31.2% of susceptible strains.[154] Recently, Hazbon tested 1,020 strains from different regions of the world and concluded that *emb*B306 mutations are associated with multi-resistance but do not directly cause resistance.[92] Shortly after wards, Plinke et al. reported that *emb*B306 mutations were only detected on EMB resistant strains, suggesting that they predict resistance.[185] Finally, Shen in Shanghai studied 10,659 strains and described *embB*306 mutations in EMB resistant and EMB susceptible strains.[207] Using site-directed mutagenesis and allelic exchange in *M. tuberculosis*, Safi[196] found that mutations leading to certain amino acid change are indeed causing EMB resistance while others have little effect on EMB resistance.

Other mutations were observed: D354A and N296Y at codon 330 (n=1)[205] and 360 (n=2) of *embB*, which produced a high level of resistance,[222] or at codons 328, 406 and 497,[272] and in *embA* and *embC* of some resistant strains.[222] However, there are substitutions of codon 981 in phenotypically susceptible strains.[222] Moreover, other mutations were observed in other genes such as *embR*, *Rv0340*, *rmlD*, and *rmlA2*, associated with resistance. Finally, for approximately 25% of low resistance strains (MIC <10 µg/ml), no mutation in the known resistance region was observed.[233] This low level resistance could result from an overexpression of EmB or mutations outside of the sequenced region (approximately 80% of the3297 bp gene).[182]

Multi-resistant strains have more *embB*306 mutants than non-MDR strains.[3,103,154,185] The *embB*306 locus appears then as a marker candidate for detecting MDR and XDR *M. tuberculosis* strains.[182]

A line probe assay is now proposed to detect ethambutol resistant strains.[97] The genotype MTBDR*sl* (Hain) test is based on amplification/reverse hybridization and provides detection of the *embB* mutations most commonly responsible for EMB resistance. Wild typeprobes have been designed that hybridize to the most important resistance region of the *embB* gene. When all wild type probes of a gene stain positive, there is no detectable mutation within the examined region. Hence, the strain tested is sensitive to EMB. When a mutation exists, the respective amplicon cannot bind to the corresponding wild type probe. The absence of a signal with at least one of the wild type probes indicates resistance to EMB. The mutation probes detect four 306 *embB* mutations. The banding pattern obtained with the *embB* probes

could allow a conclusion to be suggested about resistance to EMB.[182]

Streptomycin-resistance

Streptomycin (STR), an aminoglycoside antibiotic, was the first antibiotic used against tuberculosis in 1944 but its required parenteral administration,toxicity, and low effectiveness compared to other drugs has made it less prescribed as a first-line treatment. Its initial use in monotherapy led to the emergence of an STR-resistant *M. tuberculosis* strain (also associated with INHresistance).[182]

Aminoglycoside antibiotics act by preventing protein synthesis of bacterial ribosome. They inhibit the initiation of mRNA translation. The site of action is the small 30S ribosome subunit, a highly conserved part of the translation apparatus including S23 ribosomal protein, coded by the *rpsL* gene, and ARNr16S coded by the *rrs* gene. Changes in the amino acid sequence of *rpsL* alter RpsL and ARNr16S interaction and lead to STR resistance. S12 ribosomal protein interacts with the 530 loop of ARNr16S, and maintains its super-structure. Thus, the ARNr16S loops that interact with the S12 protein are a mutation selective site for conferring resistance to STR. In bacteria, the presence of several rRNA operons and one copy of the *rpsL* gene prevent the isolation of STR resistant mutants whose resistance is conferred by amutation on the *rrs* gene. *M. tuberculosis* only has one rRNA operon (rrn),which explains the isolation of STR resistant whose resistance is conferred by a mutation of the *rrs* gene[182].

A 50% [42% to 59%] of STR resistance strains have at least one mutation in the *rpsL* gene, which codes for the S12 ribosomal protein associated with a high level of resistance (MIC > 1000 μg/ml) [159,160].

Two *rpsL* mutations, Lys(AAG)→Arg(AGG), at codons 43 and 88, contribute to these high levels of resistance in most cases.[79,145] The *rpsL* mutants can host missense mutations at position 9 and 93 that have no obvious association to a role in STR resistance[221] since they are described in association with mutations that are already known at codons 43 and 88.

In certain strains of STR resistant *M. tuberculosis*, the *rpsL* gene is a wild type and resistance is attributed to mutations of the rrs gene coding for 16S ribosomalRNA: 8 to 21% of STR resistant strains have at least one mutation in the *rrs* gene associated with an intermediary resistance level (MIC 64 to 512 μg/ml) [205,219].

The first mutation described is A904G.[62] Resistances are associated with single mutations, mainly in one of two conserved regions of ARNr16S (around nucleotides 530 and 912), which form interactions with the S12 protein.[79,145,160] One of them, the G524C mutation, severely disrupts 524G and 507C, a Watson-Crick type action that is essential to ribosomal function.

The nucleotide mutation previously described at position 491 of the *rrs*gene, in two strains resistant to streptomycin,[221] is a polymorphism that is not related to resistance.

A 30% of STR resistant strains do not have a mutation in the *rpsL* or *rrs* genes. Resistance could be caused by membrane impermeability.[192]

Recently, it has been suggested that mutations in *gidB*, encoding a conserved 16S RNA specific 7-methylguanosine methyltransferase, or the presence of efflux pump inhibitors, appear to be involved in low-level STR resistance.[172,219]

2.2.5. Main lineages within *M. tuberculosis* species

Within the scope of this chapter is the description of the results of the molecular population approach that allowed the definition of genetically homogenous clusters of *M. tuberculosis* complex, which are now to be preferentially lined to some human hosts.[27.83] Table 4 provides the latest description of statistically, epidemiologically or phylogeographically relevant clonal complexes of *M. tuberculosis* complex based on spoligotyping signatures described in the SpolDBa database (a high resolution image can be downloaded at: http://www.biomedcentral.com/1471-2180/6/23/figure/F1?highres=y; from Brudey 2006).

Table 4. Comparision of spoligotype, Multi Locus Sequence Typing (MLST) and Large Sequence Polymorphism (LSP) nomenclature (From Palomino et al., 2007)

Spoligotyping-based (Filliol 2003)	MLST (Baker 2004)	LSP (Gagneux 2006)	Comment
East-African-Indian (EAI)	IV	Indo-Oceanic	Prevalent in Sout East Asia, East Africa and South India
Beijing	I	East-Asian	Prevalent in China, Japan, South East Asia, Russia
Central-Asian (CAS)	III	East-African-Indian	Prevalent in North India, Pakistan, Libya, Sudan
	II	Euro-American	Ubiquitous
X, Haarlem, LAM	NA	West African 1	Nigeria, Ghana
M. africanum	NA	West African 2	Senegal, Gambia
M. africanum			

Principal lineages of the *G*enetic group 1

The East African-Indian (EAI) lineage

This lineage was first described in Guinea-Bissau[105] and was shown to be fequent in South-East-Asia, India, and East Africa[118]. This group of strains is characterzied by a low number of IS6110 copies. A subgroup of these strains harboring a single copy of IS6110 was showed to be widespread in Malaysia, Tanzania, and Oman.[82] In combined datastes (i,e, pooled datasets charcterised by one ore more methods), this lineage demonstrated congruence between spoligotypes (absence of spacers 29-32, presence of spacer 33, absence of spacer 34), VNTR [extract tandem repeat A (ETR-A) allele ≥4], *katG-gyrA* grouping (Group 1), and later the presence of the TbD1 sequence.[211] More recently, the presence of an *oxyR* C37T transition was showed to be specific the lineage.[11] This lineage was showed to belong to cluster group 1 or Cluster I.[77] It harbors a specific region of difference, RD239 and was renamed as Indo-Oceanic in the work of Gagneux et al.[83] It is speculated that this lineage, which is endemic in South-East Asia, South-India, and East-Africa, may have orgirinated in Asia, where TB could have historically found favorable spreading conditions. The Manila family was first identified by Douglas in 1997, and was later thoroughly characterized by the same group.[61] This genotype was identified based on the prevalence of clustered strains isolated from Philippino immigrants in the United States (US) and was only later showed to be prevalent in the Philippines. The Manila family bears ST19 as prototypic spoligo-signature and is actually identical to EAI-2.[76] ST 89, which defines the Nonthaburi (Thailand) group of strains, is a derived clone.[161] In this family, specific variants have been also described for Vietnam (ST139 or EAI-4). Bangladesh (ST591, ST1898 or EAI-6 and 7) and Madagasca (ST109, EAI-6).

There is no precise idea about the prevalence of the EAI lineage in India and China, although it is evident that this genotype is more specifically linked with South-East Asia and South India than with Northern China.[177] This may be due to differences in civilization and agriculture histories between North and South China.[215] It is also difficult to analyze what links these clones may have with strains in the major genetic group 2, given the presence of the spacer 33 in this group of strains (a spacer that is absent in group 2 and 3). A striking discovery related to these strains was made recently when analyzing medieval human remains discovered in an English parish. TB was confirmed by amplifying multiple *M. tuberculosis* loci and EAI genotypes were apparently identified by spoligotyping.[230] Whether these spoligotyping results obtained on medieval remains are reliable or not should be confirmed independently; however, the posibility of the presence of EAI genotypes in 13th century England should not be excluded.[177]

The Beijing lineage

The Beijing genotyping belongs to the the principal genetic group 1 of Sreevatsan, and its specific spoligotype signature (absence of spacer 1-33, presence of spacer 34-43) was discovered in 1995.[248] However, a notorious outbreak due to a multidrug resistant clone of one of its offspring (New York W strain) had been characterized earlier, at the beginning of '90s.[22,184] The emergency of this family of related genotypes continues to pose a serious threat to TB control due to its high virulence and frequent association with multidrug resistance. It was hypothesized that this genotype emerged successfully in East Asia due to mass BCG vaccination during the 20th century.[1,248] However, Beijing should also be considered as a group of variant clones that evolved from a common precursor at undefined time, maybe during the Genghis Khan reign or before.[152]

These strains are characterised by the presence of an inverted IS6110 copy within the DR region, an IS6110 element at a particular insertion site (within the origin of replication) and one or two IS6110 copies in DNA region called NTF.[184,120] A characteristic Beijing lineage-defining SNP (G81A in Rv3815c) has been reported by Filliol *et al*. According to SNP analysis, the Beijing cluster was designated as SCG 2 or sSNP-II.[77] Other characteristic sSNPs of the Beijing lineage were described in putative DNA repaire genes.[186]

More recently, new phylogenetically informative specific LSP markers were found, such as RD105, which is present in all Beijing/W or RD142, RD150, and RD181. It allows a futher division of the Beijing lineage into four monophyletic subgroups.[242] The Beijing lineage was recently renamed as the East Asian Lineage by other authors.[83] Its most frequent VNTR signature is 42435.[118]

Recent evidence points to an early dispersal of the Beijing genotype in correlation to genetic haplotype diversity of the male Y chromosome (i.e. in correlation with human phylogeography). These results susgest that the spreading history of Beijing has a molecular evolutionary history that is much more intricate and more deeply rooted to human history than initially thought. Using the Beijing genotype as a model, and comparing its phylogeography to Y-chromosome-based phylogeography, Mokrousov et al. hypothesized that two events shaped the early history of this genotype: (1) its upper Paleolithic origin in the *Homo sapiens sapiens* K-M9 cluster in central Asia, and (2) a primary dispersal of the secondary Beijing NFT::IS6110 lineage by Proto-Sino-Tibetan farmers within East-Asian (human O-M214/M122 haplogroup).[152]

The Central-Asian (CAS) or Delhi lineage

The presence in India of a specific lineage of the *M. tuberculosis* complex was concomitantly and independently reported by two different groups using IS6110 RFLP and spoligotyping, respectively.[21,75] This lineage was also shown to be endemic in Sudan, other sub-Sahara countries and Pakistan.[27] Using IS6110 RFLP, the Delhi lineage shows a characteristic band pair in the high molecular weight region (12.1 and 10.1 kilobase pairs) and its specific spoligotype signature is formed in the absence of spacers 4-27 and 23-34. This spoligo-signature shows numerous variants and several subgroups such as CAS1-Kili (for Kilimanjaro) and CAS1-Dar (for Dar-es-Salaam), which have already been defined on the basis of new spoligotype-signatures that are specific for each new clonal complex.[70,143] Still, more results using other polymorphic markers should complement these data. VNTR signatures of *M. tuberculosis* complex clinical isolates from South-Asian immigrants in London and native patients in Rawalpindi, Pakistan, were identical (allelecombination 42235) and correlated with the CAS spoligotype.[85]

This genotype family could be the ancestor of the Beijing family since it clusters close to Beijing when analyzed by a combination of MIRU, spoligotyping and VNTR.[214] In India, its fequency varies from one region to another: it is more prevalent in the North than in the South, where the EAI family predominates.[227] An outbreak strains named CH was recently reported in Leicester, United Kingdom. It belongs to the CAS family and harbors a specific delection (Rv1519). In broth media, this strain was found to growmore slowly and to be less tolerant to acid and H_2O_2 than two laboratory reference strains, CDC1551 and H37Rv. Nevertheless, its ability to grow in human monocyte-derived macrophages was not impaired. This strain included more anti-inflammatory IL-10, more IL-6 gene transcription/section from monocyte-derived macrophages, and less protective IL-12p40 than CDC1551 and H37Rv strains. Thus, this strain seem to compensate the microbiological attennuation by skewing the innate response toward a phagocyte deactivation. These results suggest that the Rv1519 polymorphism confers an immune subverting *M. tuberculosis* phenotype that might contribute to the persistence and outbreak potential of this lineage.[162]

Lineages belonging to the Princilap Genetic group 2 and 3

The Haarlem family

The Haarlem family was described in the Netherland in 1999.[118] On IS6110 RFLP, these strains harbor a double band at 1.4 kb. Their spoligotype is characterized by the absence of the spacer 31, which is due to the presence of a second copy of IS6110 in the DR region.[89] Due to an asymmetric insertion within the DR locus, this second IS6110 copy hinders the detection

of spacer 31.[78,124] Three main spoligotype-signatures define the variants H1 to H3.[76] However, many Haarlem clonal complexes may harbor other Haarlem-based spoligotype-signatures that are, as yet, poorly characterized. Another characteristic of the Haarlem lineage is the frequent VNTR pattern 33233.[118] The Haarlem family is highly prevalent in Northern Europe. It is present in the Caribbean to a lesser extent and is also prevalent in Central Africa, where it is believed to have been introduced during the European colonization.[75] This family, which is highly diverse, merits futher studies to better understsand its evolutionary history. A SNP in the *mgt* gene of the *M. tuberculosis* Haarlem genotpe was discovered recently.[4] More SNPs are expected to be specific of the Haarlem lineage.[177]

The Latin American and Mediterranean (LAM) family

The LAM family was defined by the finding of linkage disequilibrium between the absence of spacers 21-24 in the spoligotyping and the presence of an ETR-A allele equal to 2.[215] However, this genotype family is more diverse and its study is more complicated than initially thought. Strains belonging to the LAM3/F11 family and the S/F28 family harbor identical spoligotypes of the shared type ST4, revealing the existence of genetic convergence between spoliotypes.[256] This phenomenon seems, however, to be rare and highly dependent on the structure of the observed spoligotype. The absence of spacer 21-24 may also have occurred more than once in tubercle bacilli evolution although no genetic evidence has suggested such a convergence event untill now. Many sub-motifs - LAM1 to LAM12 - have been suggested according to the latest international spoligotype database project SpolDB4.[27] However, the phylogenetic significance of the common absence of spacers 23-24 has not been demonstrated in this lineage. In this sense, some genotypes that showed strong geographical specificity (for example the LAM10-Cameroon or the LAM7-Turkey) were initially labeled as LAM, although there is no evidence of their phylogenetical relation to other LAM spoligo-signature.[169,276] Recently, a specific deletion designated as RD^{RIO} was showed to be linked to certain LAM spoligo-signatures present in Rio de Janeiro, Brazil (L Lazzarini, R Huard, JL Ho personal communication).[177]

The LAM clade is frequent in Mediterranean countries and its presence in Latin America is supposed to be linked to the Lusitanian-Hispanian colonization of the New World. Conversely, it may have been endemic in Africa and/or in South America, spreading to Europe later. At this stage, we must highlight that paleopathological and ancient DNA data support the existence of TB before the arrival of Spanish settler to Latin America in the 15^{th} century.[10]

The X family: the European IS6110 low banders

The X family of strains is defined by two concomitant features, a low number of IS6110 copies and the absence of spacer 18 in the spoligotyping.[204] This latter is indeed an important characteristic common to at least three spoligotype shared types: ST119, ST137, and ST92. Both characteristics are present in the CDC1551 strain, which are once suggested to be highly virulent. The X family was also the first group identified in Guadeloupe [216] and the French Polynesia.[237] Specific epidemic variants of this genotype family were described in South Africa.[223] The absence of spacer 18 bears phylogenetical significance because it is improbable that this spacer was delected more than once in this evolution of *M. tuberculosis*. This distribution of X family appears to be linked to Anglo-Saxon countries.[49] It is also highly prevalent in South Africa and to a lesser extent in the Caribbean. Currently, it is only poorly documented in India. The strong presence of this genotype family in Mexico could be explained by its close proximity to the USA.[177]

The T families and others

The ''ill-defined'' T group is characterized ''by default''. It includes strains that miss spacers 33-36 and can hardly be classified in other groups. This is a general characteristic of strains belonging to the principal genetic groups 2 and 3, together with the absence of an intact *pks* 15/1 gene.[135] The presence of intact polyketide synthase genes, active in the synthesis of the specific lipid complex of the *M. tuberculosis* complex is known to be linked to virulence.[47] Conversely, the 7 bp frameshift delection in *pks*15/1 may be considered as a phylogenetical marker specific for the modern *M. tuberculosis* strains[83] and may define the recently designatured Euro-American lineage. It is expected that the combination of spoligotype and improved MIRU signatures will be the best way to precisely define epidemiological clonal complexes.[226] Alternatively, RDs and/or SNPs may also improve the taxonomic definition of these clones.

Table 4 shows the nomenclature correspondence between the main spoligotyping-based *M. tuberculosis* complex lineage and those recently by MLST-SNPs[11] and LSP[83]. As shown, spoligotyping appears to be more discriminative than the other two typing systems since it is able to resolve clinical isolates within the branch of the modern strains that are not solved by LSP. Specific RDs are described for many individual spoligotype-signatures; however, no Table is yet available for LSP and/or SNP synthetic correspondence with spoligotype.

Even if there is consensus in the fact that the main branches of the genetic tree of the *M. tuberculosis* complex have now been found, many

uncertainties still remain with regard to the chronology of the evolution of the *M. tuberculosis* complex. For example, Gagneux et al. suggest that West African 2 diverged from an ancestral branch of *M. bovis*, whereas West African 1, characterized by a deletion of RD711, did not.[83]

Recent results[177] have shown that, in certain cases, it should be possible to reconstruct the past evolutionary history of some modern clones of the *M. tuberculosis* complex belonging to the principal groups 2 and 3. As an example, a striking identity was found recently between the MIRU typing results of the mains LAM7-Turkey clonal complex[276] and the Japanese group T3-OSA.7 The meaning of this identity is under investigation and there is no reason to believe that it is due to convergence. Similarly, an endemic clone found in Nunavik[166] was shown to be related to a clone found to be prevalent in central Europe (Poland and Germany).

Brudey et al.[27] data-mined an updated international spoligotype database of the *M. tuberculosis* complex, SpolDB4, both for improving classification of *M. tuberculosis* complex genomes, and for presenting a more reliable snapshot picture of the global and local population genetics of tubercle bacilli. Considering the known diversity of the origin of patients, SpolDB4 represents clinical isolates from a total of 141 countries.

However recent studies from different regions[15,238] have shown that several spoligopatterns could not be typed on the basis of the existing SpolBD4 database. This indicates the current absence of knowledge on the genetic diversity of *M. tuberculosis* strains from those regions. This therefore calls for more clinical epidemiological studies in different regions to clearly understand the genetic diversity of the TB epidemic on the continent.

In a more recent research, Brown et al[26] showed the strong associations between patient's country of origin and the spoligo family of the isolate: CAS and EAI families dominated in patients born on ISC (RR 2.4, 95% CI 2.02–2.74) as did Beijing and EAI families in patients born in South East Asia (RR 4.8, 95% CI 2.70–8.54). EAI families were seen in 80.4% of isolates from patients born in East Africa andhe ISC. The *M. africanum* family dominated in patientsborn in West Africa (RR 3.67, 95% CI 1.52–6.50). In contrast, LAM and Haarlem isolates were infrequently seen inpatients born on the ISC (4.5% and 5.4%) and SoutheastAsia (3.4% and 6.9%). T family isolates, one of the genetic groups determined by spoligotyping, were distributed evenly across all regions except Southeast Asia, where they were infrequently seen.

