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GENOTYPIC CHARACTERISTICS IN *RPOB* AND *KATG* OF RIF-AND/OR INH-RESISTANT *MYCOBACTERIUM TUBERCULOSIS* ISOLATES IN CENTRAL VIETNAM

Tutors:
Professor. Paola Molicotti
Associate Professor. Ngo Viet Quynh Tram

Doc. NGUYEN THI BINH NGUYEN
Ph.D Thesis

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LIST OF ABBREVIATIONS

AFB : Acid-fast bacillus
BCG : Bacille Calmette-Guérin
bp : base-pair
CSF : Cerebral spinal fluid
DST : Drug susceptibility testing
EMB : Ethambutol
IFN : Interferon
katG : catalase-peroxidase
GE : Genomic Equivalents
LAM : Latin American and Mediterranean
MGB : Minor Groove Binder
MDR : Multidrug-resistant
MDR-TB: Multidrug-resistant tuberculosis
MIC : Minimum Inhibitory Concentration
MODS : Microscopic-Observation Drug-Susceptibility
MTB : *Mycobacterium tuberculosis* complex
NCBI : National Center for Biotechnology Information
NPV : Negative Predictive Value
NTP : The National Tuberculosis Program
OADC : Oxalic acid, albumin, dextrose, and catalase
PANTA : Polymyxin, Amphotericin B, Nalidixic Acid,
PCR : Polymerase Chain Reaction
PM : Proportional method
PPV : Positive Predictive Value
RD : Direct Repeat
REMA : Resazurin Microtiter Assay
RFLP : Restriction fragment length polymorphism
rpoB : β subunit of DNA dependant RNA polymerase
RRDR : RIF-resistance determining region
SNP : Single-nucleotide polymorphism
ST : shared type
STR : Streptomycin
TB : Tuberculosis
WHO : World Health Organization
XDR-TB : Extensively drug- resistant tuberculosis
ZN : Ziehl-Neelsen stain

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ABSTRACT

Tuberculosis (TB) is a health burden over the world and one of the most challenges in TB treatment is drug-resistance tuberculosis (DR-TB). Therefore, developing methodologies for TB and DR-TB diagnosis is at the central of TB's management strategy. Vietnam is in the group of 30 countries in which the annual TB new cases is highest globally and the research on TB in Vietnam is in emergency despite many efforts in TB management during the last decade.

This study aimed to evaluate the frequency of DR-TB to Rifampicin and Isoniazid- the two important first-line drugs in TB treatment. Resazurin microtiter assay plate (REMA) - a culture-based phenotypic drug susceptibility testing (DST) was applied in this task. Then we designed a PCR TaqMan probes panel to identifying the DR-TB- associated mutations in *rpoB* and *katG* in cultured TB strains from patient's samples and clinical specimens.

In this study, a total of 468 *M. tuberculosis* isolates were subjected by REMA, 106/468 (22.6%) was resistant isolates; it was observed that of these, 69% (74 strains) were resistant to INH, while 0.94% (1 strain) was resistant to RIF and 29.24% (31 strains) were resistant to both antibiotics (MDR-TB strains). Comparing the BACTEC MGIT 960 system DST, the sensitivity for INH resistant and RIF resistant were 100% and the specificity was 99.19 % and 99.54%, respectively.

Real-time PCR TaqMan allelic discrimination assay was performed to detect the mutations in *rpoB* and *katG* of 52 *M. tuberculosis* clinical isolates and 52 *M. tuberculosis* clinical samples using three probes labelled with fluorophores. A total of 19/41(46.34%) of the phenotypic INH resistant isolates had the mutation at codon Ser315Thr (AGC→ACC). Eight different mutations were detected in the rifampicin resistance determining region (RRDR) of *rpoB* which were found in 67.7% (22/32) resistance isolates to Rifampicin. The mutations at codons 531, 526 and 516 were 37.5% (12/32), 18.75% (6/32) and 6.25% (2/32), respectively. Susceptible isolates had no mutations in *katG* and *rpoB*. Comparing DNA

sequencing results with the sensitivity and specificity of Isoniazid resistance detection by analysing *katG* codons 311-316 on the real-time PCR allelic and DNA sequencing results were found 95% and 100%, respectively.