3. RESEARCH OBJECTIVES

Tuberculosis remains a major cause of morbidity and mortality in many countries and a significant public health problem worldwide. The emergence of drug resistant strains and particularly multidrug-resistant strains of *Mycobacterium tuberculosis*, has become a significant public health problem in a number of countries and an obstacle for an effective control of tuberculosis.

Vietnam is a high burden country for tuberculosis.[260] In Vietnam, almost 30,000 people die every year from TB (one death every 18 minutes). Vietnam, with its estimated 175,000 new cases per year (or 201 cases per 100,000 population) ranks 12th among the 22 countries[262] which added together account for 80% of the global TB burden, with a prevalence of smear-positive tuberculosis of 89 per 100,000 population (WHO, unpublished data, 2008). If TB is detected early and properly treated using a combination of medicines for 6 to 9 months, the patients quickly become non-infectious and are eventually cured. Multi-drug resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB), HIV-associated TB and weak health systems are major challenges in Vietnam. There are an estimated 7,000 new MDR-TB cases and 6,400 new TB/HIV cases every year. Both of these forms of complicated TB carry a high risk of early mortality.[262] The National Tuberculosis Control Programme is well established and has nationwide coverage.[98]

Although tuberculosis is still a public health problem in Vietnam, there is little information about the genetic characteristics of the isolates. A better knowledge of the molecular characteristics of *M.tuberculosis* strains will contribute to understand the transmission dynamics of the disease within the country.

The overall objective of this study isto determine the molecular epidemiology and the phenotypic and genetic characteristics of mycobacteria isolated in central Vietnam.

The specific objectives are:

- to determine the rate of drug-resistant and multidrug-resistant *M.tuberculosis* strains.
- to detect tuberculosis by using the PCR assay and determine the rate of *M.tuberculosis* strains which do not harbor insert sequence IS6110.
- to determine with molecular epidemiology methods the distribution of genotypes and create a phylogenetic tree of isolated *M. tuberculosis* strains.
- to describe the molecular characteristics of a representative multidrug-resistant *M. tuberculosis* strain isolated in centralVietnam.

4. MATERIALS AND METHODS

4.1. Study design

This was a cross-sectional study and was conducted from July 2009 to June 2011, at the Carlo Urbani Centre, Department of Microbiology, Hue College of Medicine and Pharmacy, in Central Vietnam.

4.2. Enrollment

Patients suspected or confirmed of having tuberculosisat the Hospital of Hue College of Medicine and Pharmacy, Hue Central Hospital and Danang Lung Hospital, in Central Vietnam were enrolled into the study.

All patients were from a single ethnicity (Vietnamese Kinh).

Any patient already receiving TB therapy for more than two weeks was excluded from the study.

Samples were collected as per routine care as deemed appropriate by the treating physician. All specimen types from suspected cases were included in the study.

Data on socio-economic and demographic features, TB history, HIV status and presenting clinical features were prospectively collected on a case report form.

Written informed consent was obtained from all participants and the study was approved by the ethics committee of Hue College of Medicine and Pharmacy.

4.3. Specimen preparation and examination

Ziehl-Neelsen staining

All specimens were processed for acid fast microscopy and reported according to guidelines of WHO.[258]

The smear is flooded entirely with concentrated fuchsin solution and heated using a spirit lamp from beneath, flaming must be stopped once fumes arise and allowed to cool in 5 minutes. The solution is then poured off and washed in gentle stream of running tap water. The smear is then covered with few drops of 3% acid-alcohol and allowed to act for a maximum of 3 minutes and then washed in tap water. The process of decolorisation will be

repeated until the smear is faintly pink or almost colorless. The smear is then washed in water and counterstained with methylene blue solution and allowed to act for 60 seconds. The slide is then washed in water and dried with blotting paper and observed under oil immersion objective.

A positive sputum sample typically contain pink colored, rod shaped bacteria that are slightly curved, sometimes branching, sometimes beaded in appearance, present singly or in small clumps against a blue background of pus cells and epithelial cells.

Specimen decontamination

All specimen, except for cerebral spinal fluid (CSF), were decontaminated. Specimens were digested and decontaminated of other bacteria by the standard N-acetyl-L-cysteine-sodium hydroxide method.[119] An equal volume of fresh 4% sodium hydroxide-N-acetyl-L-cysteine (NALC -NaOH) solution was added to each specimen in a sterile 50 ml conical screw-top centrifuge tube. The tube was tightly closed, inverted and on the vortex, then incubated at room temperature for exactly 15 min, followed by dilution up to 45 ml with phosphate buffer saline (PBS). After centrifugation at 3000 g for 15 min, the supernatant was decanted. Pellets were resuspended in a final volume of 2 ml and used for the inoculation of culture media and PCR assay.

For CSF samples, the sample tubes were centrifuged at 3000 g, 4^{o}C for 20 minutes (Eppendorf). After discarding the supernatant, 2 ml pellet was used for smear and PCR assay.

4.5. The Microscopic-Observation Drug-Susceptibility (MODS) assay

The MODS assay for the detection of tuberculosis and multidrug-resistant tuberculosis, directly from sputum, relies on three principles: first, that *Mycobacterium tuberculosis* grows faster in liquid medium than in solid medium; second, that characteristic cord formation can be visualized microscopically in liquid medium at an early stage; and third, that the incorporation of drugs permits rapid and direct drug-susceptibility testing concomitantly with the detection of bacterial growth.[155]

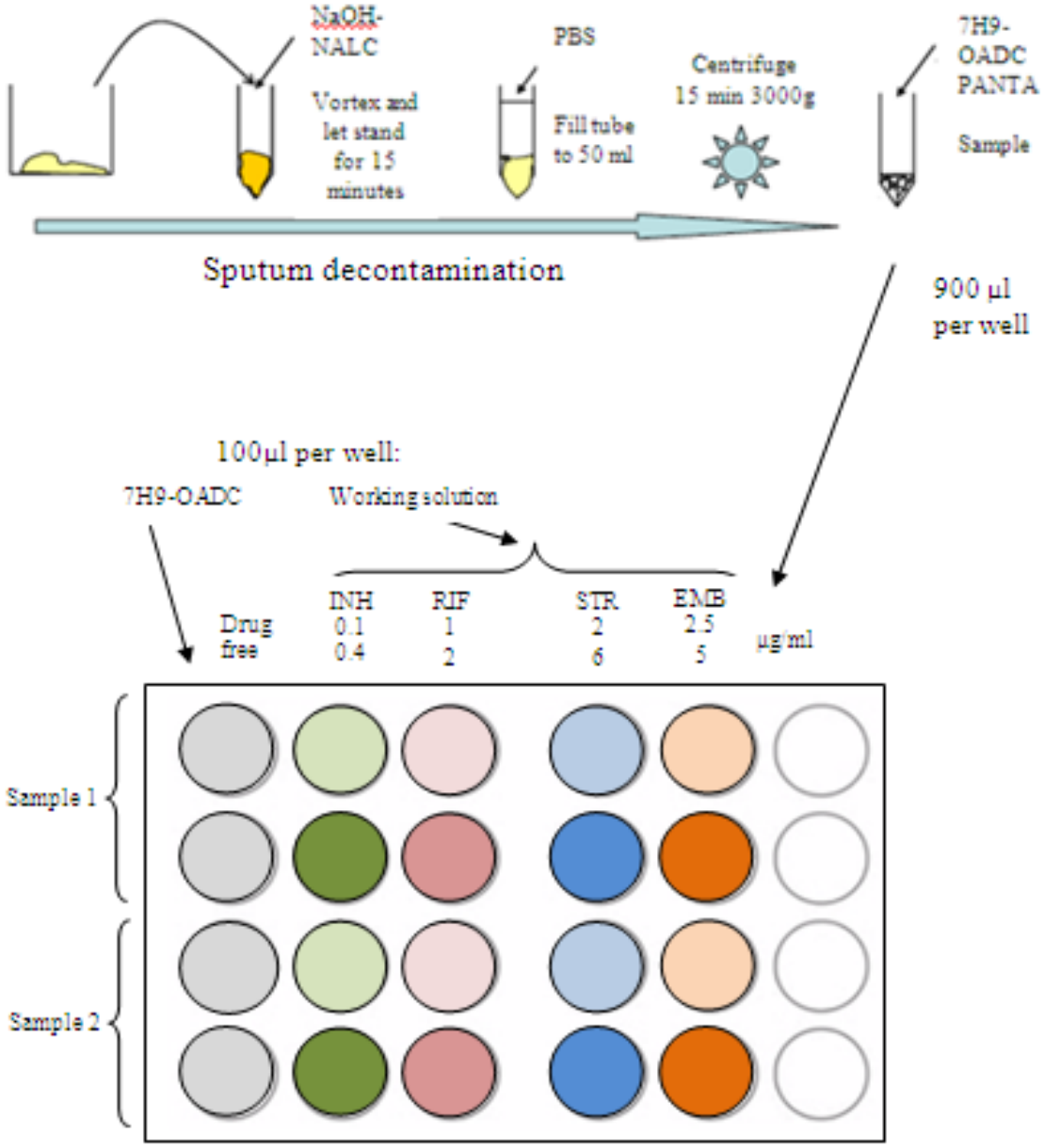


Figure 6: The MODS assay flowchart

The MODS assay was performed in 24-well tissue-culture plates (Corning) as described in previous protocols,[48,155] with some modifications. Antibiotic stock solutions were diluted from isoniazid (INH), rifampicin (RIF), streptomycin (STR) and ethambutol ((EMB) powder and added to liquid medium to give the following critical concentrations: INH, 0.1 and 0.4 µg/ml (MODS INH medium); RIF, 1 and 2 µg/ml (MODS RIF medium); STR, 2 and 6 µg/ml and EMB, 2.5 and 5 µg/ml. Specimens obtained from different subjects but processed on the same day were plated into different rows of the same plate. To minimize cross contamination and occupational

exposure, plates were permanently sealed inside plastic zip lock bags and were subsequently examined within the bag, the plates were incubated at 37°C. On each processing time, H37Rv strain was used as a drug-sensitive postive control. Wells containing decontaminated sample, 7H9-OADC, and PANTA, with or without drug, were examined daily (weekdays only) from day 5 to 15, on alternate days from day 16 to 25, and twice weekly from day 26 to 40 under an inverted light microscope at 40x magnification.

Positive cultures were identified by cord formation, characteristic of *M. tuberculosis* growth, in liquid medium in drug-free control wells, as described previously.[156]

Drug-containing wells were recorded as positive (indicating drug resistance) if corded growth was visible and negative (indicating susceptibility) if no corded growth was visible.

Nontuberculous mycobacteria could be recognized by their lack of cording or, for *M. chelonae* (which is the only nontuberculous mycobacteria that does form cords), by rapid overgrowth by day 5.[155]

The samples corded growth of which was visible by day 5 or earlier or the samples which were smear positive but no corded growth was visible, were retested by the 16S rRNA gene realtime PCR specific for *M.tuberculosis* complex.

4.6. DNA extraction

Extraction of DNA from clinical samples

DNA was extracted from the samples by DNeasy Blood & Tissue kit (Qiagen). Before performing DNA extraction, 500 µl of decontaminated samples were incubate at 95^{o}C for 20 minutes to kill bacteria. Samples are first lysed using proteinase K, incubate at 56°C overnight. Add 200 µl of buffer AL add 200 µl of ethanol (96%). Transfer this mixture into the DNeasy Mini spin column placed in a 2 ml collection tube. During centrifugation, DNA is selectively bound to the DNeasy membrane as contaminants pass through. Remaining contaminants and enzyme inhibitors are removed in two efficient wash steps with buffer AW1 and AW2. Purified DNA is then eluted in 100 µl buffer AE .

DNA extraction from isolated mycobacterial strains by using Cetyltrimethyl Ammonium Bromide (CTAB)

24 hour before harvesting the cells for genomic DNA preparation, add glycerine to a late-log-phase culture to a final concentration of 1% using a 10% (wt/vol) glycine stock, incubate at 37^{o}C.

Transfer 10 ml of culture to a 15 ml conical tube and spin in a centrifuge at 2,000 x g for 20 min. Centrifuge bacteria andre-suspended in 50 µl of GTE buffer, add 50 µl of 10mg/ml lysozyme solution, incubate at 37^{o}C

overnight, then add 100 µl of 10% SDS and 50 µl of 10 mg/ml proteinase K, incubate at 55^{o}C for 20-40 min to process the resistant cell wall. After incubation, add 200 µl of 5M NaCl and 160 µl of CTAB, this step is conducted at 65°C for 10 min, to precipitate proteins and macromolecules. Subsequently, DNA is purified by extraction with an equal volume of chloroform-isoamyl alcohol (24:1). This step was repeated twice. After the last step of extraction is completed, DNA is precipitated with isopropanol and mix gently by inversion until the DNA has precipitated out of solution, incubate at room temperature for 5 min. After centrifugation, DNA is washed from the pellet with 70% ethanol, air-dried and eluted in 50 µl of water and store at 4^{o}C to allow pellet to dissolve overnight.[93]

4.7. Polymerase chain reaction (PCR) assay

In this study, insertion sequence IS6110 based nested PCR (IS6110 PCR) assay and 16S rRNAgene TaqMan based realtime PCR (16S rDNA PCR) assay were combined for detecting the absence of IS6110 sequence in *M. tuberculosis* strain.

The performance of 16S rDNA PCR was assessed in 480clinical specimens including sputum, bronchial lavage, pleural fluid, cerebral spinal fluid (CSF), and others (gastric fluid , joint fluid, blood, abscess). All samples which were positive with the 16S rDNA PCR assay were retested in IS6110 PCR assay to detect the deletion of the insert sequence IS6110.

The 16S rRNA gene TaqMan based realtime PCR (16S rDNA PCR) assay

The amplified target is part of the 16S rRNA gene which is common to all the mycobacteria. The discrimination between the members of the *M. tuberculosis* complex and the other mycobacteria requires an additional step that involves DNA hybridization.[117,234]

LightPower iVAMTB rPCR Kit (from VietA Company, Vietnam) is a qualitative realtime PCR assay which identify the members of the *M. tuberculosis* complex; the target used in this procedures is the 16S rRNA gene in association with the insertion sequence IS6110 to increase the sensitivity of the assay.

For the target 16S rRNA gene, a pair of unlabeled *Mycobacteria* specific PCR primers, the product of which is 95 bp in length, and a *M. tuberculosis* specific TaqMan probe with a FAM dye label on the 5' end, and non-fluorescent quencher dye (TAMRA) on the 3' end are designed.

PCR amplifications were carried out in 25µl reaction mixtures by adding 5µl of template DNA to 20µl of a premade amplification master mixture. Target DNAs were amplified in a thermal cycler (Stratagene Mx 3000, USA) as follows: initial denaturation at95°C for 5 minute, this was followed by 40 cycles, each cycle consisting of 15 seconds at 95°C, 1 minute at 50°C and 20 seconds at 72°C and then 10 minutes at 72°C.

The IS6110 based two-stepnested PCR (IS6110 PCR) assay

The IS6110 nested PCR assay was performed with an in-house kit, following the protocol of tuberculosis laboratory of Section of Experimental and Clinical Microbiology, Department of Biomedical Science, Sassari University.

Two sets of primers IS1, IS2 and IS3, IS4 (as described in the appendix) were used to amplify 300 bp fragment of insertion element IS6110 of *M. tuberculosis* complex. The extracted DNA was amplified by two step PCR assay. The amplification was carried out in a thermocycler (Veriti™ 96-Well Thermal Cycler, Applied Biosystems).

- In the first step, a DNA segment was amplified by external primer (IS1, IS2). The first amplification carried out in 30µl final volume, in which:

PCR Master Mix (2X)	12.5 µl
IS1 primer (10 µM)	1.0 µl
IS2 primer (10 µM)	1.0 µl
MQ water	5.5 µl
DNA template	10.0 µl

Target DNAs were amplified in a thermal cycler (Stratagene Mx 3000, USA) as follows: after initial denaturation at 94°C for 5 minute, 36 amplification cycles were performed within thermocycler. Each cycle consisting of denaturation at 94°C for 50 seconds, annealing of primer at 58°C for 50 seconds and primer extension at 72°C for 50 seconds; then and a final extension at 72°C for 10 minutes. After complete amplification, the amplified product was used as DNA template for second amplification of 300 bp segments.

- In the second step, the first PCR product was amplified by internal primer (IS3, IS4). The second amplification carried out in 25µl final volume, in which:

PCR Master Mix (2X)	12.5 µl
IS3 primer (10 µM)	1.0 µl
IS4 primer (10 µM)	1.0 µl
MQ water	5.5 µl
The first PCR product	5.0 µl

The second amplification was carried out as follows: at94°C for 5 minute, 36 amplification cycles of at 94°C for 50 seconds, at 60°C for 50 seconds and primer extension at 72°C for 50 seconds; and then at 72°C for 10 minutes.

The second PCR products were analyzed by gel electrophores is in 2% agarose prepared in TBE buffer containing 0.5µg/ml of ethidium bromide (Sigma). The gel was examined in a gel documentation system (Bio-Rad) for a 300 bp using standard molecular marker (100 bp DNA Ladder). Presence of a band equivalent to 300 bp was taken a positive result when negative control gave no reaction. All the false negative and doubtful results were retested.

Throughout PCR processing the three room procedure and other recommended stringent precautions were followed and the results were evaluated in the light of the performance of appropriate positive and negative controls, to avoid cross-contamination and false positive reactions.

4.8. Spoligotyping (spacer oligonucleotide typing)

This is a PCR-based method, which depends on the amplification of a highly polymorphic Direct Repeat (DR) locus in *M. tuberculosis* genome. The DR region in *M. bovis* BCG contains direct repeat sequences of 36 bp, which is interspersed by the non-repetitive DNA spacers of 35-41 bp in length. In other *M. tuberculosis* complex strains, the number of DR elements vary significantly and majority of the *M. tuberculosis* strains contain one or more IS6110 elements in DR region.

The performance of spoligotyping was assessed in 122 DNA samples. *M. tuberculosis* strain H37Rv and *M.bovis* BCG P3 were used as positive controls and water was used as a negative control.

M. tuberculosis strains were genotyped by spoligotyping using the standardized method as follows:

Amplification of spacer DNA by PCR

Prepare the reaction mixture:

2 µl template DNA

3 µl primer DRa (0.2 µmol/µl)

3 µl primer DRb (0.2 µmol/µl)

20 µl 2×TaqPCR MasterMix

12 µl MQ water (to a final volume of 40 µl)

Perform the amplification in a thermocycler by the following temperature cycling: at 94°C for 3 minutes, then 25 cycles, each cycle consisting of at 94°C for 1 minute, at 55°C for 1minute and at 72°C for 30 seconds; and then a final extension at 72°C for 1 minutes.

Hybridization with PCR product and detection

- Add 25 µl of the PCR products to 150 µl 2×SSPE/0.1 % SDS.Heat-denature the diluted PCR product for 10 min at 99°C and cool on ice immediately.
- Wash the membrane for 5 min at 60 °C in 250 ml 2×SSPE/0.1 % SDS.
- Place the membrane and a support cushion into the miniblotter, in such a way that the slots are perpendicular to the line pattern of the applied oligonucleotides.
- Remove residual fluid from the slots of the miniblotter by aspiration.
- Fill the slots with the diluted PCR product (avoid air bubbles) and

hybridize for 60 min at 60 °C on a horizontal surface (no shaking). Avoid contamination of neighbouring slots.

- Remove the samples from the miniblotter by aspiration and take the membrane from the miniblotter using forceps.
- Wash the membrane twice in 250 ml 2×SSPE/0.5 % SDS for 10 min at 60 °C.
- Place the membrane in a rolling bottle and allow it to cool down to prevent inactivation of the peroxidase in the next step.
- Add 2.5 µl streptavidin-peroxidase conjugate (500 U/ml) to 10 ml of 2×SSPE/0.5 % SDS, and incubate the membrane in this solution for 60 min at 42°C in the rolling bottle.
- Wash the membrane twice in 250 ml of 2×SSPE/0.5 % SDS for 10 min at 42 °C.
- Rinse the membrane twice with 250 ml of 2×SSPE for 5 min at room temperature.
- For chemiluminiscent detection of hybridizing DNA, incubate the membrane for 1 min in 20 ml ECL detection liquid.
- Cover the membrane with a transparent plastic sheet and expose a light sensitive film to the membrane for 3hours.
- Develop the film using Fuji Hunt Chemistry (Automatic Developer Replenisher).

Result analysis

Genotypes were defined as spoligotyping signatures described in the SpolDB4 (a high resolution image can be downloaded at: http://www.biomedcentral.com/1471-2180/6/23/figure/F1?highres=y; from Brudey 2006).

Spoligotypes were assigned to families and subfamilies by using the Spoligodb Lookup Tools, which was established by Nguyen Hoang Bach, at Carlo Urbani Center, Hue College of Medicine and Pharmacy (http://khdn-yhue.edu.vn/modules.php?name=Spoligodb).

4.9. Whole genome sequencing

- One strain of *M. tuberculosis* (MTB_HUE_20 strain) was choosen to re-sequencing whole genome based on paired-end sequencing on the Illumina GAIIx platform.
- This strain had the following characteristics:
  - was isolated from a smear-positive sputum specimen of a 47-year-old male patient with typical clinical features of tuberculosis. This patient didnot have previous tuberculosis disease.
  - resistance to rifampicin, isoniazid (multidrug resistance)
  - positive with IS6110 PCR
  - its spoligotyping pattern does not match with any described genotye in the SpolDB4 database (unknown genotype)

- Thirty microgram of the genomic DNA was purified by CTAB method and sent to BaseClear Co. DNA Sequencing Service in the Netherlands to perform the sequencing.
- The type of sequence run is paired-end with 75 bp read length.
- We received about 300 MB of DNA sequencing data in paired-end reads from the Illumina platform. That is the raw sequence data in FASTQ format.
- Analysing: De Novo genomic assembly was performed with Velvet program.[271] Gene prediction and translate predicted genes into proteins with GeneMarkS.[19] Annotate all ORFs obtained with Blast2Go program [blast2go]. Create GFF file to annotate circular genome MTB_HUE_20 strain with Geneious software.[63]

4.10. Data processing

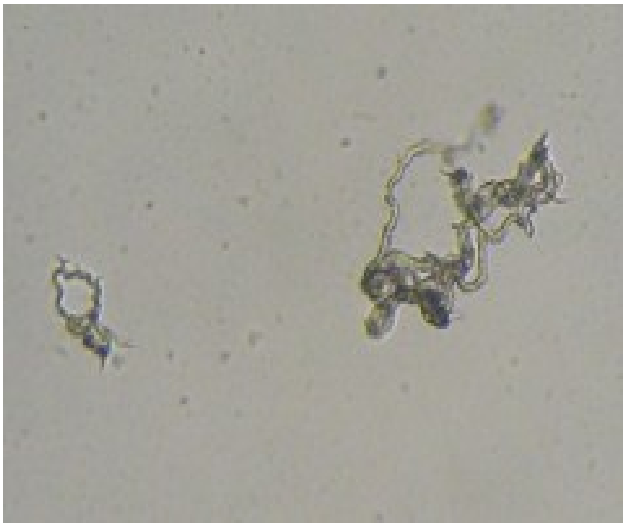
- Statistical analysis was performed using Medcalc software.
- Comparison of proportions between two groups of patient was done with Fisher's exact test.
- Odds ratio was used to predict the ratio of the odds of drug resistance occurring in spoligotype groups.
- All reported confidence interval are two-sided 95% confidence intervals and p-values <0.05 were regarded as statistically significant.

5. RESULTS

5.1. Proportion of culture positivity and drug susceptibility of *M. tuberculosis* isolates from the MODS assay

The MODS assay was performed for 305 sputum samples of patients with suspected or confirmed tuberculosis. A total of 53 (17.4%) samples, in which contamination in the cultured sample was observed, were excluded from the study. Data of 252 remaining patients were included in the analysis. In brief, the male: female ratio was 1: 2.5, the median age was 47.5 ± 19.3 (min 18, max 93). In 224 (98.9 %) patients new tuberculosis was suspected and 28 (11.1%) were previous tuberculosis patients. Ziehl-Neelsen AFB staining was positive in 155 (61.5%) of samples.

Among 252 MODS cultured samples 153 (60.7%) were positive for *M. tuberculosis* which was identified by the cord formation (Figure 7). The proportion of culture positive samples in the group of patients suspected of new tuberculosis and in the group of previous tuberculosis patients were 57.9% and 85.7%, respectively. The prevalences of smear - positive and MODS culture - positive samples is shown in Table 5 and Figure 8.



Fifure 7. Corded growth of *M. tuberculosis* in MODS culture observed at 400 x magnification

Table 5. The proportion of smear - positive and MODS culture – positive samples

Positive samples	Patients (N=252) No. of samples	%	Previous TB patients(N =28) No. of samples	%	New TB suspected Patients(N =224) No. of samples	%
AFB smear P>0.05	155	61.5	21	75.0	134	59.8
MODS culture P<0.01	153	60.7	24	85.7	129	57.9
We used Fisher' exact test for comparison of proportions.						

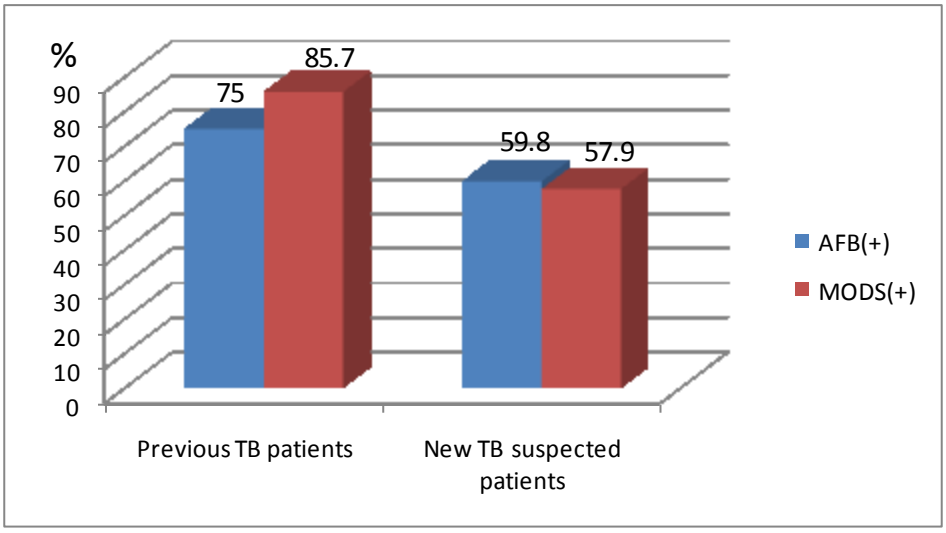


Figure 8. The proportion of smear - positive and MODS culture - positive samples according to the groups of patient

In our study, 32.0% (31/97) of samples were culture positive but negative for AFB and 21.3% (33/155) of samples were AFB positive, but negative for culture (Table 6 and Figure 9).

The mean time for culture positivity and drug susceptibility testing using MODS assay were 9 days (range 5 to 17 days for culture and range 6 to 14 days for drug susceptibility testing).

Table 6. Proportion of positive samples with MODS culture according to AFB smear

MODS culture	AFB	
	Positive	Negative
	No. of samples	No. of samples	No. of samples
Positive	153 (60.7%)	122 (78.7%)	31 (32%)
Negative	99 (39.3%)	33 (21.3%)	66(68%)
Total	252 (100%)	155 (100%)	97 (100%)

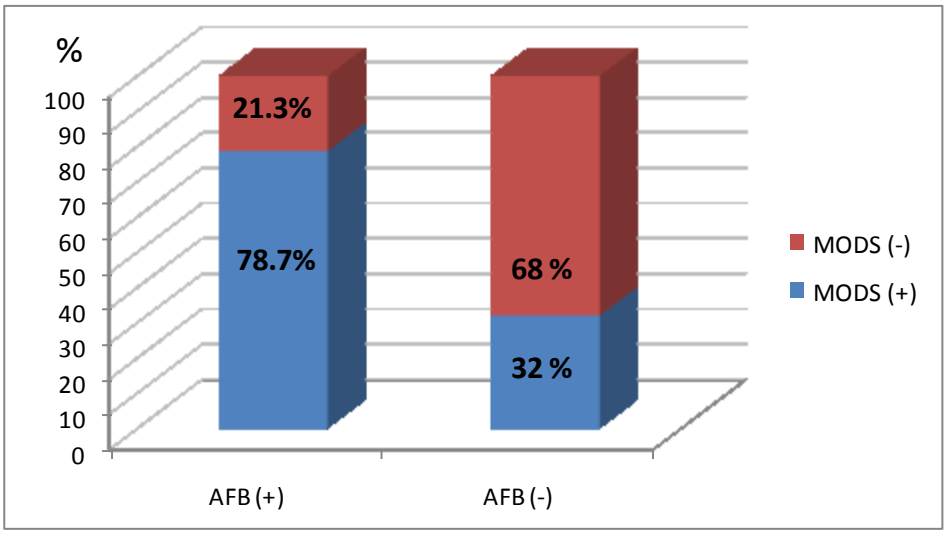


Figure 9. Proportion of positive samples with MODS culture according to AFB smear

Drug susceptibility

Among 153 culture-positive samples, the proportion of *M. tuberculosis* strains demonstrating sensitivity to all antibiotics (INH, RIF, STR and EMB) was 107 (69.9 %) and any drug resistance was 46 (30.1%). One drug resistance was observed in 30 strains (19.6%): 3.9% for INH, 11.8% for RIF, 2.0% for STR and EBM. Multidrug resistant *M. tuberculosis* as defined by WHO (resistant to RIF and INH) was observed in 8.5%. There were 9.2% of strains showing resistance to two or more drugs. The results of drug susceptibility of *M. tuberculosis* strains is shown in Table 7 and Figure 10.

Table 7. Drug susceptibility of *M. tuberculosis* strains from MODS sssay

Drug susceptibility			*M.tuberculosis* strains (N=153)		Previous TB patients (N =25)		New TB suspected patients (N =128)		P 95% CI
			No. of sample	%	No. of sample	%	No. of sample	%	
Any drug resistance			46	30.1	14	56.0	32	25.0	<0.01
Resistance to	INH	0.1µg/µl	6	3.9	4	16.0	2	1.6	<0.01
	RIF	1.0 µg/µl	18	11.8	2	8.0	16	12.5	>0.05
	EMB	2.5µg/µl	3	2.0	0	-	3	2.3	-
	STR	2.0 µg/µl	3	2.0	0	-	3	2.3	-
		MDR*	13	8.5	8	32.0	5	3.9	<0.01
		≥ 2 drugs	14	9.2	5	20.0	9	7.0	<0.01

*MDR -/+ EMB and -/+ STR

Two concentrations were examined for each drug, the second concentration, with no useful information gained, were not shown

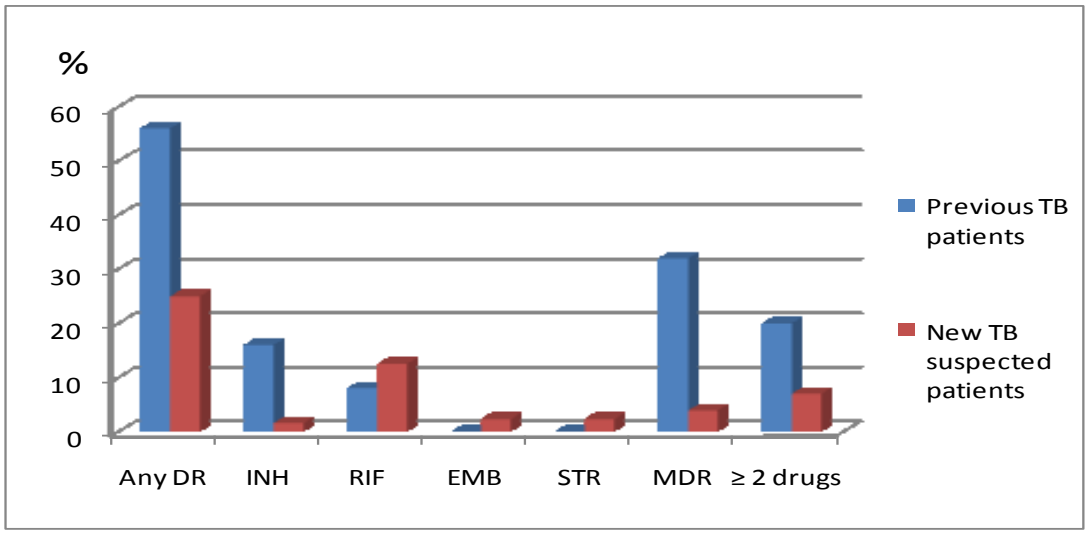


Figure 10. Proportion of drug resistant *M. tuberculosis* according to patient group (DR: drug resistant)

There was a statistically significant difference of drug resistance between the two groups of patients: the proportion of the strains which were resistant to any drug, INH, multidrug resistance and ≥ 2 drugs in the group of previous TB patients was higher than in the group of new tuberculosis suspected patients (p<0.01): 56% versus 25%, 16% versus 1.6%, 2% versus 3.9% and 20% versus 7%, respectively.

There was no significant difference of the drug resistance to RIF alone between the two groups of patients. Resistance to EMB and STR was not determined in the group of previous TB patients.

5.2. Results of PCR assay

A total of 480 clinical samples of patients with suspected tuberculosis including sputum, bronchial lavage, pleural fluid, cerebral spinal fluid (CSF), and others (gastric fluid, joint fluid, blood, abscess) were assessed. The sputum specimens were the most common type of specimen (76 %). Positivity with AFB staining was in 192 samples (40% of all types). No CSF specimen was positive with AFB. The AFB positivivity was detected in approximately 50% of the sputum specimens (186/365) and in lower percentage in bronchial lavage (1/30 - 3.3%), pleural fluid (2/50 - 4%) and other specimens (3/25 - 12%). The proportion of AFB positive samples is shown in Table 8.

Table 8. Proportion of AFB positive samples

Specimens			AFB positive samples	
	no.	%	no.	%
Sputum	365	76.0	186	50.9
Bronchial lavage	30	6.3	1	3.2
Pleural fluid	50	10.4	2	4.0
CSF	10	2.1	0	-
Others	25	5.2	3	12.0
Total	480	100	192	40.0

The 16S rDNA PCR assay for 480 clinical samples detected 258 positive cases (53.8%). A 3.6 % (7/192) of AFB-positive samples were negative. Furthermore the 16S rDNA PCR detected 73 cases (25.3%) of tuberculosis in AFB-negative samples, of which 54 (30.2%) were from sputum and 19 (17.4%) from the other type of specimens (Table 9 and Figure 11).

Table 9. Results of the 16S rDNA PCR according to AFB smear

16S rDNA		AFB smear	
		Positive	Negative
	no. of sample	no. of sample	no. of sample
Sputum	365 (100%)	186 (100%)	179 (100%)
Positive	233 (63.8%)	179 (96.2%)	54 (30.2%)
Negative	132 (36.2%)	7(3.8%)	125 (69.8%)
Fluid and others	115 (100%)	6 (100%)	109 (100%)
Positive	25 (21.3%)	6(100%)	19 (17.4%)
Negative	90 (78.7%)	0 -	90 (82.6%)
Total	480 (100%)	192 (100%)	288 (100%)
Positive	258 (53.8%)	185 (96.4%)	73 (25.3%)
Negative	222 (46.2%)	7 (3.6%)	215 (74.7%)

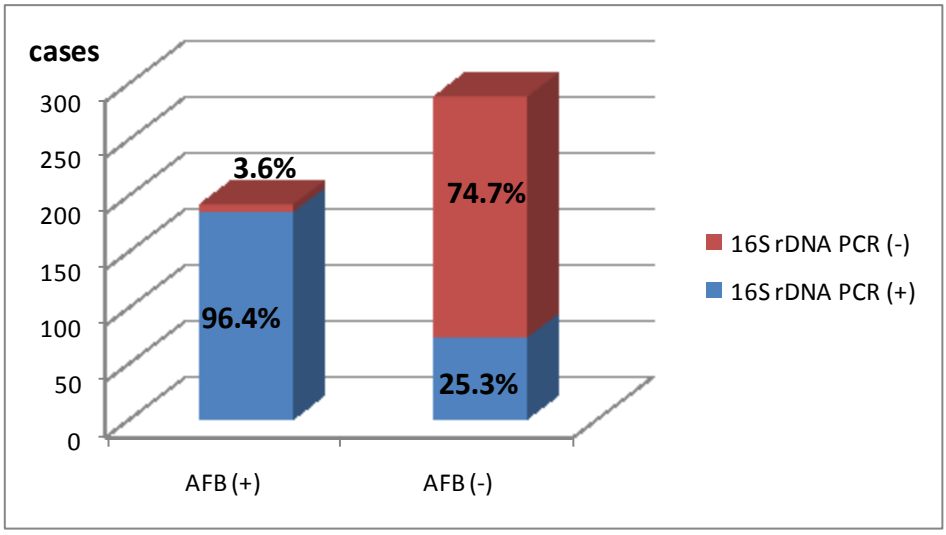


Figure 11. Proportion of positive samples with the 16S rDNA PCR according to AFB smear

The performance of IS6110 PCR assay was assessed in a total of 258 samples positive with 16S rDNA PCR. The result indicated that most of the processed samples (255, 98.8%) was positive with the IS6110 PCR product of 300 bp in lengh (Figure 12), and only 3 samples (1.2 %) were negative with IS6110 PCR.

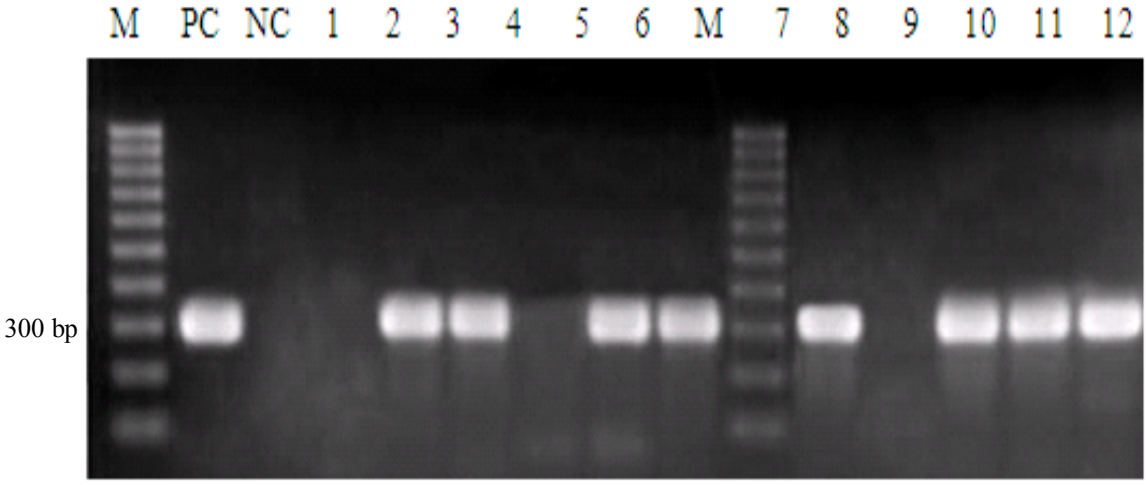


Figure 12. IS6110 PCR products of electrophoresed on 2% agarose
M:DNA ruler of 100 bp, PC: positive control (H37Rv), NC: negative control, 1-12: patient samples

5.3. The distribution of genotypes of isolated *M. tuberculosis* strains and the association between the genotypes and drug susceptibility

The performance of spoligotyping assay for 122 samples which were identified as *M.tuberculosis* strains showed that EAI families dominated in our study. EAI genotypes were seen in 65.6% of isolates, of which 15.6% were of the EAI4-VNM genotype, and 46.7 % were of EAI5. Beijing genotype was observed in 12.3 % of isolates. There were 4 isolates (3.3%), of which spoligotype patterns did not match with any of the updated international spoligotype database of the *M.tuberculosis* complex - SpolDB4, and were called Unknown (Unk) from 1 to 4. The remaining 22% were of other genotypes (Table 10 and Figure 13). Spoligotyping produced a total of 36 different patterns for the 122 strains studied (Figure 14).