At the same time, the sensitivity and specificity detection by mutations analysis in the RIF-resistance determining region (RRDR) of *rpoB* to RIF-resistance by the real-time PCR allelic and DNA sequencing results, there were found 95.5% and 100%, respectively. Using real-time PCR TaqMan probe for detecting mutations in the *rpoB* and *katG* of *M. tuberculosis* from clinical samples with the sensitivities 100% of *katG* and *rpoB*, the specificities were 96.88% of the *rpoB* and 93.94% of the *katG*.

This study results suggest a potential application of real-time PCR TaqMan allelic discrimination in TB/DR-TB diagnosis which will not replace the culture-based method and conventional DST but will allow more rapid detection method for a large-scale screening.

1. INTRODUCTION

1.1 Tuberculosis

1.1.1. Briefly history of tuberculosis

Tuberculosis (TB) is an old infectious disease caused by the *Mycobacterium tuberculosis (M. tuberculosis),* which manifests as a multisystemic mortal disease worldwide for thousands of years and has been one of the top 10 causes of death worldwide since 2007 [1]. TB is the leading cause, ahead of HIV/AIDS responsive for deaths caused by infectious disease [1]. Nowadays, there are tremendous advances in biomedical research, TB infection rates have dropped dramatically until almost eradicated. However, TB is still a significant health burden in many parts of the world, including developing countries like Vietnam. Not only in a large number of accumulated TB patients year by year, the increasing prevalence of drug-resistant and multidrug-resistant TB is a major challenge in treatment in these countries [1]. Therefore, strategies for TB prevention, treatment and research remain an urgent obstacle for global healthcare.

According to archaeological studies on human skeletons, tuberculosis is a mysterious disease that has appeared and affected human health for thousands of years. Tuberculosis was discovered except before BC in India, Egypt, Greece and the countries of Central Asia. Until 1882, the cause of tuberculosis was discovered when Dr Robert Koch announced the finding of a bacillus called *Mycobacterium tuberculosis* [2,3].

Since the discovery of *Mycobacterium tuberculosis* until now, TB research has been continuously carried out and developed into methods of diagnosis, prevention and treatment. In 1908, Mantoux optimized a method of subcutaneous tuberculin injection to detect tuberculosis allergy. During the period from 1908-1919, Léon Charles Albert Calmette and Jean-Marie Camille

Guérin generated an attenuated strain of *Mycobacterium bovis* called Bacillus Calmette- Guérin or BCG by multiple passing the original strain [2, 3].