Table 10. Distribution of spoligotype families and subfamilies of *M.tuberculosis* isolates

Spoligotype families and subfamilies	No. of isolates N = 122	
	n	%
EAI	80	65.6
EAI4-VNM	19	15.6
EAI5	57	46.7
EAI1-SOM	3	2.5
EAI2-NTB	1	0.8
Beijing	15	12.3
U	10	8.2
T	6	4.9
MANU	3	2.4
H	3	2.4
CAS	1	0.8
Unknown	4	3.3

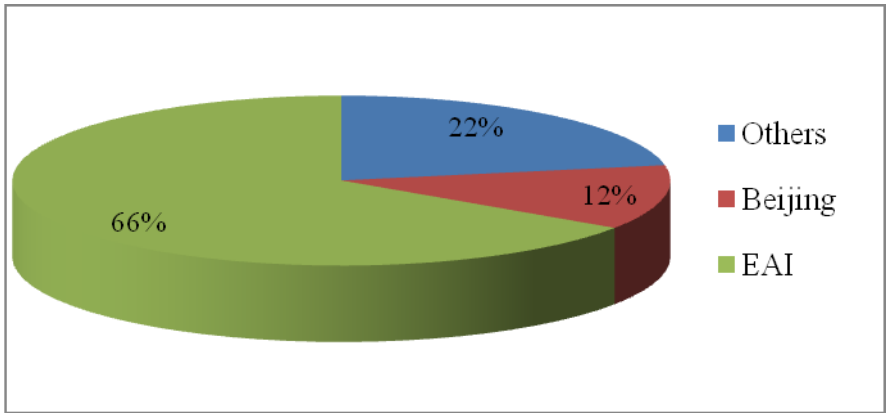


Figure 13.. The distribution of spoligotype families and subfamilies of *M.tuberculosis* isolates

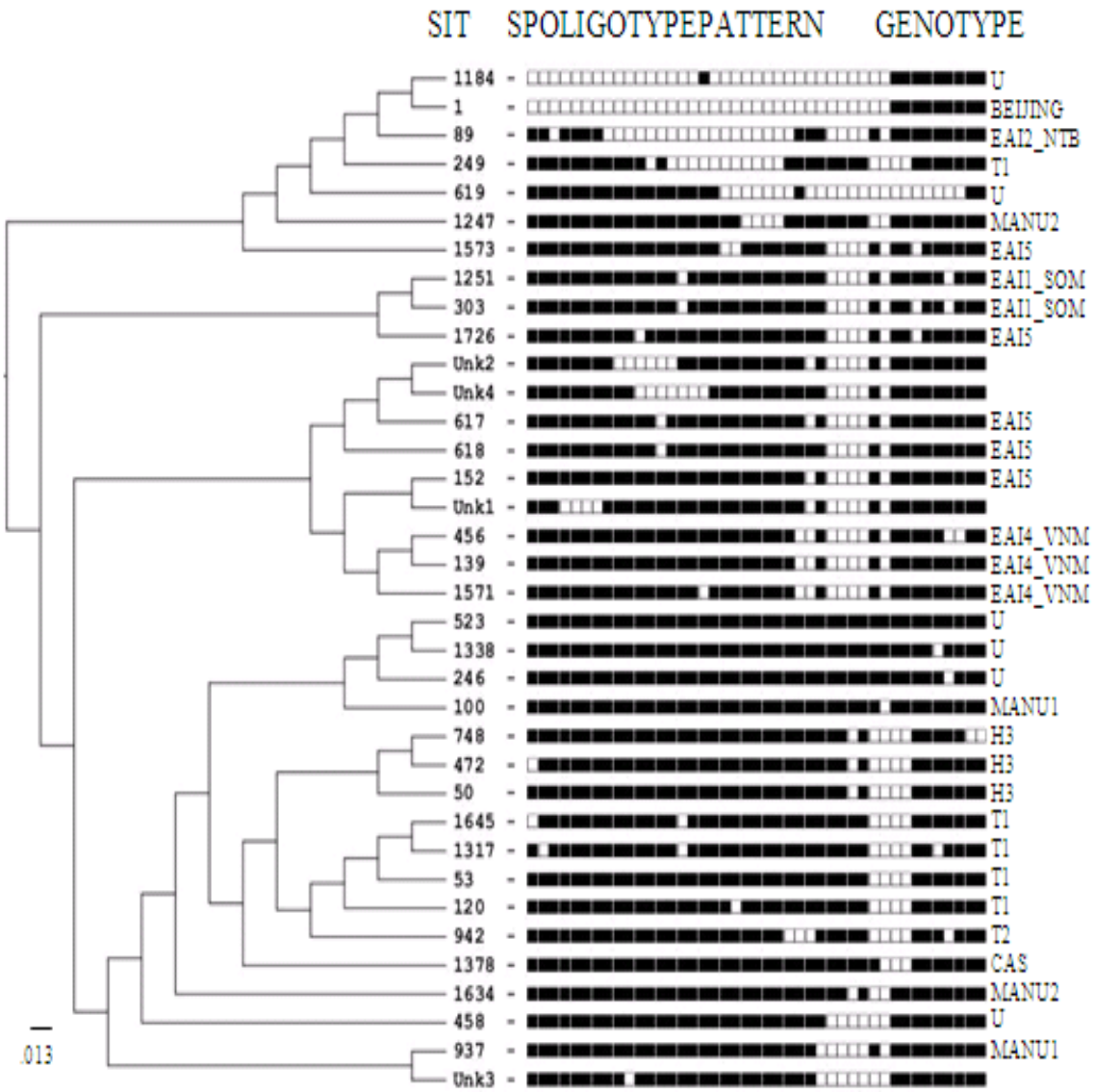


Figure 14. Spoligotype patterns of *M. tuberculosis* isolates. Spacers 1 to 43 are represented from left to right. A dendrogram created with Bionumerics software (neighbor joining; Euclidean distance) is presented on the left. Genotypes are identified on the right.

In the present study, there was no significant association of the Beijing genotype with resistance to drug since 20 % of Beijing isolates and 28.9% of non Beijing isolates were resistant to any first-linedrug (OR = 0.6 [95% confidence interval {CI}, 0.2 to 2.3]; P=0.47); 6.7% of Beijing isolates and 10.3% of non Beijing isolates could be defined as multidrug resistance (OR = 0.6 [95% confidence interval {CI}, 0.1 to 5.2]; P=0.66).

Similarly, there was no significant association of the EAI genotype with resistance to drug: 28.8 % of EAI isolates and 26.2% of non EAI isolates being resistant to any first-linedrug (OR = 1.1 [95% confidence interval {CI}, 0.5 to 2.6]; P=0.76); 8.6% of EAI isolates and 11.9% of non EAI isolates being multidrug resistance (OR = 0.7 [95% confidence interval {CI}, 0.2 to 2.4]; P=0.58).

Table 11. Proportion of drug resistance of Beijing genotype and EAI genotype of *Mycobacterium tuberculosis*

Resistance	Anydrug resistance (1)		MDR (2)		Total	OR (1) 95% CI	OR (2) 95% CI
Genotype	n	%	n	%			
Beijing	3	20.0	1	6.7	15	0.6 0.2- 2.3	0.6 0.1 - 5.2
Non Beijing	31	28.9	11	10.3	107	1	1
EAI	23	28.8	7	8.6	80	1.1 0.5- 2.6	0.7 0.2 - 2.4
Non EAI	11	26.2	5	11.9	42	1	1

5.4. Molecular characteristics of the MTB_HUE_20 strain

De Novo and re-sequencing of *M. tuberculosis* genome (MTB_HUE_20 strain) have been performed. We selected the Illumina GAIIx platform. Using 20 µg of purified genomic DNA of *M. tuberculosis* HUE_20 strain, we have obtained about ~300Mb of high quality sequencing data in paired-end reads with 75bp reads length.

For De Novo assembly, Velvet currently takes in short read sequences, removes errors then produces high quality unique contigs. It then uses paired-end read to retrieve the repeated areas between contigs. With kmer=31, we obtained 1074 nodes with range length 94,000-100bp.

We performed gene prediction and annotation on the obtained contigs, gene translation to protein and prediction of gene function with GenMarkS server, blast all open reading frames (ORFs) with NBCI server via Blast2Go Software. With a GFF file including the annotation, we built a full sequence with annotation on all loci.

The full length of our strain is 4,397,928 bp with a high GC content of 64.8% (Table 12, Figure 15).

Table 12. Statistics of complete genome *M. tuberculosis* MTB_HUE_20 strain

Base	Frequency	Percentage %
A	776,371	17.7%
C	1,434,609	32.6%
G	1,416,809	32.2%
T	770,139	17.5%
GC	2,851,418	64.8%
Length	4,397,928	

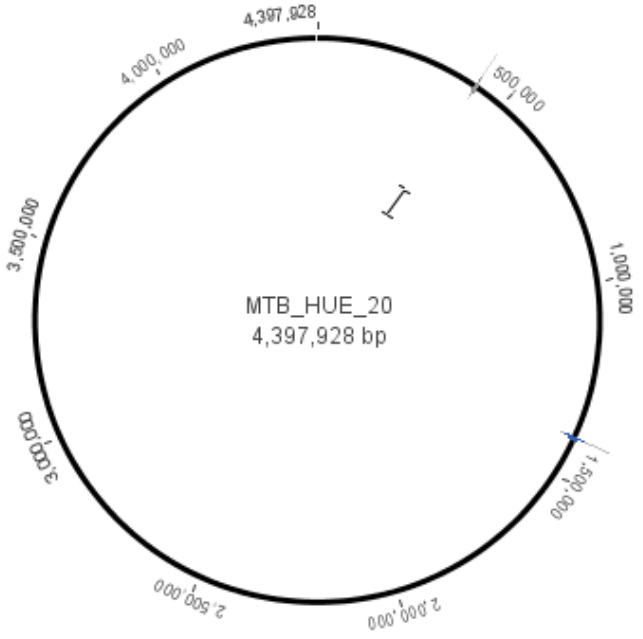


Figure 15. Visual full sequence of MTB_HUE_20 strain in circular mode

Mapping reads to reference – Resequencing assembly

Use CLC Genomics Workbench 4.7.2 for mapping 3,857,132 reads to refseq number NC_00962 (accession number PRJNA57777, ID: 57777). We obtained a consensus sequence of MTB_HUE_20 strain whichwas annotated as table 13.

Table 13. Annotation types table on consensus sequence of MTB_HUE_20 strain

Annotation types	Quality
Gene	4062
CDS (coding sequence)	4,003
Mobile_element	56
Repeat region	262
rRNAgene (16S, 23S, 5S)	3
tRNA gene	45

We performed the SNP and DIP detection on mapped sequence. We found 2329 SNPs, including 1257 SNPs lead change of amino acid with coverage 4-221 and 159 DIPs with coverage range 4-123, of which 105 DIPs lead change of amino acid (Table 14).

Table 14. Statistic of variation between MTB_HUE_20 and H37Rv strain

Variation type	Quality	Length (bp)	Coverage range	Amino acid changed
SNP	2329	1	4-221	1257
DIP	159	1-6	4-123	108

When mapping reads to the reference sequence (*M. tuberculosis* H37Rv complete genome with refseq number NC_00926), we found that there are 16 copies of IS6110 in H37Rv strain genome (Table 3 in appendix). Comparing the whole genomic DNA of MTB_HUE_20 strain with that of H37Rv strain, we determined the MTB_HUE_20 strain harbors one copy of IS6110 at position appropriate with locus_tagRv1755c of H37Rv strain, annotation of which is *plcD* gene, this ORF has been interrupted by insertion of IS6110 element (Figure 16).

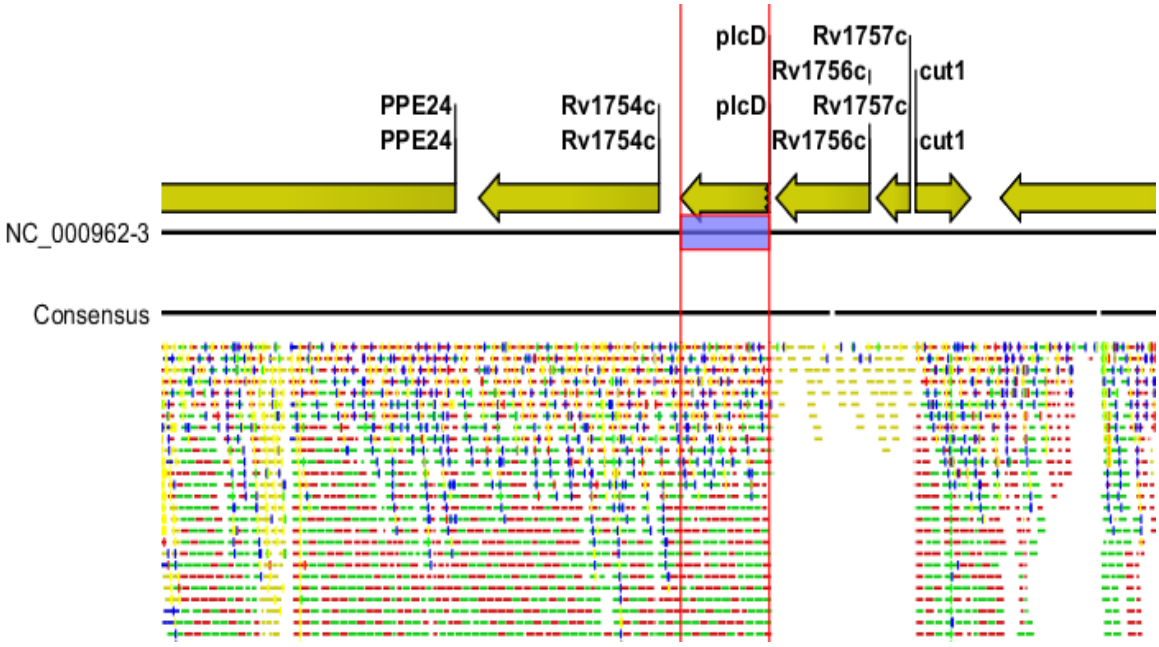


Figure 16. Mapped IS6110 insertion of MTB_HUE_20 strain at locus_tag Rv1755c (*plcD* gene)

MTB_HUE_20 strain is a MDR strain which was comfirmed by the MODS assay. Mutations in DNA-dependent RNA polymerase (*rpoB*) gene is among the most frequent in RIF-resistant strain [231,232,272] and catalase-peroxidase (*katG*) gene in INH-resistant strain. [92,102,188,192,272,274]

So in this present study, we focused on analyzing *rpoB* gene and *katG* gene.

Export *rpoB* sequence from full length genome obtained, the *rpoB* gene length of MTB_HUE_20 is 3518bp. We performed alignment with locus_tag Rv0667 of reference genome NC_000962.2 (Rv0667 is locus of *rpoB* gene of *M. tuberculosis* H37Rv). The percent of homology between 2 sequences is ~99.98%, with 1 nucleotide different at position 3225: C in the MTB_HUE_20 strain versus T in H37Rv strain (Figure 17)

Translate *rpoB* sequence of our strain to protein and do alignment of 2 amino acid sequences, the percent of homology is 100%. Amino acid at position 1075 is alanine on both sequences (Figure 18) .

Figure 17. Nucleotide alignment of *rpoB* gene

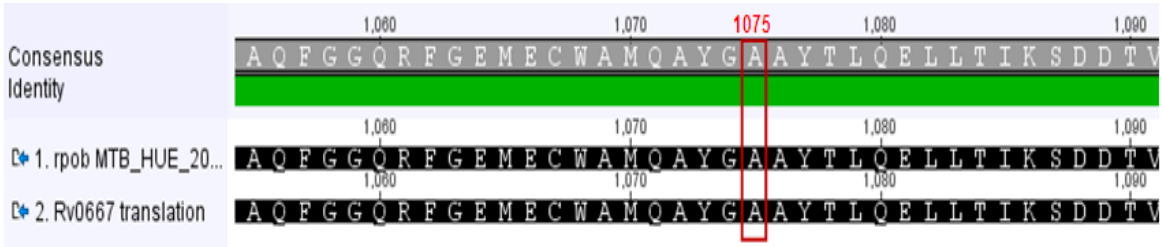


Figure 18.Protein alignment of *rpoB* gene

When doing alignment of *rpoB* sequence of the MTB_HUE_20 strain with partial CDS of *rpoB* sequence of two *M.tuberculosis* strains (MDR-TB2 and 331122070013) which were MDR strains with the mutation in *rpoB* gene, we found that there are 2 different nucleotides at position 1333 in strain 331122070013 (locus HQ844251.1) and at position 1334 (locus FJ915188.1) in MDR-TB2 strain (Figure 19).

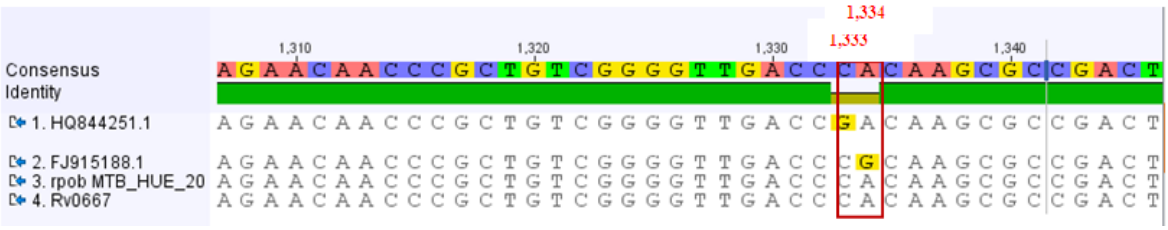


Figure 19.Nucleotide alignment of *rpoB* gene of 4 strains

Protein alignment of 4 sequences showed difference at position 445 of 1 amino acid: histidine (H) of MTB_HUE_20 and H37Rv strains, versus arginine (R) of MDR-TB2 strain and aspartic acid (D) of 331122070013 strain (Figure 20).

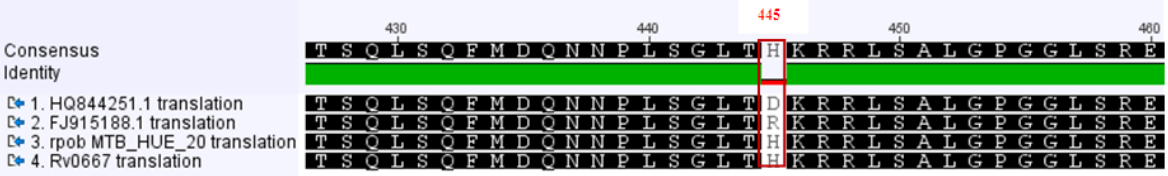


Figure 20.Protein alignment of *rpoB* gene of 4 strains

The *katG* sequence, obtained from full length genome, shows that the *katG* gene length of MTB_HUE_20 is 2223bp. We performed alignment with locus_tagRv1908c of reference genome NC_000962.2 (Rv1908cis locus of *katG* gene of *M. tuberculosis* H37Rv) and *M. tuberculosis* INH-resistant strain H0169/93 (locus U41311.1). The percent of homology of 3 sequences is ~99.96%, with 1 nucleotide different at position 1388, T in the MTB_HUE_20 and H0169/93 strain versus G in H37Rv strain (Figure 21).

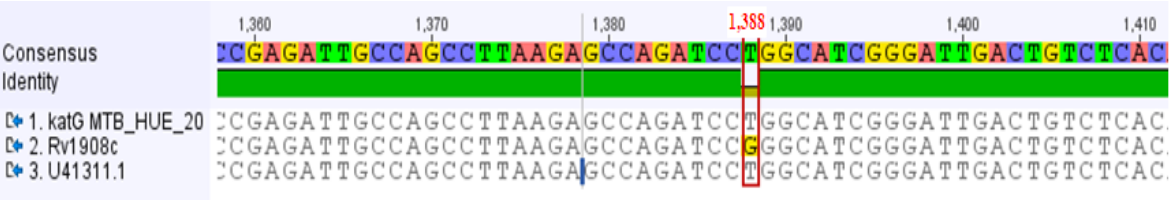


Figure 21. Nucleotide alignment of *kat*G gene

Protein alignment of 3 sequences showed there is difference of 1 amino acid at position 463: leucine (L) in the MTB_HUE_20 and H0169/93 strains, versus arginine (R) in H37Rv strain (Figure 22).