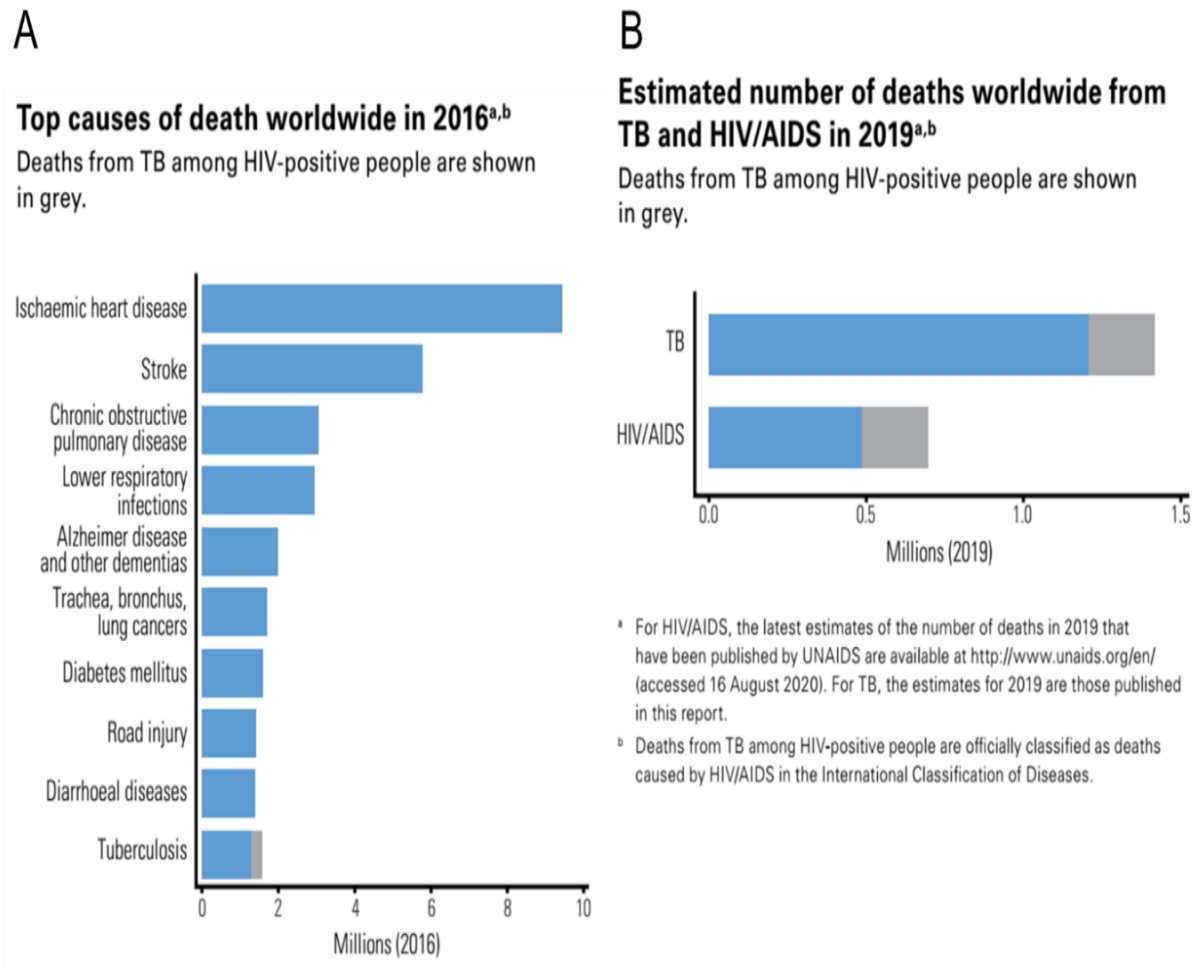

Figure 1: (A) Top causes of death worldwide in 2016. (B) Estimated number of deaths worldwide from TB and HIV/AIDS in 2019 [1].

BCG has developed 100 years ago and was first tested as a TB vaccine in humans in 1921. Interestingly, the BCG is the only licensed vaccine to prevent TB and continues to be widely used for childhood immunization nowadays. There is still no efficient vaccine to prevent adult TB before or after exposure to a TB infection [1].

TB drugs have also been focused on development continuously since World War II because TB bacteria showed rapid drug resistance. First, streptomycin, an antibacterial agent extracted from the fungus Streptomyces griseus in 1944. Then, it was first tested by Selman A. Waksman and Albert Schart in the

treatment of tuberculosis in humans in Europe and the United States in late 1944[4]. Although TB patients initially reported significant improvement within the first few months of streptomycin therapy, resistance began to emerge. Many trials of new tuberculosis drugs such as para-aminosalicylic acid (PAS), isoniazid or a combination of the two and streptomycin were conducted soon after 1944 [5]. Along with that, many anti-tuberculosis drugs were also tested. were found as rifampicin, pyrazinamide, ethambutol, cycloserine and ethionamide [5]. Anti-TB drug regimens together and the TB vaccination with the BCG vaccine have reduced the TB burden worldwide in many ways. Despite this, the increasing incidence of drug-resistant TB and transmission of those strains bring many work remains to be done to reduce TB globally.

M. tuberculosis can be transmitted from person to person through airborne droplets containing the bacteria. Each of these droplets has a diameter of 1-5μm and for each cough, a person with active pulmonary TB can release 3000 infectious particles. Only about 10 TB bacilli entering the lungs are sufficient for initial infection [6]. However, after entering the airspaces of the lung, *M. tuberculosis* activates immune responses and can be (i) destroyed, (ii) inhibited to an inactive form called latent tuberculosis infection (LTBI) or (iii) develop tuberculosis [7]. TB bacteria grow mainly in the lungs and up to 85% of TB patients develop pulmonary tuberculosis [1]. In addition to the lungs, they can also develop in other organs such as the mediastinal, retroperitoneal, cervical (scrofula) lymph nodes, meninges, bone or GI tract [7,8]. In these organs, *M tuberculosis* induces immune reaction to the antigenic cell wall proteins and causes tissue damage, then cause lymph node tuberculosis, tuberculous pleural effusion, tuberculous pericardial effusion, tuberculous peritoneal effusion, tuberculous encephalomyelitis, tuberculosis of the joints and bones or urinary and genital tuberculosis. [7,9].