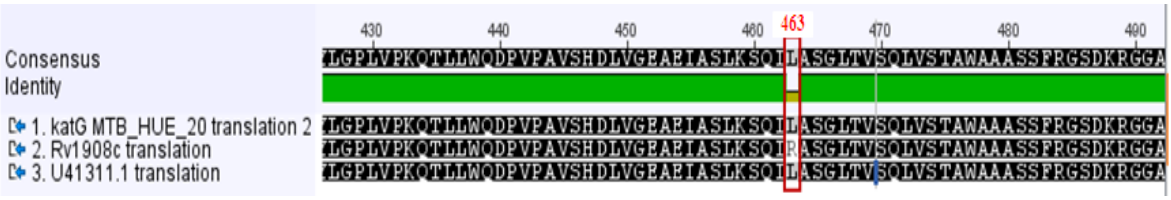


Figure 22. Protein alignment of *katG* gene of 3 strains

6. DISCUSSION

6.1. Proportion of culture positivity and drug susceptibility of *M. tuberculosis* isolates from the MODS assay

Early detection of drug resistance is the keypoint in effective control of drug resistant tuberculosis. Culture and drug susceptibility testing (DST) using conventional methods are time-consuming while automated methods tend to be expensive, limiting their use in resource constrained setting.[189,264] Hence there is need for a rapid, reliable and cost effective test for detection of drug resistance. MODS assay has the advantage of simultaneous detection and DST of *M. tuberculosis*.[129] The greater sensitivity and speed of detection of MODS culture in comparison to gold standards were predicted on the basis of previous studies.[35,156]

In the present study, among 252 MODS cultured samples, 153 (60.7%) were positive for *M.tuberculosis* and they were identified by the cord forming (Figure 7). The proportion of culture positive samples of the group of previous tuberculosis patients (85.7%) was higher than the group of patients suspected of new tuberculosis (57.6%). This difference was statistically significant (P<0.01; 95% CI)). The proportion of smear positive samples was 61.5% (155/252), similar to the proportion of MODS positive samples (60.7%). Interestingly, among the smear positive samples 21.3% (33/155) were negative for MODS culture, and among the smear negative samples 32% (31/97) were positive for MODS culture but negative for AFB. Of 33 culture negative and smear positive samples, 7 samples were negative for 16S rDNA PCR indicating non tuberculous mycobacteria. A previous study found that in sputum, the sensitivity of MODS was significantly higher than smear (P<0.001; 95%CI).[53] The 26 remaining samples collected from patients receiving TB therapy for less than two weeks were positive for 16S rDNA PCR, indicating ability of killed bacteria. In a study of Kashmira Limaye et al.[129] on new smear positive patients, the culture positivity rate was 100% (excluding contamination) for both MODS assay as well as Lowenstein medium. The high culture positivity rate in that study is due to the inclusion of only known smear positive patients.

In a recent research in patients with confirmed or probable tuberculosis in South Vietnam[52], MODS detected 72.8% (40/55) of Lowenstein-Jensen culture positive and smear negative tuberculosis cases. According to Moore et al.[156] the detection rate of MODS among smear negative tuberculosis cases was 17.85%. In our study, we detected 32% (31/97) of tuberculosis cases with smear negative samples. These results showed that MODS is useful for

detecting tuberculosis of smear negative cases.

Time to detection was defined as the number of days from sample processing (day 1) to identification of positivity. Our results of MODS culture showed that the median time for culture positivity and drug susceptibility testing were 9 days (range 5 to 17 days for culture and range 6 to 14 days for drug susceptibility testing). Our results are similar to those reported by Ejigu et al. (9 days).[69] The median time to culture positivity and drug susceptibility testing in the study of Moor et al. [155] and of Limaye et al. [129] were 7 days and 10 days, respectively. Our results confirm a greater sensitivity and speed of detection with MODS culture in comparison to the gold standard of Lowenstein-Jensen culture as predicted on the basis of previous studies.[35,156,53,129,69]

Contamination is a common problem with all liquid cultures and MODS is not an exception; careful manipulation and rigorous aseptic technique must be maintained to minimize contamination. In the present study, contamination with fungi and other bacteria was 17.4%, higher than that in the other studies (1.4% [53], 5.26 %[129],8.1% [155]). The MODS assay was recently set up in our laboratory for the first time for tuberculosis confirmation and drug susceptibility testing. It is possible that the lack of previous experience may have caused the relatively high rate of contamination. Michaelet et al., have also reported that more extensive training and standardization is required before conducting any study using MODS assay.[147]

In the present study, among 153 culture-positive samples, the proportion of *M. tuberculosis s*trains demonstrating sensitivity to all tested antibiotics (INH, RIF, STR and EMB) was 107 (69.9 %) and any drug resistance was 46 (30.1%). One drug resistance was observed in 30 strains (19.6%): INH 3.9% RIF 11.8%, STR 2.0% and EBM 2.0%. Multidrug resistant *M. tuberculosis* as defined by WHO (resistant to RIF and INH)was observed in 8.5%. There were 9.2% of isolates showing resistance to two or more drugs. It is well known that there are large variations of the resitance percentages according to geographical areas and socio-economical conditions. In a similar study by Moor et al.[155] done in Peru multidrug resistance was 10.1% and resistance to single drugs were as following: INH, 19.5%, RIF, 10.7%, EMB, 10.1%, STR, 21.4%. The percentage of samples showing agreement between MODS culture and the gold-standard methods was 100% for RIF, 96.7% for INH, 98.8% for multidrug resistance, 95.4% for EMB, and 91.7% for STR.

In our study, there was a statistically significant difference of drug resistance between the two groups of patients. The proportion of strains resistant to any drug, to INH, multidrug resistance and ≥ 2 drugs in the group

of previous TB patients was higher than in the group of new tuberculosis suspected patients ($p<0.01$). However, there was no significant difference of resistance to RIF alone between the two groups of patients and this may be due to the longer exposure to anti-mycobacterial drugs of the previous TB patient group. Unfortunately, resistance to EMB and STR could not be determined in the group of previous TB patients due to the limited number of strains.

The growing problem of drug resistance in *M. tuberculosis* is accompanied by increasing demand for quick, cheap, and easy techniques to detect resistance.[268] Several methods to detect drug resistance are available, but none clearly satisfies the demand for quick, cheap and easy testing. Traditional agar-based methods can take months for results. Commercial drug susceptibility testing with liquid culture decreases turnaround times but requires expensive equipment. Molecular detection of gene mutations associated with drug resistance has also been developed, with variable sensitivity reported especially for in-house methods.[148]

The MODS assay is a rapid, sensitive, specific direct method for simultaneous culture detection and drug susceptibility of *M. tuberculosis* [9,155,156] and was only recently introduced in our laboratory, therefore our results are still limited and difficult to compare with those of other groups. More work is needed to increase the patient sample and reach statistically significant numbers.

6.2. The proportion of detected tuberculosis by using the PCR assay and the rate of *M. tuberculosis*s trains which do not harbor insert sequence IS6110

A total of 480 clinical samples of patients with suspected tuberculosis including sputum, bronchial lavage, pleural fluid, cerebral spinal fluid (CSF), and others (gastric fluid, joint fluid, blood, abscess) were examined. Sputum was the most common type of specimen (76%). Positivity with AFB staining was in 192 samples (40% of all types). No CSF specimen was positive with AFB. The AFB positivity was detected in approximately 50% of the sputum specimens (186/365) and in lower percentage in bronchial lavage (1/30 - 3.3%), pleural fluid (2/50 - 4 %) and other specimens (3/25 - 12%).

The 16S rDNA PCR assay for 480 clinical samples detected 258 positive cases (53.8%). A 3.6 % (7/192) of AFB-positive samples were negative. Furthermore the 16S rDNA PCR detected 73 cases (25.3%) of tuberculosis in AFB-negative samples, of which 54 (30.2%) were from sputum and 19 (17.4%) from the other type of specimens.

In previous studies, different amplification methods (PCR as well as isothermal) have been found to be positive in 95-100% of smear/culture positive specimens whereas the positivity ranges from 40-60% in smear negative paucibacillary pulmonary disease.[107]

According to Katoch et al.[107] PCR methods for detection of *M. tuberculosis* from clinical specimens represent the ultimate in sensitivity and under optimum conditions are expected to detect 1-10 organisms. In this study, the 16S rRNA gene target is amplified by using a pair of unlabeled Mycobacterium genus specific PCR primers and a *M. tuberculosis* species-specific TaqMan probe, meaning that all 258 samples positive with the16S rDNA PCRwere infected with *M.tuberculosis*. The 73 cases, negative with AFB, which were detected by this PCR assay indicated its useful role inearly confirmation of diagnosis in paucibacillary diseases. Futhermore the 16S rDNA PCR found that 7 cases among 192 AFB-positive samples (3.6%) were nontuberculous mycobacteria.

Among the first proposed genomic targets for tuberculosis diagnostic PCR was the insertion element IS6110 which, being present in multiple copies (from 4 to 20 in more than 95% of *M. tuberculosis* strains), appeared to have the potential for an enhanced sensitivity.[177]Using a nested PCR assay protocol for the IS6110 target could further increase sensitivity of PCR.

The results of a Yuen's study [270]demonstrate that IS6110 is not present in the genome of all strains of *M. tuberculosis* and that the deletion of IS6110 would occurs more frequently in the group of Vietnamese patients.

In our study, the performance of IS6110 PCR assay was assessed in a total of 258 samples positive with 16S rDNA PCR. The result indicated that most of processed samples (98.8%) were positive with the IS6110 PCR (only 3 samples corresponding to 1.2 %, were negative). This showed that the proportion of deletion of IS6110 sequence in *M. tuberculosis* strain in our study is lower than that in the above study.

PCR has the potential to provide a more rapid, sensitive, and specific diagnostic assay for *M. tuberculosis* in clinical specimens.[30,68] The IS6110 nested PCR is an in-hous kit, performing in a conventional thermal cycler, so that this assay can be applied more widely than realtime PCR for the 16S rRNA gene. In case of using IS6110 PCR assay, results may be false-negative with a low proportion.

6.3. The distribution of genotypes of isolated *M. tuberculosis* strains and the association between the genotypes and drug susceptibility

In the present study, EAI genotypes were seen in 65.6% of isolates, of which 15.6 % were of the EAI4-VNM genotype, and 46.7 % were of EAI5. This analysis indicates a dominance in Central Vietnam of the *M. tuberculosis* EAI family, with a strong supremacy of the EAI5 spoligotypes.

Previous studies showed that the EAI lineage is more prevalent in South-East Asia, particularly in the Philippines(73%), in Myanmar and Malaysia (53%), in Vietnam and Thailand (32%). In this family, specific variants have been described for Vietnam (ST139 or EAI-4).[27] EAI family represents an "ancient" strain type in some classification schemes, whereas the Bejing genotype is classified as "modern." It is likely that this genotype was circulating in Vietnam prior to the introduction of the Beijing genotype and that East Asian Indian (EAI) clade genotypes represent the endemic *M. tuberculosis* strains, circulating in all age groups, while the Beijing "epidemic" strain has been relatively recently introduced or achieved selective advantage and is being actively transmitted among the younger population group.[52]

In our study, Beijing genotype was observed in 12.3 % of isolates. Dang Duc Anh et al. reported in a previous study that Beijing genotypes represent approximately 40% of *M. tuberculosis* strains and are apparently endemic and stable in the community.[51] In a more recent study of Nguyen van Hung et al.[167], Beijing genotype was identified overall in Vietnam in 35.1% of isolates, and there was statistically significant difference of the distribution of Bejing genotype in the three regions of Vietnam ($P < 0.01$): in the South, 39%, in the North, 30.1%, and in the Centre, 28.6%. Our result indicated a lower proportion of Beijing genotype, however, because our sample sized was small, it may not be fully representative of all *M. tuberculosis* strains in central Vietnam.

The association of the Beijing strain with drug resistance and MDR has been documented in other settings, though this has not been a consistent finding. It has been postulated that in much of Asia, non association is probably due to the endemic nature of the Beijing genotype, whereas in areas where the proportion of Beijing strains is increasing, and therefore is "epidemic", associations with drug resistance are found.[8] The association with drug resistance is particularly strong in Vietnam,[51] unlike most parts of Asia.

According to Nguyen van Hung et al.[167] among new pulmonary tuberculosis patients, Beijing genotype with any-drug resistant tuberculosis

was 43.4% and MDR-TB was 5%. In a group of patients with recurrent pulmonary tuberculosis, Beijing genotype with resistance to any-drug was 74% and MDR-TB was 26%.

Sun et al.[224] reported that the multidrug-resistant isolates (n = 41, OR 2.66, 95%CI 1.28-5.50), rifampicin resistant isolates (n = 48, OR 2.88, 95%CI 1.44-5.76), and streptomycin resistant isolates (n = 103, OR 3.35, 95%CI 1.99-5.62) were more common among Beijing genotype strains than among non-Beijing strains, while streptomycin-resistant isolates were less common in East-African-Indian (EAI) genotype strains than in non-EAI strains (OR 0.30, 95%CI 0.14-0.64).

In the present study, there was no significant association of the Beijing genotype with resistance to drug: 20 % of Beijing isolates and 28.9% of non Beijing isolates being resistant to any first-linedrug (OR = 0.6 [95% confidence interval {CI}, 0.2 to 2.3]; P=0.47); 6.7% of Beijing isolates and 10.3% of non Beijing isolates being multidrug resistance (OR = 0.6 [95% confidence interval {CI}, 0.1 to 5.2]; P=0.66).

Similarly, there was no significant association of the EAI genotype with resistance to drug:28.8 % of EAI isolates and 26.2% of non EAI isolates being resistant to any first-linedrug (OR = 1.1 [95% confidence interval {CI}, 0.5 to 2.6]; P=0.76); 8.6% of EAI isolates and 11.9% of non EAI isolates being multidrug resistance (OR = 0.7 [95% confidence interval {CI}, 0.2 to 2.4]; P=0.58).

In our study, however, the small sample (15 strains) of Beijing genotype cannot be considered a representative strain of Beijing genotype of *M. tuberculosis* in Central Vietnam; further analysis should be performed with a large sample.

6.4. Molecular characteristics of the MTB_HUE_20 strain

This is first time that a next generation sequencing for the whole genome of a mycobacterium was performed in Viet Nam. In “de novo” assembly process, we only assembled all reads to have the contigs, annotated all ORFs and made a full length genome with annotation on all loci.

The full length of our strain is about 4,397KB, less than H37Rv strain, of 4,411KB. The diffrence in length is about 14KB. MTB_HUE_20 strain has only one copy of IS6110 versus 16 copies of H37Rv, each IS6110 is 1355 bp in length (see Table A4 in appendix) and the lack of 15 copies of IS6110 combining 159 DIPs may account for the shorter length of MTB_HUE_20 strain.

Percentages of A, T, C and G in genome of the MTB_HUE_20 strain were similar to those of H37Rv strain and of the other sequenced *M. tuberculosis* strains which are submitted on NCBI (BioProject). The MTB_HUE_20 strain contains a high percentage (64.8%) of G and C similar to that of H37Rv strain (65.6%) (table 15).

Table15. Statistics of complete genome *M. tuberculosis* H37Rv strain

Base	Frequency	Percentage %
A	758,565	17.2%
C	1,449,985	32.9%
G	1,444,603	32.7%
T	758,379	17.2%
GC	2,851,418	65.6%
Length:	4,411,532	

Following the annotation of reference assembly, the MTB_HUE_20 strain has 4062 genes and 4003 protein coding sequences, 3 rRNA genes and 45 tRNA genes. This strain also has 2329 SNPs, 1257 of which lead change of amino acid and 159 DIPs, 105 of which lead change of amino acid.

After completing with the annotation of full length sequences, we will focus on analyzing the different genes in genome, such as genes for resistance, mobile elements, virulence genes, etc.

As in most bacteria, various mobile genetic elements (MGEs) or 'jumping genes' have been detected in the *M. tuberculosis* genome.[87] These elements are capable of moving from one chromosomal location to another in a process called transposition and their dynamic nature has been implicated in the phenotypic characteristics of several pathogenic bacteria.[179,28] Due to its extensive numerical and positional polymorphism in *M.tuberculosis*, the mobile genetic elements known as IS6110 has been used extensively as a genotypic marker in epidemiological studies.[142]

The insertion element IS6110 is present in multiple copies (from 4 to 20) in more than 95 % of *M. tuberculosis* strains[177]. Yuen et al.[270] reported *M. tuberculosis* isolates from Vietnamese patients have an average of 8 ± 7 copies of IS6110.

Comparing the whole genomic DNA of MTB_HUE_20 strain with that of H37Rv strain, we determined it harbors only one copy of IS6110 versus 16 copies of IS6110 in genome of H37Rv strain. MTB_HUE_20 strain was also confirmed positive with IS6110 PCR.

An unusual feature of *M. tuberculosis* in Vietnam is the presence of a second highly prevalent spoligotype, ST319, or the Vietnam genotype.[51] This genotype has a single IS6110 copy; it represents around 20% of the isolates from southern Vietnam. Although the MTB_HUE_20 strain has only one

IS6110 copy, has not the same spoligotype pattern as the Vietnam genotype or any spolygotype pattern of the updated international spoligotype database of the *M.tuberculosis* complex - SpolDB4.

Rifampicin (RIF) is an important component in treating tuberculosis. It is a broad spectrum rifamycin derivative that interferes with the synthesis of mRNA synthesis by binding to the ß subunit of RNA polymerase (*rpo*B) in bacterial cells. The RIF binding site is a pocket in the upper wall of the main channel for double-stranded DNA entry just upstream of the polymerase catalytic center. The various RIF-resistant mutations are clustered around this pocket.[273] Mutations of the *rpoB* gene are found in nearly all of the RIF resistant strains (>95%).[272]

Comparing our result with the locus Rv0667 of *rpo*B gene of *M.tuberculosis* H37Rv,the percent of homology between 2 sequences is ~99.98%, with 1 nucleotide different at position 3225, C in the MTB_HUE_20 strain versus T in H37Rv. Translation of *rpoB* sequence of our strain to protein and alignment with 2possible amino acid sequences shows that the percent of homology of amino acid is 100%. Amino acid at position 1075 is alanine on both sequences.

When doing alignment of *rpoB* sequence of the MTB_HUE_20 strain with partial CDS of *rpoB* sequence of two MDR *M. tuberculosis* strains (MDR-TB2 and 331122070013) with mutation in *rpoB* gene, we found that there are 2 different nucleotides at position 1333 and 1334. Protein alignment of 4 sequences showed difference at position 445 of 1 amino acid: histidine (H) in the MTB_HUE_20 and H37Rv and arginine (R) in MDR-TB2 strain and aspartic (D) in strain 331122070013.

Although the MTB_HUE_20 strain has one nucleotide at position 3225 in *rpo*B gene which differs from strain H37Rv, the percent of homology of amino acid is 100%. Amino acid at position 1075 is alanine (A) on both sequences. Conversely, two above MDR strains differ from H37Rv one amino acid at position 1075. This indicated that there is no mutation on *rpo*B gene in the MTB_HUE_20 strain. As reported above, the MTB_HUE_20 strain is a MDR strain and less than 5% of resistant strains do not show a mutation in the *rpoB* resistance region.[134,192] Pierre-Audigier et al.[182] showed rare inconsistencies of mutated *Mtb* strains, one of them may be clinically resistant, which could suggest other mechanism for resistance. One of them may even be clinically resistant, which could suggest greater genotypic test sensibility. Maybe the MTB_HUE_20 strain is one of these strains.

Isoniazid (INH) has been one of the most effective and specific agents for treating *M. tuberculosis* infections. It is a simple structure made up of a pyridine ring and a hydrazide group, two essential components to its powerful activity against *M. tuberculosis*.[272] It has one of the most complex modes of action among all antibiotics. INH is a prodrug that requires activation of the catalase-peroxidase enzyme (*KatG*) coded by the *katG* gene. There are many molecular mechanisms causing INH resistance, but only some of them have been characterized.[251]

Mutations in the *katG* gene, which hinder activation of the INH prodrug, are among the most frequent in INH-resistant strains: 20 to 95% INH-resistant strains have at least one mutation in the *katG* gene; its position varies according to their geographical origin.[92,102,188,192,272,274]

In the present study, we performed alignment with locus_tagRv1908c of reference genome NC_000962.2 (Rv1908cis locus of *katG* gene of *M.tuberculosis* H37Rv) and INH-resistant *M.tuberculosis*strain H0169/93. The percent of homology of 3 sequences is ~99.96%, with 1 nucleotide different at position 1388, T in the MTB_HUE_20 and H0169/93 strain versus G in H37Rv strain. Protein alignment of the 3 sequences showed 1 amino acid different at position 463: Leucine (L) in both of MTB_HUE_20 and H0169/93 strains versus Arginine (R) in H37Rv strain.

The MTB_HUE_20 strain is MDR resistant, this is consistant with the analysis of mutation of *katG* gene.

7. CONCLUSION

The MODS assay is a rapid, sensitive, specific direct method for simultaneous culture detection and drug susceptibility of *M. tuberculosis,* we will continue to research and improve the routine procedure of MODS assay at Carlo Urbani Centre, in Hue College of Medicine and Pharmacy.

PCR is a more rapid, sensitive, and specific diagnostic assay for *M. tuberculosis* in clinical specimens. The IS6110 nested PCR is an in-hous kit, performing in a conventional thermal cycler, so that this assay can be applied more widely than the 16S rDNA realtime PCR. In case of using IS6110 PCR assay, results may show a low proportion of false negative. Combining 16S rDNA PCR with the IS6110 based PCR allowed detection of deletion of IS6110sequence in a limited number of *M. tuberculosis* isolates.

Our results of spoligotype were inconsistent with those of previous studies indicating that spoligotyping should be performed with a large size of sample to determine the association between genotype and drug resistance of *M. tuberculosis*.

Whole genomic DNA sequence of the MTB_HUE_20 strain should be completed with the annotation of full length sequences and with focus on analyzing different group of genes such as those involved in resitance, mobile elements and virulence genes.

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9. APPENDIX

Appendix 1: Media and buffers

Reagents for Ziehl-Neelsen staining

- Fuchsin solution

Fuchsin

Basis fuchsin	3g
95% ethanol (technical grade)	100ml

Dissolve basis fuchsin in ethanol…….Solution 1

Phenol

Phenol crystals	5g
Distilled water	100ml

Dissolve phenol crystals in distilled water (gentle heat)…….Solution 2

Working solution: combine 10ml of solution 1 with 90ml of solution 2.

- Decolorising reagent: 3% acid-alcohol

Concentrated hydrochloric acid (technical grade)	3ml
95% ethanol (technical grade)	97ml

- *Counterstain:* Methylene blue

Methylene bleu chloride	0.3g
Sterile distilled water	100ml

Reagents for sputum decontamination

- N-acetyl-L-cysteine-sodium hydroxide

Solution A: NaOH 4%

NaOH	4 g
Distil- led water	100 ml

Solution B: sodium citrate 2.9%

sodium citrate 2 H2O	2.9 g
distilled water	100 ml

Mix equal parts of solution A and solution B. Distribute in screw-cap flasks. Autoclave at 121 °C for 20 minutes. On the day of use, add N-acetyl-L-cysteine at 1% concentration in the flask.

- Phosphate Buffered Saline (PBS)

10x PBS Solution (Biobasis)

Reagents for Microscopic Observation Drug Susceptibility (MODS) assay

- Middlebrook 7H9 liquid medium with casitone and glycerol (7H9)
  - Middlebrook 7H9 broth base (Becton Dickinson) 5.9 g
  - Glycerol (Sigma, St Louis, MO, USA) 3.1ml
  - Casitone (Becton Dickinson) 1.25g
  - Sterile distilled water 900ml
- *OADC* (Becton Dickinson)
- *PANTA* (BBL MGIT PANTA, Becton Dickinson)

Table A1. Reconstitute a lyophilized vial of PANTA with 3 ml of sterile distilled water

Antibiotic	Formulation per vial	Concentration per ml after reconstitution with 3ml sterile	Final concentration in the well with sample and 7H9-OADC media
Polimixin B	6000 units	2000 units /ml	40 units/ml
Amphotericin B	600 µg	200 µg /ml	4 µg/ml
Nalidixic acid	2,400 µg	800 µg /ml	16 µg/ml
Trimethoprim	600 µg	200 µg /ml	4 µg/ml
Azlocillin	600 µg	200 µg /ml	4 µg/ml

- Antibiotic solutions
  - Isoniazid stock (0.1 mg/ml)
    - Isoniazid (Sigma) 1mg
    - Sterile distilled water 10ml
  - Rifampicin stock (1 mg/ml)
    - Rifampicin (Sigma) 10mg
    - Dimethyl sulphoxide 5 ml
    - Sterile distilled water 5ml
  - Streptomycin stock (2 mg/ml)
    - Streptomycin (Sigma) 20mg
    - Sterile distilled water 10ml
  - Ethambutol stock (2.5 mg/ml)
    - Ethambutol (Sigma) 25mg
    - Sterile distilled water 10ml

Working solutions of each drug are prepared on the day of use from the storage stock. Dilutions are made using 7H9 with 10% OADC (7H9-OADC).

Table A2. Dilution of antibiotic working solutions

Antibiotic stock	Dilution of stock in 7H9-OADC to generate stock 2	Dilution of stock 2 in 7H9-OADC to generate working solution	Final concentration in well when added to sample (µg/ml)	
Isoniazid 100 µg/ml	100 µl stock / 900 µl 7H9-OADC (10 µg/ml)	1/10 1 µg/ml	INH	0.1
	400 µl stock / 600 µl 7H9-OADC (40 µg/ml)	1/10 4 µg/ml	INH	0.4
Rifampicin 1000µg/ml	100 µl stock / 900 µl7H9-OADC (100 µg/ml)	1/10 1 0µg/ml	RIF	1
Streptomycin 2000µg/ml	200 µl stock / 800 µl7H9-OADC (200 µg/ml)	1/10 20 µg/ml	RIF	2
	100 µl stock / 900 µl7H9-OADC (200 µg/ml)	1/10 20 µg/ml 1/10 60 µg/ml	STR	2
Ethambutol 2500 µg/ml	300 µl stock / 700 µl7H9-OADC (600 µg/ml)	1/10 25 µg/ml	STR	6
	100 µl stock / 900 µl7H9-OADC (250 µg/ml)	1/10 50 µg/ml	EMB	2.5
	200 µl stock / 800 µl7H9-OADC		EMB	5

Reagents for DNA extraction

- *DNeasy Blood & Tissue kit* (Qiagen) for extraction of DNA from clinical samples.
- Reagents for extraction of DNA from isolated mycobacterial strains by using Cetyltrimethyl Ammonium Bromide (CTAB)

+ Glycine stock 10% (wt/vol)
+ Sodium dodecyl sulfate (SDS) 10%
+ Proteinase K 10 mg/ml (Sigma)
+ NaCl 5 M
+ Chloroform-isoamyl alcohol (24:1)
+ GTE Solution

Tris-HCl (pH 8.0)	25 mM
EDTA	10 mM
Glucose	50 mM

+ Lysozyme Solution

For optimal lysozyme activity, make a fresh 10 mg/ml solution of lysozyme (Sigma) in 25 mM Tris-HCl (pH8.5).

+ CTAB Solution (Sigma)

Sodium cloride	4.1 g
distilled water	90 ml
Cetrimide	10 g

(Cetrimide is hexadecyltrimethylammonium bromide; Sigma)

Reagents for PCR assay

- PCR Master Mix (2X) (Fermentas)
- Two sets of primers for the IS6110 based nested PCR assay (following the protocol of tuberculosis laboratory of Section of Experimental and Clinical Microbiology, Department of Biomedical Science, SassariUniversity):

+ External primers:

IS1: 5- GCA CCG CCC GGG ACC -3'
IS2: 5- CTG CCC AGG TCG ACA CAT AG -3'

+ Internal primers:

IS3: 5'- GAT GGT TTG CGG TGG GGT GTC GAG T-3'
IS4: 5'- GAT CGT GGT CCT GCG GGC TTT GC-3'

- Tris-Borate EDTA buffer: TBE 10X buffer (Bio Rad).
- Loading 6X (Fermentas)
- GeneRuler™ 100 bp DNA Ladder (Fermentas)
- Agarose 2%
- LightPower iVAMTB rPCR Kit (VietA company, Vietnam) for 16S rDNA sequence based realtime PCR.

Reagents for Spoligotyping

- Spoligotyping kit for detection of the *M.tuberculosis* complex of Ocimum Biosolutions. This kit contains:

+ One activated Biodyne C membrane - to which oligonucleotides derived from the known spacers in the DR cluster are covalenttly linked in parallel line.

+ One set of primers for the PCR based on the ''Direct Repeat'' (DR) region:

• Primer DRa (biotinylated at 5' end):
5' -GGT TTT GGG TCT GAC GAC- 3'
• Primer DRb: 5' -CCG AGA GGG GAC GGA AAC- 3'

+ Positive control 1(*M. tuberculosis strain* H37Rv)
+ Positive control 2 (*M. bovis BCG* P3)

- Streptavidin - Horseradish Peroxidase 500U/ml (GE Healthcare)
- ECL detection reagents (GE Healthcare)
- Buffers

+ 20X SSPE - Stock Solution

0.2M $Na_2HPO_4.2H_20$	35.6 g/L
3.6M NaCl	210.24 g/L
20mM EDTA	7.4 g/L
Demineralised water up to	1000 mL

+ 10% SDS

SDS	10g
Demineralised water up to	100 mL

+ 2X SSPE/0.1% SDS

20X SSPE	100 mL
10% SDS	10 mL
DW	890mL

+ 2X SSPE/0.5% SDS

20X SSPE	100 mL
10% SDS	50 mL
Demineralised water	850mL

+ 2X SSPE

20X SSPE	100 mL
Demineralised water	900mL

+ 1% SDS

10% SDS	100mL
Demineralised water	900 mL

+ 20 mM EDTA, pH8

EDTA	7.4g
Demineralised water up to	1000Ml

Appendix 2: Spoligotype of *M.tuberculosis* isolates in Central Vietnam

Table A3: Spoligotype of *M.tuberculosis* isolates in Central Vietnam

no.	Strain	Spoligotype Description Binary	SIT No	Spoligotype
1	13	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	1	**BEIJING**
2	28	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	1	**BEIJING**
3	29	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	1	**BEIJING**
4	335	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	1	**BEIJING**
5	438	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	1	**BEIJING**
6	479	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	1	**BEIJING**
7	592	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	1	**BEIJING**
8	CM12	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	1	**BEIJING**
9	CT3	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	1	**BEIJING**
10	DN840	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	1	**BEIJING**
11	DN846	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	1	**BEIJING**
12	DN858	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	1	**BEIJING**
13	DN867	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	1	**BEIJING**
14	DN955	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	1	**BEIJING**
15	DN970	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	1	**BEIJING**
16	DN922	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□■■■■■■■■	1378	**CAS**
17	406	■■■■■■■■■■■■■□■■■■■■■■■■■■□□□□■□■■□■■□■■■	303	**EAI1_SOM**
18	535	■■■■■■■■■■■■■□■■■■■■■■■■■■□□□□■□■■□■■□■■■	303	**EAI1_SOM**
19	CM14_2	■■■■■■■■■■■■■□■■■■■■■■■■■■□□□□■□■■■■■□■■■	1251	**EAI1_SOM**
20	DN769	■■□■■■■□□□□□□□□□□□□□□□□□■■■□□□□■□■■■■■■■■	89	**EAI2_NTB**
21	12	■■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■■■	139	**EAI4_VNM**
22	376	■■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■■■	139	**EAI4_VNM**
23	381	■■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■■■	139	**EAI4_VNM**
24	526	■■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■■■	139	**EAI4_VNM**
25	558	■■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■■■	139	**EAI4_VNM**
26	852	■■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■■■	139	**EAI4_VNM**
27	CM22	■■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■■■	139	**EAI4_VNM**
28	16	■■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■■■	139	**EAI4_VNM**
29	261	■■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■■■	139	**EAI4_VNM**
30	301	■■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■■■	139	**EAI4_VNM**
31	350	■■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■■■	139	**EAI4_VNM**
32	470	■■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■■■	139	**EAI4_VNM**
33	CM2(2)	■■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■■■	139	**EAI4_VNM**
34	CT4	■■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■■■	139	**EAI4_VNM**
35	DN750	■■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■■■	139	**EAI4_VNM**
36	DN796(1)	■■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■■■	139	**EAI4_VNM**
37	DN15(NA)	■■■■■■■■■■■■■■■□■■■■■■■■□□■□□□□■□■■■■■■■■	1571	**EAI4_VNM**
38	PK31	■■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■■■	139	**EAI4_VNM**
39	PK4	■■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■□□■■	456	**EAI4_VNM**
40	5	■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■	152	**EAI5**
41	21	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■■■■	236	**EAI5**

no.	Strain	Spoligotype Description Binary	SIT No	Spoligotype
42	23	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■■■■■■	236	**EAI5**
43	25	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■■■■■■	236	**EAI5**
44	26	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■■■■■■	236	**EAI5**
45	30	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
46	33	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■■■■■■	236	**EAI5**
47	34	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
48	37	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
49	131	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
50	269	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
51	276	■■■■■■■■■■□■■■■■■■■■■■■■■■■■□□□□■□■■□■■■■■■	1726	**EAI5**
52	285	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
53	288	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
54	313	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
55	318	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■■■■■■	236	**EAI5**
56	322	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
57	361	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■■■■■■	236	**EAI5**
58	372	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
59	386	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
60	397	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
61	414	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
62	416	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■■■■■■	236	**EAI5**
63	419	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■■■■■■	236	**EAI5**
64	451	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
65	480	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
66	561	■■■■■■■■■■■■■■■■■■□□■■■■■■■■■□□□□■□■■□■■■■■■	1573	**EAI5**
67	572	■■■■■■■■■■■■□■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	617	**EAI5**
68	280	■■■■■■■■■■■■□■■■■■■■■■■■■■■■□□□□■□■■■■■■■■■■	618	**EAI5**
69	555	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■■■■■■	236	**EAI5**
70	BT6	■■■■■■■■■■■■□■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	617	**EAI5**
71	CM17	■■■■■■■■■■■■■■■■■■□□■■■■■■■■■□□□□■□■■□■■■■■■	1573	**EAI5**
72	CM18	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
73	CM20	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
74	CM21	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
75	DN819	■■■■■■■■■■■■■■■■■■□□■■■■■■■■■□□□□■□■■□■■■■■■	1573	**EAI5**
76	DN874	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
77	DN923	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■■■■■■	236	**EAI5**
78	PK1	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
79	PK16	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**

no.	Strain	Spoligotype Description Binary	SIT No	Spoligotype
80	PK19	■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
81	PK3	■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
82	591	■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
83	599	■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
84	527	■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
85	CM14	■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
86	CM25	■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
87	CT2	■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
88	DN745	■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
89	DN861	■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
90	DN974	■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
91	BM11	■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
92	CM10	■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
93	CM15	■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
94	DN10	■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
95	DN865	■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
96	DN870	■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■■	152	**EAI5**
97	DN67	□■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■■■■■	472	**H3**
98	531	■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■■■□□	748	**H3**
99	CM3	■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■■■■■	50	**H3**
100	302	■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■■■■■	937	**MANU1**
101	565	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■■■■■■■■■	100	**MANU1**
102	BM9	■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□■■■■■■■■■	1247	**MANU2**
103	8	■■■■■■■■■■■■□■□□□□□□□□□□□■■■■■■■□□□□■■■■■■■	249	**T1**
104	18	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	53	**T1**
105	463	■■■■■■■■■■■■■■■■■□■■■■■■■■■■■■□□□□■■■■■■■	120	**T1**
106	597	□■■■■■■■■■■■■■□■■■■■■■■■■■■■■■■□□□□■■■■■■■	1645	**T1**
107	DN63	■□■■■■■■■■■■■■□■■■■■■■■■■■■■■■■□□□□■■□■■■■	1317	**T1**
108	461	■■■■■■■■■■■■■■■■■■■■■■■□□□■■■■■■□□□□■■■□■■■	942	**T2**
109	3	■■■■■■■■■■■■■■■■■■□□□□□□□■□□□□□□□□□□□□□□■■	619	**U**
110	24	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■■■■■■■■	458	**U**
111	27	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■■■■■■■■	458	**U**
112	286	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■■■■■■■■	458	**U**
113	404	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■■■	246	**U**
114	415	■■	523	**U**
115	547	□□□□□□□□□□□□□□□□□■□□□□□□□□□□□□□□□□□■■■■■■■	1184	**U**
116	CM16	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■■■■■■■■	458	**U**
117	CM9	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■■■■	1338	**U**
118	DN815	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■■■■■■■■	458	**U**
119	MTB_HUE_20	■■■□□□□■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■		**Unk**
120	278	■■■■■■■■□□□□□□■■■■■■■■■■■■□■□□□□■□■■■■■■■■■		**Unk**
121	289	■■■■■■■■■□■■■■■■■■■■■■■■■■□□□□□□□■■■■■■■■■		**Unk**

Appendix 3: Insertion sequence IS6110 in genome of H37Rv

Table A4. Sixteen copies of IS6110 in genome H37Rv strain

Name	Type	Region
IS6110-1, len: 1355 bp. Insertion sequence IS6110.	mobile_element	889021..890375
IS6110-2, len: 1355 bp. Almost identical toInsertion sequence IS986 element.	mobile_element	complement(1541952..1543306)
IS6110-3, len: 1355 bp. Insertion sequence IS6110.	mobile_element	complement(1987703..1989057)
IS6110-4, len: 1355 bp. Insertion sequence IS6110.	mobile_element	1996101..1997455
IS6110-5, len: 1355 bp. Insertion sequence IS6110.	mobile_element	2365414..2366768
IS6110-6, len: 1355 bp. Insertion sequence IS6110.	mobile_element	complement(2430117..2431471)
IS6110-7, len: 1355 bp. Insertion sequence IS6110.	mobile_element	2550014..2551368
IS6110-8, len: 1355 bp. Insertion sequence IS6110	mobile_element	2635577..2636931
IS6110-9, len: 1357 bp. Insertion sequence IS6110.	mobile_element	complement(2784614..2785970)
IS6110-10, len: 1355 bp. Insertion sequenceIS6110.	mobile_element	2972109..2973463
IS6110-11, len: 1375 bp. Insertion sequenceIS6110.	mobile_element	complement(3120523..3121897)
IS6110-12, len: 1355 bp. Insertion sequenceIS6110.	mobile_element	3551230..3552584
IS6110-13, len: 1355 bp. Insertion sequenceIS6110.	mobile_element	3552713..3554067
IS6110-14, len: 1355 bp. Insertion sequenceIS6110.	mobile_element	3710382..3711736
IS6110-15, len: 1355 bp. Insertion sequenceIS6110	mobile_element	3795058..3796412
IS6110-16, len: 1355 bp. Insertion sequenceIS6110	mobile_element	3890779..3892133

Appendix 4: Ethic approval

To:
The Chairman
The Scientific Committee of Hue College of Medicine and Pharmacy
Hue College of Medicine and Pharmacy

APPLICATION FOR ETHIC APPROVAL FOR IMPLEMENTATION OF SCIENTIFIC RESEARCH

Name of investigator: NGO VIET QUYNH TRAM

The title of study project:

Molecular epidemiology and phenotypic and genetic characterization of mycobacteria isolated in central Vietnam.

This study is a part of my study project for my PhD thesis of the doctorate programme in Biomolecular and Biotechnological Sciences in Sassari University, Italy.

Supervisor: Prof. Piero Cappuccinelli, University of Sassari and honorable professor of Hue College of Medicine and Pharmacy

Objectives of the project:

- to determine the rate of drug-resistant and multidrug-resistant *M.tuberculosis* strains.
- to detect tuberculosis by using the PCR assay and determine the rate of *M.tuberculosis* strains which do not habor insert sequence IS6110.
- to determine with molecular epidemiology methods the distribution of genotypes and create a phylogenetic tree of isolated *M.tuberculosis* strains.
- to describe molecular characteristics of a representative *M.tuberculosis* strain isolated in central Vietnam.

The intended period of time for the study project:
from July 2009 to June 2011

The participants will be enrolled:
Patients suspected or confirmed of having tuberculosis at the Hospital of Hue College of Medicine and Pharmacy, Hue Central Hospital and Danang Lung Hospital, in Vietnam were enrolled into the study.

The procedures will be taken:
The study will be carried out in order to implement the following steps:
- Selecting the patients, giving consultations to the patients to obtain the agreement of their involvements in the study.
- Performing clinical examinations and completing the patient recording form.

- Collecting the samples (such as sputum, bronchial lavage, etc.).

Declaration of responsibility:

- Agreement (in writing or oral) from patients or through patient s' guardians will be taken before entering them into study.
- I am aware that any medical procedure, which can be used on patient, may bring all the potential risks to patient s' health and I also know with certainty that the procedure performed for collection of specimen will make no harm to patient.
- The patient data, record and results of examination and laboratory analysis will be kept only for scientific purpose and not for anything else.

I confirm that the information contained in this application is correct and true.