M. tuberculosis proliferates rapidly in subpleural regions of the lung of a typical active TB patient. The standard TB lesion is an epithelioid granuloma with

central caseation necrosis within alveolar macrophages. Then, the bacillus spreads to the surrounding lymphatics vessel to a hilar node, induce host immune responses by recruiting lymphocytes, plasma cells and macrophages to form early tubercles.

Name	Clinical form
Phthisis	Original Greek name for TB
Lung Sickness	TB
Consumption	TB
Lupus vulgaris	TB of the skin
Mesenteric disease	TB of the abdominal lymph nodes
Pott's disease	TB of the spine
Scrofula	TB of the neck lymph nodes
King´s evil	TB of the neck lymph nodes
White Plague	TB especially of the lungs
White swelling	TB of the bones
Milliary TB	Disseminated TB

Table 1: Disease names related to different clinical forms of TB [9]

The initial nodules with a size of 0.5-3.0mm usually have 3-4 regions, which are (i) central necrotic zone (ii) intracytoplasmic region consisting of macrophages and Langhans giant cells, (iii) middle zone with immature epithelial cells, lymphocytes, and macrophages and (iv) outer fibrous rim [7].

This structure causes the central necrotic zone to have a high concentration of fatty acids, low pH and inhibits the growth of tuberculosis bacteria [7]. The initial small lesions may heal or the infection is suppressed and becomes latent. However, when the enzymes hydrolyze the fibrosis, the nodule dissolves, causing larger areas of damage. These large lesions are often filled with TB bacilli and can be reactivated for a long time. Submucosal granulomas can rupture into the pleural or pericardial space, and cause an effusion. If not treated in time, TB bacteria will multiply and spread into the surrounding environment [7,9]. A small percentage (5-10%) of people infected with TB will develop active

TB during life, but this rate is much higher in certain groups of people such as HIV-positive people or people with other diseases [1,7,9]. Risk factors such as undernutrition, diabetes, smoking and heavy use of alcoholic beverages[1,9]. Diagnostic tests for the presence of TB bacteria may include sputum smear microscopy, rapid molecular tests, and culture-based methods. If left untreated, the rate of mortality in TB patients is very high. Research shows that, before the appearance of anti-TB drugs, 70% of sputum smear-positive people died within 10 years [9].

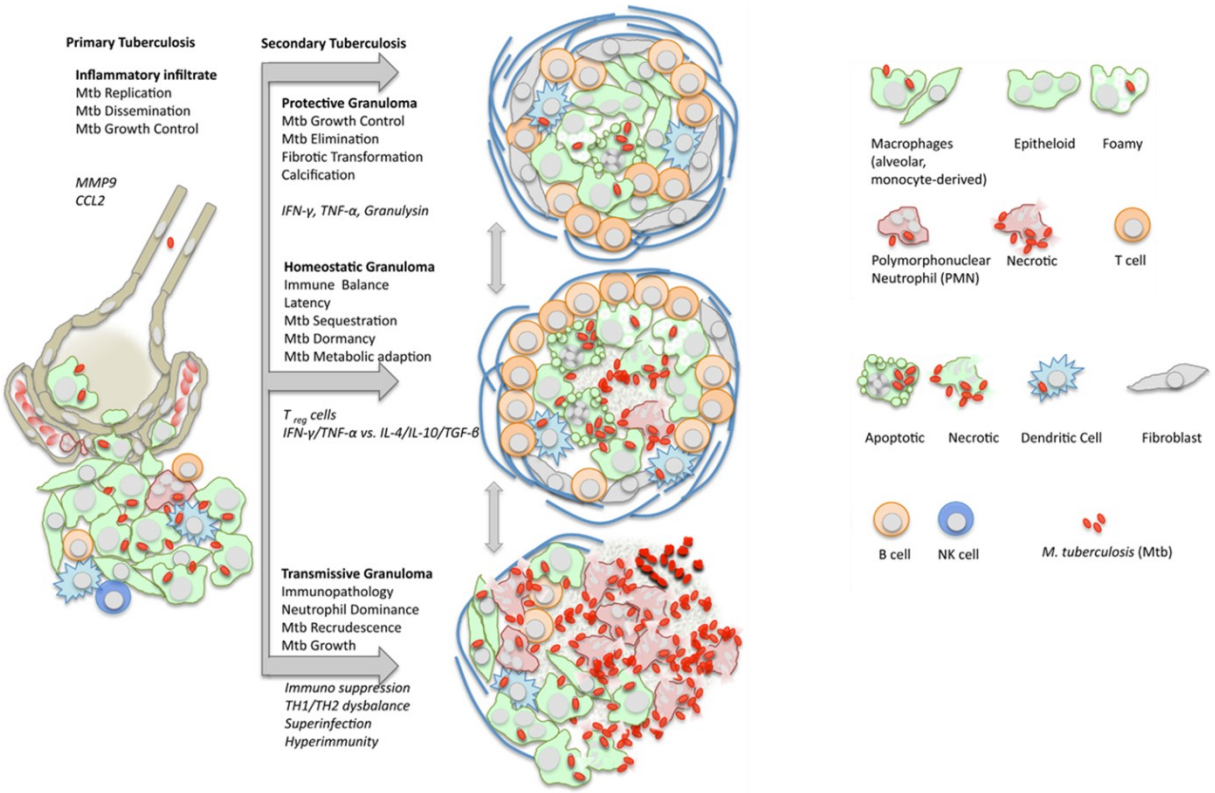

Figure 2. Dynamics of granuloma formation and pathology in tuberculosis [8]

1.1.2. Global burden of tuberculosis

According to the estimated data by the World Health Organization (WHO), about a fourth of the global population has been infected with *M. tuberculosis,* and about 10% of them develop TB. The estimated TB incidence was generated by (i) case notifications with expert opinion, (ii) case notifications with standard adjustment, (iii) results from inventory studies and (iv) prevalence survey. In

2019 alone, the world has about 10.0 million more people infected, and 1.2 million people die from TB. People infected with *Mycobacterium tuberculosis* are mainly in Southeast Asian countries (accounting for 44%) and Africa (25%) [1]. Specifically, the total number of TB cases in India, Indonesia, China, the Philippines, Pakistan, Nigeria, Bangladesh and South Africa accounts for two-thirds of cases worldwide. Meanwhile, North America and Europe have almost eliminated TB with less than 10 cases per 100,000 populations per year. Therefore, TB is also seen as a disease of poverty and economic distress [1].