Hue 05th, July, 2009
Investigator Signature

Ngo Viet Quynh Tram

Approval

The Scientific Committee of Hue College of Medicine and Pharmacy
Hue College of Medicine and Pharmacy
Chairman and Rector

Prof. Dr. Cao Ngoc Thanh

Appendix 5:

PARTICIPANT INFORMATION
FOR SAMPLE COLLECTION AND INTERVIEWING

Study title: Molecular epidemiology and phenotypic and genetic characterization of mycobacteria Isolated in central Vietnam.

Research Team Contacts	
Ngo Viet Quynh Tram, Dr, Msc PhD student, Sassary University , Italy Lecturer, Department of Microbiology HueCollege of Medicine and Pharmacy Hue, Vietnam Phone: +84 914202317	Nguyen Thi Chau Anh, Dr Lecturer, Department of Microbiology HueCollege of Medicine and Pharmacy Hue, Vietnam Phone: +84 984570083

This study is being taken as part of PhD of Ngo viet Quynh Tram, the principal investigator of this study, with the guidance and supervision of researcher from SassaiUniversity (Prof. Piero Capuccinelli).

This study aims to identify Molecular epidemiology and phenotypic and genetic characterization of mycobacteria Isolated in central Vietnam. Your participation in this study is voluntary. Your refusal to participate will not affect your diagnosis and treatment in any way. If you agree to participate in this study, you can withdraw from your participation at any time during the interview with no comment or penalty.

If you agree to participate, we will take your specimens (such as sputum or bronchial lavage, etc.) for for study on *M.tuberculosis* and you will be asked to answer some questions about tuberculosis. The questions will be about history of tuberculosis, its symptoms and treatment.

Information provided will be treated confidentially and we will not identify you in any of our publications or reports. What we learn from you will be used as the basis of our university research project. The Ethics Committee of Hue College of Medicine and Pharmacy has provided formal ethics approval for this study.

You are very welcome to ask us anything about what we are doing and what we are studying. Please contact the researcher team members named above to have any questions answered or if you require further information about the project.

If there are issues related to the study and/or you would like to have further discussion on ethics issues, please contact A/Prof. Tran Van Huy, Head of Office for Research and International Relations, Level 3, Building A, Hue College of Medicine and Pharmacy, Phone number: 054 3822873.

Thank you for your time and for helping us in our study.

Ngo Viet Quynh Tram

Appendix 5: Publish in vietnamese

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NGHIÊN CỨU Y HỌC

Journal of Medical Research

Supplement, Volume 68. N°3. May, 2010

SỐ ĐẶC BIỆT KỶ NIỆM
1000 NĂM THĂNG LONG - HÀ NỘI

TOÀN VĂN BÁO CÁO
HỘI NGHỊ KHOA HỌC CÔNG NGHỆ TUỔI TRẺ CÁC TRƯỜNG Y DƯỢC VIỆT NAM LẦN THỨ XV

BỘ Y TẾ - ĐẠI HỌC Y HÀ NỘI

MINISTRY OF HEALTH HANOI MEDICAL UNIVERSITY

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Summary

THE PROPORTION OF MALARIA PARASITE INFECTION AND KAP IN THE PEOPLE SLEEPING AT FIELD HUTS IN EA LOP COMMUNE, EA SOUP DISTRICT, DAKLAK PROVICE 2009

The proportion of malaria parasite infection and KAP in the people sleeping at field huts in Ea Lop commune, Ea soup district, Dak Lak province 2009 . Although malaria morbidity and mortality rates in central Vietnam have reduced considerably compared with the previous years, the risk of malaria infection is still high at the people sleeping at field huts. A cross-sectional study using blood examination and KAP survey conducted in Ea Lop commune was to determine proportion of malaria parasite infection and to evaluate KAP on malaria disease of the people sleeping at field huts. ***The results*** *of cross-sectional studies in April, 2009 showed that the infection of malaria parasite on the people sleeping at field huts was 5,81%, P.falciparum was dominant with 78.57% of malaria parasite total.The KAP survey showed that 74.27% of them believed that malaria could be transmitted through the bites of the mosquitoes. By using direct observation method, it showed that there were 58.41% of them sleeping under bed nets at night. There were only about 21.58% of them sleeping under bed nets during staying in the field huts with the interview method. The reasons why they don't sleep under bed nets were small houses (50.53%), lack of the bed nets (25.26%) and some others.In conclusion, we found that the risk of malaria infection on the people sleeping at field huts was high, the proportion of sleeping under bed nets was low. So we should apply appropriate malaria control solutions for these communities.*

BƯỚC ĐẦU TRIỂN KHAI PHƯƠNG PHÁP MODS ĐỂ NUÔI CẤY VI KHUẨN LAO VÀ PHÁT HIỆN VI KHUẨN LAO ĐỂ KHÁNG THUỐC TẠI BỘ MÔN VI SINH – TRUNG TÂM CARLO URBANI, TRƯỜNG ĐẠI HỌC Y DƯỢC HUẾ

Ngô Viết Quỳnh Trâm, Nguyễn Thị Châu Anh
Trường Đại Học Y Dược Huế

Mục tiêu: *Triển khai phương pháp MODS (Microscopic Observation Drug Susceptibility) để phát hiện vi khuẩn lao và lao đề kháng thuốc cùng một thời gian - một phương pháp chẩn đoán lao nhanh, nhạy, chính xác nhưng rẻ tiền - tại Bộ môn Vi sinh, Trung tâm Carlo Urbani, Trường Đại học Y Dược Huế.* ***Phương pháp nghiên cứu:*** *Các mẫu đàm dương tính với vi khuẩn kháng acid cồn (n=36) được cấy trên môi trường lỏng Middlebrook 7H9 theo phương pháp MODS và kỹ thuật phát hiện độ nhạy cảm với kháng sinh (INH: 0,1 và 0,4 µl/ml; rifampicin: 1 và 2 µl/ml). Kỹ thuật realtime PCR 16S được sử dụng để bổ sung kết quả nuôi cấy bằng phương pháp MODS.* ***Kết quả:*** *Thời gian trung bình của phương pháp MODS cho kết quả cấy dương tính và độ nhạy cảm với kháng sinh của vi khuẩn lao là 9 ngày (tối thiểu 7 ngày, tối đa 12 ngày). Trong số 36 mẫu đàm AFB dương tính có 33 mẫu (91,7%) cấy dương tính với Mycobacteria, 3 mẫu (8,3%) âm tính. Trong 3 mẫu âm tính có 2 mẫu dương tính với kỹ thuật realtime PCR 16S và 1 mẫu âm tính với realtime PCR 16S (vi khuẩn kháng acid cồn không phải lao). Trong số*

máy real time PCR (STRATAGENE)

● Buồng an toàn sinh học cấp 2 (Nuaire).

2.4. Tiến hành:

●Xử lý đờm bằng NaOH 4%.

● Nuôi cấy và làm test nhạy cảm kháng sinh theo phương pháp MODS [9, 10]: pha môi trường lỏng bằng Middlebrook 7H9 thêm OADC và PANTA theo quy trình đã được mô tả [guideline], nồng độ kháng sinh cuối cùng trong môi trường nuôi cấy là INH: 0,1 và 0,4 µg/ml, Rifampicin: 1 và 2 µg/ml. Với mỗi mẫu đờm, cấy vào 5 giếng của khay 24 giếng, trong đó 1 giếng chứa môi trường không có kháng sinh, 1 giếng chứa môi trường có INH 0,1µg/ml, 1 giếng chứa môi trường có INH 0,4 µg/ml, 1 giếng chứa môi trường có Rifampicin 1µg/ml và 1 giếng chứa môi trường có Rifampicin 2 µg/ml. Ủ 37°C, xem kết quả mọc hàng ngày dưới kính hiển vi đảo ngược vật kính 20X từ ngày thứ 4 đến ngày thứ 15, cách ngày từ ngày thứ 16 đến 25 và một tuần 2 lần từ ngày thứ 26 đến 40. Để tránh ngoại nhiễm và an toàn các khay giếng được bọc kín trong bao nilon có khóa.

Kết quả dương tính được xác định khi ở các giếng không có kháng sinh có sự tạo thành dây (cord), đặc tính mọc trong môi trường lỏng của vi khuẩn lao. Các vi khuẩn kháng acid cồn khác không phải lao khi mọc sẽ không tạo dây, trừ M. chelona có tạo dây nhưng sớm vào ngày thứ 5. Nấm và các vi khuẩn tạp nhiễm khác mọc rất sớm và làm đục các giếng. Trường hợp bị tạp nhiễm các mẫu đờm được xử lý và thực hiện lại. Nếu vi khuẩn mọc tạo dây chỉ ở giếng không có kháng sinh và không mọc ở giếng có kháng sinh chứng tỏ vi khuẩn nhạy cảm với kháng sinh, nếu vi khuẩn mọc tạo dây ở cả giếng có kháng sinh chứng tỏ vi khuẩn đề kháng với kháng sinh [2, 9].

● Tách chiết DNA từ mẫu đờm theo quy trình của QiaGen và thực hiện kỹ thuật real time PCR 16S theo quy trình của công ty Việt Á để bổ sung kết quả.

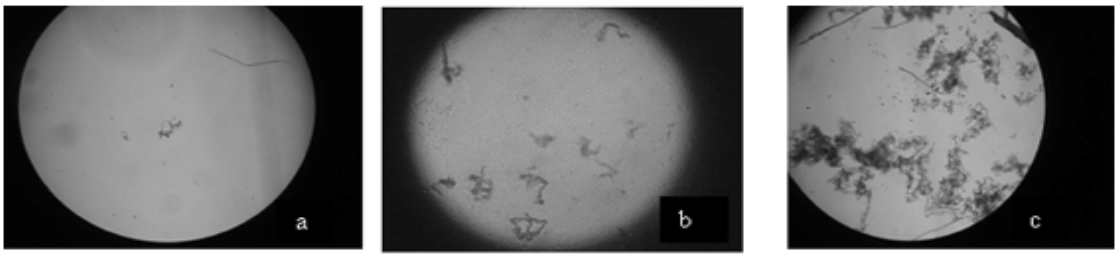
III. KẾT QUẢ

Qua thực hiện nuôi cấy theo phương pháp MODS và real time PCR cho 36 mẫu đàm AFB dương tính, chúng tôi có được các kết quả như sau:

Bảng 1. Tỷ lệ cấy dương tính theo phương pháp MODS:

Kết quả MODS	n	%
Dương tính	33	91,7
Âm tính	3	8,3
Tổng cộng	36	100

Trong số 36 mẫu đàm AFB dương tính, có 33 mẫu (91,7%) cấy dương tính với vi khuẩn lao, có 3 (8,3%) trường hợp cấy lao không mọc.



Hình 1: Kết quả cấy dương tính theo phương pháp MODS: vi khuẩn lao mọc tạo thành sợi xem sau 9 ngày (a), 12 ngày (b) và 18 ngày (c) từ một mẫu đàm AFB dương tính (vật kính X20)

Bảng 2: Kết quả cấy theo phương pháp MODS so với kết quả của real time PCR 16S

Real time PCR 16S / Kết quả cấy theo phương pháp MODS	Dương tính n	Âm tính n	Tổng cộng n
Dương tính	33	0	33
Âm tính	2	1	3
Tổng cộng	**35**	1	36

Ngo Viet Quynh Tram - Molecular epidemiology and phenotypic and genetic characterization of Mycobacteria isolated in central Vietnam - Ph.D thesis in Clinical and Molecular Microbiology of Ph.D School in Biomolecular and Biotechnological Science - University of Sassari

Tất cả 33 mẫu cấy dương tính đều có kết quả dương tính với kỹ thuật real time PCR 16S và PCR phát hiện thêm 2 mẫu dương tính trong 3 mẫu cấy âm tính. Độ nhạy phát hiện là 94,3% và độ đặc hiệu là 100%.

Bảng 3. Thời gian cấy dương tính theo phương pháp MODS:

Thời gian	Số lượng	Tỷ lệ %	Thời gian trung bình cấy dương tính
cấy dương tính			
Ngày thứ 5	0	-	
Ngày thứ 6	0	-	
Ngày thứ 7	10	30,3	
Ngày thứ 8	5	15,2	
Ngày thứ 9	6	18,1	
Ngày thứ 10	4	12,1	
Ngày thứ 11	3	9,1	ngày thứ 9
Ngày thứ 12	5	15,2	
Ngày thứ 13	0	-	
Ngày thứ 14	0	-	
Ngày thứ 15	0	-	
Ngày thứ 16-25	0	-	
Ngày thứ 26-40	0	-	
Tổng cộng	33	100%	

Trong số 33 mẫu cấy lao dương tính có 10 mẫu (30,3%) mọc vào ngày thứ 7; 5 mẫu (15,2%) mọc vào ngày thứ 8 và 12; 6 mẫu (18,1 %) mọc vào ngày thứ 9; 4 mẫu (12,1%) mọc vào ngày thứ 10 và 3 mẫu (9,1) mọc vào ngày thứ 11. Thời gian trung bình cấy dương tính là 9 ngày.

Bảng 4: Kết quả về độ nhạy cảm với kháng sinh của vi khuẩn lao cấy dương tính theo phương pháp MODS

Kháng sinh	Đề kháng (N=33)	
	n	%
INH 0,1 µg/ml	**1**	**3,0**
INH 0,4 µg/ml	**0**	-
Rifampicin 1 µg/ml	**8**	**24,2**
Rifampicin 2 µg/ml	**5**	**15,2**
INH và Rifampcin*	**4**	**12,1**

Trong 33 mẫu cấy lao dương tính, có 1 mẫu (3,0%) là vi khuẩn lao đề kháng chỉ với INH, 13 mẫu (39,4%) là vi khuẩn lao đề kháng chỉ với Rifampicin và 4 mẫu (12,1%) là vi khuẩn lao đa đề kháng (đề kháng với cả hai loại kháng sinh).

IV. BÀN LUẬN

Theo Moore và cs. phương pháp MODS được xem như là phương pháp nuôi cấy phát hiện vi khuẩn lao và lao đa đề kháng thuốc nhanh và nhạy hơn các phương pháp chuẩn vàng đang được sử dụng: độ nhạy phát hiện của MODS là 97,8%, của cấy tự động là 89,0% và của cấy trên môi trường Lowenstein-Jensen là 84,0% (p<0,001) [9]. Theo Arias và cs. phương pháp MODS có độ nhạy là 97,5% và độ đặc hiệu là 94,4% [1]. Kết quả của chúng tôi có độ nhạy phát hiện là 94,3% và độ đặc hiệu là 100%. Vì mới triển khai phương pháp MODS nên chúng tôi chỉ thực hiện với 36 mẫu đờm AFB dương tính, có 33 mẫu (91,7%) cấy mọc vi khuẩn lao, không mọc là 3 mẫu (8,3%). Vi khuẩn không mọc có thể vì đây là mẫu đờm của các bệnh nhân đã được điều trị và vi khuẩn đã bị chết nên không thể phát triển được mặc dù vẫn cho thấy hình ảnh AFB trên tiêu bản nhuộm Ziehl-Neelsen; nhưng cũng có thể AFB thấy trên tiêu bản không phải là vi khuẩn lao mà là một loại vi khuẩn kháng acid cồn khác. Bảng 2. cho thấy tất cả 33 mẫu cấy dương tính đều có kết quả dương tính

với kỹ thuật real time PCR 16S và PCR phát hiện thêm 2 mẫu dương tính trong 3 mẫu cấy âm tính. Kỹ thuật PCR phát hiện đoạn gen 16S đặc hiệu cho các vi khuẩn gây bệnh lao ở người (Mycobacterium tuberculosis complex) cho kết quả dương tính 35 mẫu (97,2%) và 1 trường hợp âm tính cùng với mẫu cấy MODS âm tính. Điều này có thể chỉ ra rằng trong 3 mẫu cấy âm tính có 2 mẫu có vi khuẩn lao đã chết (khả năng do được điều trị) và 1 mẫu có vi khuẩn kháng acid cồn nhưng không phải lao.

Thời gian dương tính trung bình cho kết quả mọc vi khuẩn lao của chúng tôi bằng phương pháp MODS là 9 ngày, kết quả của các nghiên cứu khác là 7 ngày [1],[9]. So với phương pháp cấy trên môi trường Lowenstein-Jensen là 21 ngày [1] và 26 ngày [9] thì phương pháp MODS cho kết quả nhanh hơn nhiều và so với phương pháp cấy tự động là 8 ngày [1] và 13 ngày [9], thì phương pháp MODS cho kết quả trong thời gian tương đương hoặc nhanh hơn vài ngày.

Trong 33 mẫu cấy lao dương tính, có 1 mẫu (3,0%) là vi khuẩn lao đề kháng với INH, 13 mẫu (39,4%) là vi khuẩn lao đề kháng với Rifampicin và 4 mẫu (12,1%) là vi khuẩn lao đa đề kháng. Ở Việt Nam tỷ lệ lao đa đề kháng trong số các trường hợp lao mới là 2,7% và trong số các trường hợp lao đã được điều trị trước đó là 19,3% [7]. Nghiên cứu của Moore trên đối tượng bệnh nhân nghi ngờ lao đã phát hiện 14,4% trường hợp lao kháng INH 0,4 µg/ml; 11,0% trường hợp lao kháng rifampicin và 10,5% trường hợp lao đa đề kháng [9]. Kết quả của chúng tôi khó có thể so sánh với kết quả của các nghiên cứu trên do số mẫu của chúng tôi còn quá ít, chúng tôi sẽ tiếp tục thực hiện trên lượng mẫu lớn hơn. Ngoài ra chúng tôi chưa có các chủng vi khuẩn lao chứng H37Rv là các chủng vi khuẩn lao đề kháng với một loại kháng sinh, chúng tôi đang liên hệ để có các chủng này để có thể có kết quả chính xác hơn.

Tuy nhiên, điều đáng quan tâm ở đây là thời gian cho kết quả độ nhạy cảm kháng sinh của vi khuẩn lao bằng phương pháp MODS rất sớm, nghiên cứu của chúng tôi là 9 ngày, của Moore là 7 ngày trong khi bằng phương pháp cấy máy tự động là 22 ngày và cấy trên môi trường Lowenstein-Jensen là 68 ngày [9].

V. KẾT LUẬN

Nuôi cấy 36 mẫu đờm AFB dương tính với phương pháp MODS cho kết quả cấy dương tính và độ nhạy cảm với kháng sinh của vi khuẩn lao nhanh (sau 9 ngày) bằng cách xem tính chất mọc tạo thành dây của vi khuẩn lao trong môi trường lỏng dưới kính hiển vi đảo ngược. Có 33 mẫu (91,7) cấy dương tính, 3 mẫu (8,3%) âm tính trong đó 2 mẫu dương tính với kỹ thuật realtime PCR 16S và 1 mẫu âm tính với realtime PCR 16S (AFB không phải lao). Trong số 33 mẫu cấy dương tính, có 1 mẫu (3,0%) là vi khuẩn lao đề kháng với INH, 13 mẫu (39,4%) là vi khuẩn lao đề kháng với Rifampicin và 4 mẫu (12,1%) là vi khuẩn lao đa đề kháng.

Phương pháp cấy MODS cho mẫu đờm cho phép chẩn đoán nhanh và nhạy, chính xác vi khuẩn lao và lao kháng thuốc, thích hợp cho những nơi không có điều kiện mua máy cấy tự động.