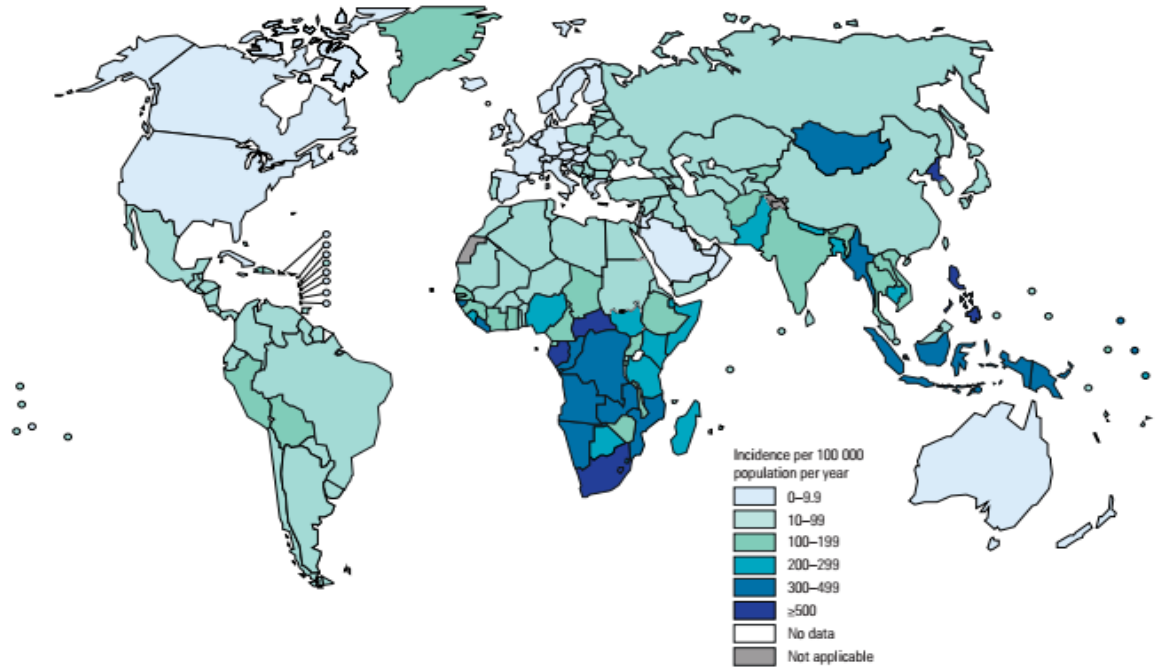

Figure 3: Estimated TB incidence rates, 2019[1].

Tuberculosis can affect anyone in any region, but people who get TB are mostly adults. Among them, men accounted for 56%, women accounted for 32% and children under 15 years old accounted for 12% [1,9]. The data show that TB affects more men than women, and the difference between the estimated data and the number of cases detected and recorded is also more significant in men than in women in adulthood. The male: female ratio in TB incidents in all ages ranged from 1.3 to 2.1 but in children under 15, the global male: female is close to 1 [1].

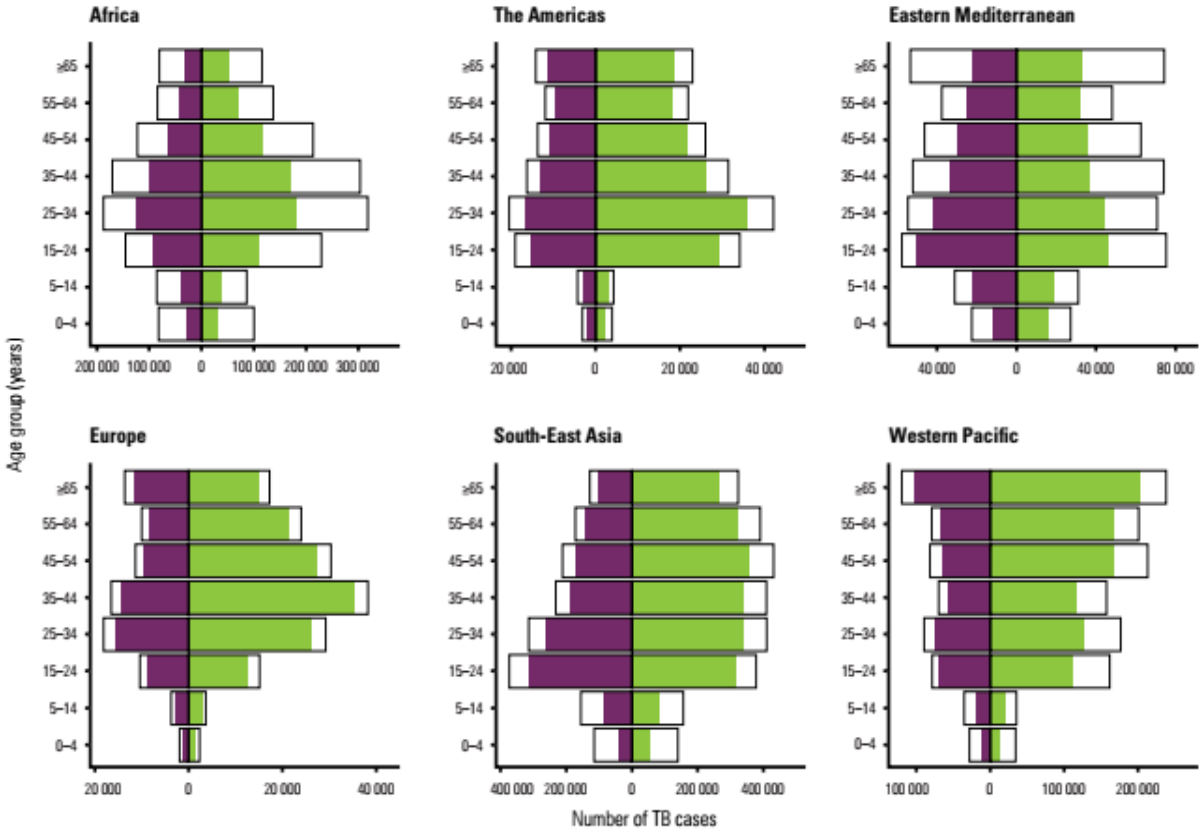

Figure 4: Regional estimates of TB incidence (black outline) and case notifications disaggregated by age and sex (Female in purple, male in green), 2019[1]

The TB incidence rate shows a tendency to decrease slowly globally, with an average rate of 1.7% per year in the 2000-2019 periods. The cumulative reduction between 2015 and 2019 was 9% and much too slow to reach the milestone of a 20% reduction in this period as agreed in the long-term vision of WHO's End TB Strategy. In particular, the data showing an increasing trend in TB relapse shows a severe scenario of drug resistance in TB. In addition to the heavy impact of the COVID-9 pandemic, the global TB situation may become more severe, and the goals are challenging to achieve.

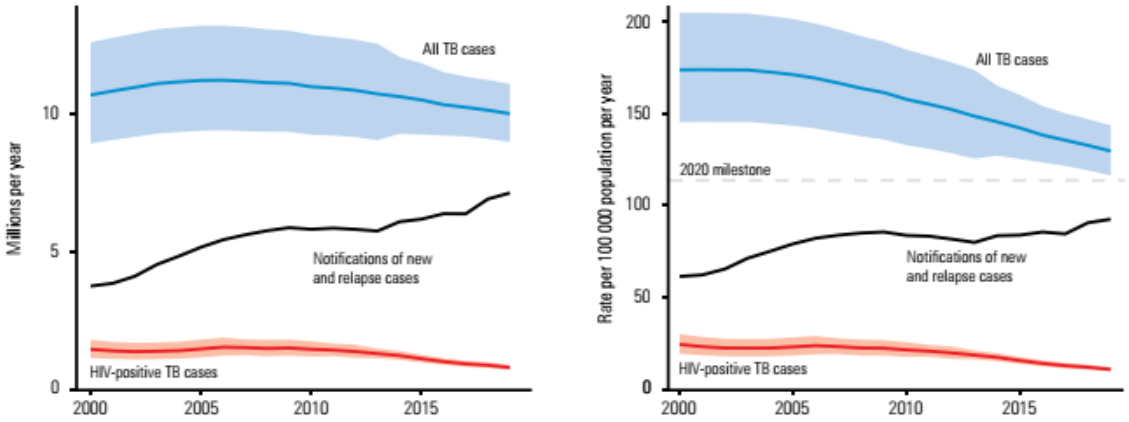

Figure 5. Global trends in the estimated number of incident TB case (left) and the incidence rate (right), 2000-2019. *Shade areas represent uncertainty intervals. The horizontal dashed line shows the 2020 milestone of the End TB Strategy* [1].

1.1.3. Tuberculosis in Vietnam

Vietnam is one of 30 countries with the highest number of annual new reported TB cases and deaths globally. According to a WHO report in 2019, Vietnam is estimated to have a total of 170.000 TB cases, of which the number of HIV-positive TB incidence is 5.500 cases. The incidence rate per 1.000.000 populations of total TB incidence and HIV-positive TB incidence are 176 and 5.8, respectively. Of the new cases, men accounted for 71% of the total, women 28% and children 0-14 years old accounted for 1% [1].

In 2018, 100% of relapse cases after treatment were resistant to at least rifampicin, and the cure rate of this group was only 85%. The total number of deaths nationwide is estimated at 11,400 cases in 2019, accounting for 11.8 per 100.000 populations. However, the detection and reporting rate of new TB patients with the national TB program and putting them on treatment is only 60% of the estimated total, at about 100.000 per year. Thus, there are many people with TB in the community who have not been diagnosed and continue to spread TB to those around them[1].