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Summary

SETTING-UP THE MODS ASSAY FOR DETECTING MYCOBACTERIUM TUBERCULOSIS COMPLEX AND DRUG RESISTANT TUBERCULOSIS AT DEPARTMENT OF MICROBIOLOGY - CARLO URBANI CENTRE, HUE COLLEGE OF MEDICINE AND PHARMACY

Subject: *Setting-up the MODS (Microscopic observation drug susceptibility) assay for detecting Mycobacterium tuberculosis complex bacteria and the drug susceptibility at the same time - a rapid, sensitive and specific low-cost method for microbiological diagnosis of tuberculosis– at the Department of Microbiology-Carlo Urbani Centre, Hue College of Medicine and Pharmacy.* ***Methods:*** *Microscopic positive samples of sputum (n=36) for AFB were cultured on Middlebrook 7H9 liquid medium according to the MODS method and, at the same time, drug-susceptibility testing (INH: 0,1 and 0,4 µl/ml; rifampicin: 1 and 2 µl/ml) was performed. A 16S realtime PCR for Mycobacterrium tuberculosis complex was used to complement the results of MODS..* ***Results:*** *The median time of MODS culture positivity and the results of susceptibility tests were 9 days (min 7 and max 12). Of 36 smear-positive samples, 33 samples (39.4%) yielded cultures positive for Mycobacterium tuberculosis complex and cultures were negative in 03 samples (8.3%). Of these 3 cullture negative, 2 samples were positive with 16S realtime PCR and 1 sample was negative with this technique (nontuberculous mycobacteria). Of 33 culture positive samples, resistance to INH alone was detected in 01 sample (3.0%); to rifampicin alone in 13 samples (39.4%), to INH and rifampicin (multidrug resistance) in 4 samples (12.1%).* ***Conclusions:*** *MODS culture of sputum samples offer rapid, sensitive and specific microbiological diagnosis of tuberculosis and multidrug-resistant tuberculosis and it is suitable for resource–limited environments.*


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Website: http://www.cimsi.org.vn và Email:xuatban@cimsi.org.vn

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NUÔI CẤY VI KHUẨN LAO VÀ PHÁT HIỆN LAO ĐA KHÁNG THUỐC BẰNG PHƯƠNG PHÁP MODS

Ngô Viết Quỳnh Trâm, Nguyễn Thị Châu Anh**, Lê Hữu Dũng**, Lê Nữ Xuân Thanh**, Piero Cappuccinelli****

TÓM TẮT

***Mục tiêu**: xác định tỷ lệ và thời gian cấy lao dương tính và phát hiện các trường hợp lao kháng thuốc và đa kháng thuốc. **Đối tượng và phương pháp**: mô tả cắt ngang, 201 mẫu đờm của 201 bệnh nhân (BN) có triệu chứng nghi lao hoặc được chẩn đoán lao nhưng chưa được điều trị, được nuôi cấy bằng phương pháp MODS (Microscopic Observation Drug Susceptibility Assay) và phát hiện độ nhạy cảm với kháng sinh (isoniazid [INH]: 0,1 và 0,4µg/ml; rifampicin: 1 và 2µg/ml), tại Bệnh viện Trường Đại học Y Dược Huế (153 BN) và Bệnh viện Trung ương Huế (48 BN), từ tháng 01/2009-4/2011. Sử dụng kỹ thuật realtime PCR 16S để kiểm tra kết quả nuôi cấy trong trường hợp dương tính trước 7 ngày hoặc âm tính nhưng AFB dương tính. **Kết quả**: tỷ lệ nuôi cấy MODS dương tính chung là 43,3% và trong các mẫu AFB dương tính là 90,6%. Thời gian nuôi cấy MODS dương tính trung bình là 9 ngày (ngày 5-14). Tỷ lệ lao kháng thuốc chung là 47,1% (đề kháng với INH là 5,7%, với rifamycin là 31% và lao đa kháng thuốc là 10,4%). **Kết luận**: MODS là phương pháp nuôi cấy vi khuẩn lao và phát hiện lao kháng thuốc nhanh, nhạy và rẻ tiền.*

SUMMARY

Culture of Mycobacterium tuberculosis and MDRTB detection by the MODS method

***Objectives**: to determine the growth rate of Mycobacterium tuberculosis (M. tuberculosis) and time taken for positive culturing and to detect the drug resistant and multidrug resistant to M.tuberculosis. **Methods**: a cross sectional study was carried out on 201 sputum samples from the suspected tuberculosis patients or the untreated tuberculosis ones of the Hospital of Hue College of Medicine and Pharmacy (153 patients), and the Tuberculosis Department of Hue Central Hospital (48 patients) from January 2009 to April 2011. Culturing and doing susceptibility test by the microscopic-observation drug-susceptibility (MODS) assay (isoniazid (INH): 0.1 and 0.4µg/ml; rifampicin: 1 and 2µg/ml). Real-time PCR (16S-rRNA fragment) was performed for the MODS culture-positive samples before 7 days and the samples with MODS culture-negative but AFB positive. **Results**: MODS cultured positive samples were 43.3% in total and 90.6% of the AFB positive cases. The median time to MODS positivity culture was 9 days (interquartile range 5-14). Drug resistant M.tuberculosis was 47.1% (the resistance to antibiotic INH alone was 5.7%, to rifampicin alone 31% and to INH and rifampicin (multidrug resistance) was 10.4%). **Conclusions**: the MODS assay is a rapid, sensitive, specific and relatively low cost culture method.*

I. ĐẶT VẤN ĐỀ

Tình hình lao kháng thuốc gia tăng đòi hỏi có những kỹ thuật phát hiện nhanh, rẻ và dễ dàng thực hiện. BN nhiễm các chủng vi khuẩn lao đa kháng thuốc (MDR-TB) hoặc siêu kháng thuốc (XDR-TB) cần điều trị bằng các thuốc kháng sinh thế hệ hai và thời gian điều trị kéo dài. Ngay cả khi điều trị đúng, MDR-TB và XDR-TB đôi khi vẫn cho kết quả xấu và thời gian lây nhiễm kéo dài hơn lao nhạy cảm với kháng sinh. Vì vậy, việc ngăn ngừa sự lây lan của MDR-TB và XDR-TB rất quan trọng. Xác định sớm những trường hợp này là bước đầu tiên và quan trọng nhất [5]. Các phương pháp xác định độ nhạy cảm kháng sinh trên môi trường đặc (Lowenstein Jensen) thường được sử dụng có giá rẻ, nhưng cho kết quả chậm hoặc trong môi trường lỏng bằng máy tự động (như BACTEC MGIT 960) cho kết quả nhanh, nhưng đòi hỏi phải có máy móc và đắt tiền [7]. Các phương pháp sinh học phân tử cũng cho kết quả nhanh, nhưng đắt tiền và đòi hỏi nguồn nhân lực được đào tạo tốt. Ngoài ra, không phải tất cả trường hợp đột biến liên quan đến kháng thuốc đều được biết [5].

MODS là phương pháp xác định độ nhạy cảm với kháng sinh của vi khuẩn lao bằng kính hiển vi, dựa trên 3 nguyên lý cơ bản:

*ThS; **BS; ***GS.TS., Bộ môn Vi sinh - Trung tâm Carlo Urbani – Trường Đại học Y Dược Huế

Ngo Viet Quynh Tram - Molecular epidemiology and phenotypic and genetic characterization of Mycobacteria isolated in central Vietnam - Ph.D thesis in Clinical and Molecular Microbiology of Ph.D School in Biomolecular and Biotechnological Science - University of Sassari

(1.) Vi khuẩn lao mọc nhanh hơn trong môi trường lỏng so với ở môi trường đặc; (2.) Có thể xem dưới kính hiển vi sự tạo thành dây của vi khuẩn lao ở môi trường lỏng trong thời gian sớm; (3.) Cùng với sự phát triển của vi khuẩn lao, cho phép phát hiện nhanh tính nhạy cảm với kháng sinh của vi khuẩn. Đây là phương pháp giúp phát hiện vi khuẩn lao và lao kháng thuốc trong đờm nhanh, nhạy, rẻ tiền, dựa vào tính chất mọc của vi khuẩn lao [2,9]. Vì vậy, chúng tôi tiến hành nghiên cứu này, nhằm xác định tỷ lệ và thời gian nuôi cấy lao dương tính và phát hiện các trường hợp lao kháng thuốc và đa kháng thuốc.

II. ĐỐI TƯỢNG VÀ PHƯƠNG PHÁP

1. Đối tượng nghiên cứu

201 mẫu đờm của 201 BN có triệu chứng nghi lao hoặc được chẩn đoán lao, nhưng chưa được điều trị. Trong đó, 153 mẫu là của các BN ở Bệnh viện Trường Đại học Y Dược Huế và 48 mẫu là của BN ở Bệnh viện Trung Ương Huế, từ tháng 01/2009-4/2011.

2. Phương pháp nghiên cứu

2.1. Thiết kế nghiên cứu: mô tả cắt ngang.

2.2. Vật liệu nghiên cứu:

* Môi trường, hóa chất:

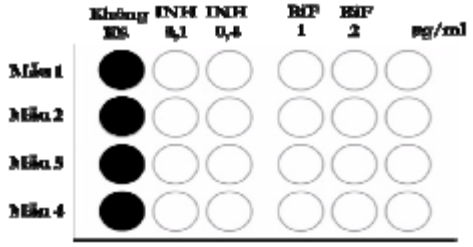
- NaOH 4%, dung dịch đệm photphat PBS 1X để xử lý đờm.
- Môi trường lỏng Middlebrook 7H9 (Becton Dickinson), OADC (oxalic acid, albumin, dextrose và catalase) (Becton Dickinson), PANTA (polymyxin, amphotericin B, acid nalidixic, trimethoprim và azlocillin) (Becton Dickinson) để nuôi cấy vi khuẩn lao.
- INH (Sigma-Aldrich), rifampicin (Sigma-Aldrich) để phát hiện độ nhạy cảm kháng sinh của vi khuẩn lao.
- Bộ kít tách chiết ADN (QiaGen), hai cặp primer IS1, IS2 và IS3, IS4 (IDT) đặc hiệu cho đoạn gen IS6110 của vi khuẩn lao, PCR Mastermix (2X), dung dịch tải loading và thang chuẩn ADN vạch 100bp (đôi base) của Fermentas, dung dịch điện di TBE (BioRad), agarose (BioRad), ethidium bromide (Merk).

* Dụng cụ và phương tiện:

- Ống quay ly tâm 1,5ml và 50ml, ống nghiệm chứa môi trường, khay 24 giếng, pipet định mức 2ml và 5ml, pipet Pasteur 3ml, bộ micropipet và các đầu micropipet có lọc với các thể tích khác nhau, lam kính.
- Kính hiển vi quang học vật kính dầu, kính hiển vi đảo ngược, tủ ấm 37^{0}C.
- Máy quay ly tâm (Hettich) và siêu ly tâm 13.000 vòng/phút (Eppendorf), máy ủ nhiệt (Eppendorf), máy real time PCR (Stratagene Mx3000).
- Buồng an toàn sinh học cấp 2 (Nuaire).

2.3. Tiến hành:

- Xử lý đờm bằng NaOH 4%.
- Nuôi cấy và thực hiện kỹ thuật nhạy cảm kháng sinh bằng phương pháp MODS, theo quy trình được thực hiện tại Trung tâm Carlo Urbani [1]. Nồng độ cuối cùng của INH là 0,1µg/ml và 0,4µg/ml và của rifampicin là 1µg/ml và 2µg/ml.



Hình 1: Sơ đồ khay giếng nuôi cấy bằng phương pháp MODS

- Tách chiết ADN từ mẫu đờm sau khi đã xử lý theo quy trình của QiaGen và thực hiện kỹ thuật PCR IS6110, theo quy trình được thực hiện tại Trung tâm Carlo Urbani cho những trường hợp cấy MODS dương tính ≤7 ngày hoặc cấy MODS âm tính nhưng AFB dương tính để kiểm tra.
- Xác định AFB dương tính bằng phương pháp nhuộm Ziehl-Neelsen.

2.4. Xử lý số liệu: bằng phần mềm Microsoft excell 2007.

III. KẾT QUẢ

Trong số 201 mẫu đờm của BN, có 96 mẫu AFB (+) (47,8%).

Qua thực hiện nuôi cấy theo phương pháp MODS và nested PCR IS6110 cho 201 mẫu đờm, chúng tôi thu được các kết quả sau:

1. Kết quả cấy MODS dương tính

Bảng 1: Tỷ lệ cấy dương tính theo phương pháp MODS

MODS	BV Trường*		BV TW**		Chung	
	SL	TL%	SL	TL%	SL	TL%
Dương tính	55	36	32	66	87	43,3
Âm tính	98	64	16	34	104	56,7
Tổng	153	100	48	100	201	100

*(*Bệnh viện Trường Đại học Y Dược Huế; **Bệnh viện Trung ương Huế)*

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Tỷ lệ nuôi cấy MODS dương tính chiếm 43,3%. Trong đó, của Bệnh viện Trung ương Huế là 66%, Bệnh viện Trường Đại học Y Dược Huế là 36%.

Bảng 2: Tỷ lệ cấy MODS dương tính trong số các mẫu AFB (+)

MODS	BV Trường*		BV TW**		Chung	
	SL	TL%	SL	TL%	SL	TL%
Dương tính	55	93,2	32	86,5	87	90,6
Âm tính	4	6,8	5	13,5	9	9,4
Tổng	59	100	37	100	96	100

*(*Bệnh viện Trường Đại học Y Dược Huế; **Bệnh viện Trung ương Huế)*

Có 87 trường hợp MODS dương tính trong số 96 mẫu AFB dương tính (90,6%). Tỷ lệ cấy MODS dương tính trong số các mẫu AFB (+) của Trường Đại học Y Dược Huế cao hơn Bệnh viện Trung ương Huế (93,2% so với 86,5%).

Bảng 3: Thời gian cấy dương tính theo phương pháp MODS

Thời gian	SL	TL%	Thời gian trung bình (ngày)
Ngày thứ 5	7	8,0	8,58±2,24
Ngày thứ 6	8	9,2	
Ngày thứ 7	16	18,4	
Ngày thứ 8	12	13,8	
Ngày thứ 9	11	12,6	
Ngày thứ 10	20	23,0	
Ngày thứ 11	2	2,3	
Ngày thứ 12	4	4,6	
Ngày thứ 13	5	5,7	
Ngày thứ 14	2	2,3	
Ngày thứ 15	0	-	
Ngày thứ 16-25	0	-	
Ngày thứ 26-40	0	-	
Tổng	87	100	

Thời gian cấy dương tính trung bình là 8,58±2,24 ngày. Trong số 87 mẫu cấy dương tính, có 20 mẫu (23%) mọc vào ngày thứ 10, 16 mẫu (18,4%) mọc ngày thứ 7, 23 mẫu (26,4%) mọc ngày thứ 8 và 9. Thời gian mọc sớm nhất là 5 ngày, muộn nhất là 14 ngày.

Bảng 4: Kiểm tra kết quả bằng kỹ thuật nested PCR IS6110

Thông số kiểm tra	(+)		(-)	
	SL	TL%	SL	TL%
Kết quả MODS dương tính trước 7 ngày	31	100	0	0
MODS âm tính nhưng AFB (+)	9	100	0	0

Tất cả các mẫu cấy MODS dương tính sớm trước 7 ngày và các mẫu cấy MODS không mọc nhưng AFB (+) đều cho kết quả PCR dương tính với vi khuẩn lao.

2. Tính nhạy cảm với kháng sinh

Bảng 5: Tính nhạy cảm với kháng sinh

Kháng kháng sinh		SL	TL%
Sự nhạy cảm	Kháng	41	47,1
	Nhạy cảm	46	52,9
Phân bố	Kháng với INH 0,1µg/ml	1	1,1
	INH 0,4µg/ml	4	4,6
	Kháng với rifampicin 1µg/ml	16	18,4
	Rifampicin 2µg/ml	11	12,6
	Đa kháng thuốc*	9	10,4
	Nhạy cảm	46	52,9
Tổng		87	100

*(*Kháng với cả 2 loại kháng sinh ở bất kỳ nồng độ nào)*

Có 41 trường hợp kháng kháng sinh (kháng INH hoặc kháng rifampicin hoặc đa kháng) (47,1%). 5 mẫu kháng INH (5,7%), 27 mẫu kháng rifamycin (31%), 9 mẫu (10,4%) đa kháng thuốc.

IV. BÀN LUẬN

1. Kết quả cấy MODS dương tính

Tỷ lệ cấy MODS dương tính của chúng tôi là 43,3% trong tất cả 201 mẫu đờm. Tỷ lệ dương tính ở mẫu của Bệnh viện Trung ương Huế cao hơn Bệnh viện Trường Đại học Y Dược Huế (66% so với 36%), do đối tượng đến khám và điều trị tại Bệnh viện Trung ương Huế là những người có triệu chứng rõ hoặc đã được chẩn đoán lao.

Phương pháp MODS được xem là một phương pháp nuôi cấy phát hiện vi khuẩn lao và lao đa đề kháng thuốc nhanh, nhạy (theo nghiên cứu của Arias M. và cộng sự là 97,5% [2], Moore D.A.J và cộng sự là 97,8% [9]) và đặc hiệu cao (theo Arias M. và cộng sự là 94,4%) [2]. Nghiên cứu của Ejigu G.S. và cộng sự trên 262 BN lao phổi AFB (+) tại Ethiopia cho thấy, phương pháp MODS dương tính trên 254 trường hợp (96,9%) [4]. Có 87 trường hợp MODS dương tính trong số 96 mẫu AFB (+) (90,6%) (bảng 2). Tỷ lệ cấy MODS dương tính trong số các mẫu AFB (+) của Bệnh viện Trường Đại học Y Dược Huế cao hơn Bệnh viện Trung ương Huế (93,2% so với 86,5%), không loại trừ khả năng trong mẫu nghiên cứu có lẫn vào một số BN lao đã được điều trị.

Theo kết quả nghiên cứu của chúng tôi, thời gian nuôi cấy của phương pháp MODS trung bình là 9 ngày (8,58±2,24). Thời gian sớm nhất là 5 ngày và muộn nhất là 14 ngày. Kết quả của Moore D.A.J. (2007) về thời gian trung bình của MODS là 7 ngày, BACTEC-MGIT là 13 ngày và cấy trên môi trường Lowenstein-Jensen là 26 ngày [11]. Nghiên cứu của Arias

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M. và cộng sự (2010) cũng cho kết quả MODS là 7 ngày (5-10 ngày), nhưng BACTEC-MGIT là 8 ngày và nuôi cấy trên môi trường Lowenstein-Jensen là 21 ngày [2]. Kết quả cấy MODS của chúng tôi tương đương với kết quả cấy MODS trong nghiên cứu của Ejigu G.S. và cộng sự (9 ngày) [3]. Như vậy, thời gian cho kết quả của phương pháp MODS là khá nhanh. Nếu so với thời gian của phương pháp nuôi cấy trên môi trường Lowenstein-Jensen thì rất có ý nghĩa trong việc chẩn đoán sớm lao và lao kháng thuốc. Chi phí cho mỗi mẫu bằng phương pháp này cũng rất thấp [9].

Những trường hợp mọc sớm trước ngày thứ 5 có thể không phải vi khuẩn lao, mà là các trực khuẩn kháng acid cồn khác [11]. Giá trị của PCR không phụ thuộc vào vi khuẩn còn sống hay đã chết, mà chỉ cần có vi khuẩn lao ở trong mẫu đờm. Kiểm tra bằng kỹ thuật nested PCR IS6110 cho 31 mẫu cấy MODS mọc sớm trước 7 ngày và 9 mẫu không mọc nhưng AFB (+) đều cho kết quả dương tính, cho phép xác định tất cả các mẫu này đều có vi khuẩn lao.

2. Tính nhạy cảm với kháng sinh

Trong 87 mẫu dương tính với MODS thì tỷ lệ kháng kháng sinh chiếm 47,1% (41 mẫu). Trong đó, 5 mẫu (5,7%) kháng với INH, 27 mẫu kháng với rifampicin (31%) và 9 mẫu (10,4%) đa kháng thuốc. Theo Tổ chức Y tế Thế giới và Chương trình Chống lao Quốc gia, ở Việt Nam, tỷ lệ MDR-TB là 2,7% trong số những trường hợp lao mới và 19,2% trong số những trường hợp lao được điều trị [12]. Trên thế giới, năm 2006, tỷ lệ MDR-TB chung trong tất cả các trường hợp mắc lao là 20%, kháng với INH là 13,3% và đa kháng thuốc là 5,3% [12]. Nghiên cứu của Moore và cộng sự (2006) trên BN nghi bị lao, đã phát hiện 34,9% kháng thuốc, trong đó, 14,4% kháng INH với 0,4µg/ml, 11% kháng với rifampicin và 10,5% trường hợp lao đa kháng thuốc [10].

V. KẾT LUẬN

Thực hiện phương pháp cấy MODS cho 201 mẫu đờm, chúng tôi xác định được tỷ lệ cấy MODS dương tính chung là 43,3% và trong các mẫu AFB (+) là 90,6%. Thời gian cấy dương tính trung bình là 9 ngày (5-14 ngày). Tỷ lệ lao kháng thuốc chung là 47,1%, trong đó, kháng INH là 5,7%, kháng rifamycin là 31,0% và MDR-TB là 10,4%. Do đó, MODS là phương pháp nuôi cấy vi khuẩn lao và phát hiện lao kháng thuốc nhanh, nhạy và rẻ tiền.

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the qualification of any other degree or diploma of a university or other insititution of higher learning, except where due acknowledgement is made in the acknowledgements

Name: **Ngo Viet Quynh Tram** Signed:

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