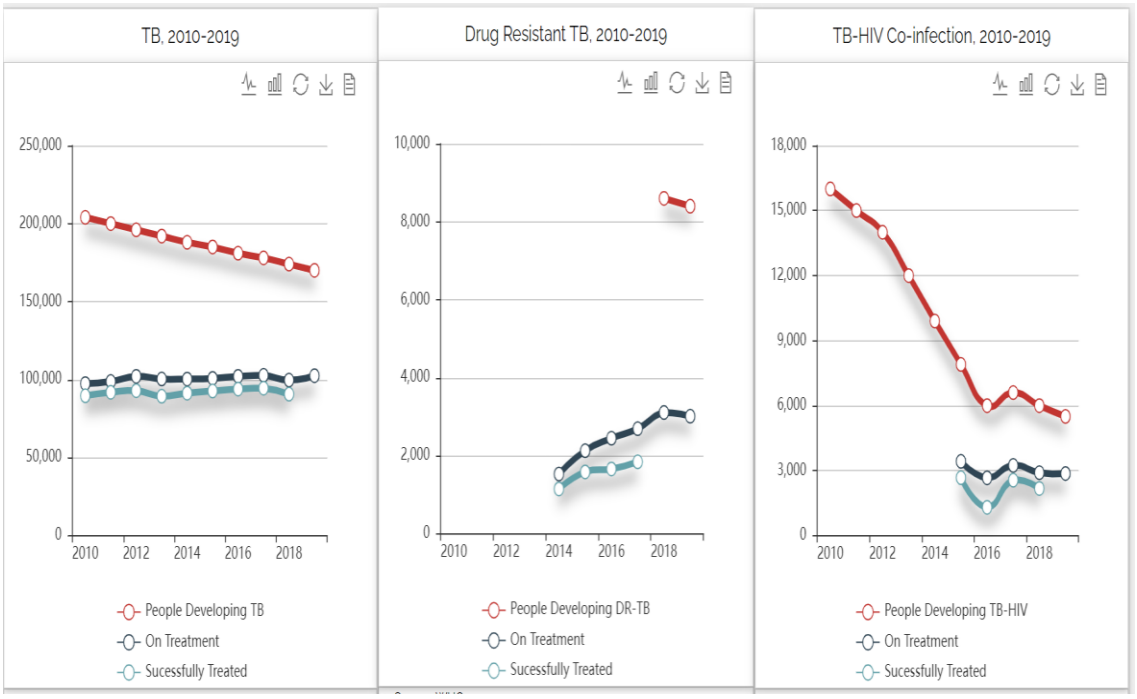

Figure 6: Estimated epidemiological burden of TB, in Vietnam (2010-2019) from WHO for the *Global Tuberculosis Report* [1].

1.2. Mycobacterium tuberculosis

1.2.1. The bacillus

Tuberculosis in humans, is caused by the *M. tuberculosis*, a member of the *Mycobacterium* genus. The species in this *Mycobacterium* genus are diverse, widely distributed in the natural ecosystem, and most are not capable of causing disease. Even so, a few species in this genus are successful pathogens that cause disease invertebrates [2,9]. A special feature is that these host-dependent mycobacteria cannot be replicated outside the natural environment but are obligate intracellular parasites in host cells. The most notable of this group are the members of the *M. tuberculosis* complex, which are generically called the tubercle bacillus. Despite many similar cellulars and molecular biological features, these species differ with distinct host, zoonotic and reservoir. *Mycobacterium tuberculosis* and the regional variants of *M. africanum* and *M. canettii* cause tuberculosis in humans. Other species that cause disease in animals but can be transmitted to humans include *M.*

bovis and *M. microti* [9]. Some *M. bovis* subspecies or variants such as *M. caprae* and *Mycobacterium pinnipedii* are isolated from goats and seals. Although there are specific adaptations for each type of host, the above-mentioned strains of *M. tuberculosis* have high similarity on genomic DNA as well as phenotype [2,9].

1.2.2. Morphology

Depending on the conditions and time of culture, TB bacteria have different sizes and shapes from short coccobacilli to long rods. Morphological examination under the microscope makes it difficult to distinguish species of *Mycobacterium tuberculosis* complex from other mycoplasmas[10]. Van Soolingen described the morphology of *M. microti* with a typical curved shape with dimensions of 1-10µm in length (usually 3-5um) and 0.2-0.6µm in width. This size is comparable with the lymphocyte nucleus' diameter. The bacillus is non-motile, non-spore-forming and difficult to stain with conventional dyes. When stained with Gram, the TB bacillus are weak or do not retain the dye colour [9]. By the Ziehl-Neelsen staining method, the tubercle bacillus picks up the red colour of carbon fuchsin, is not discoloured by alcohol and acids, and can be observed under the microscope [9].

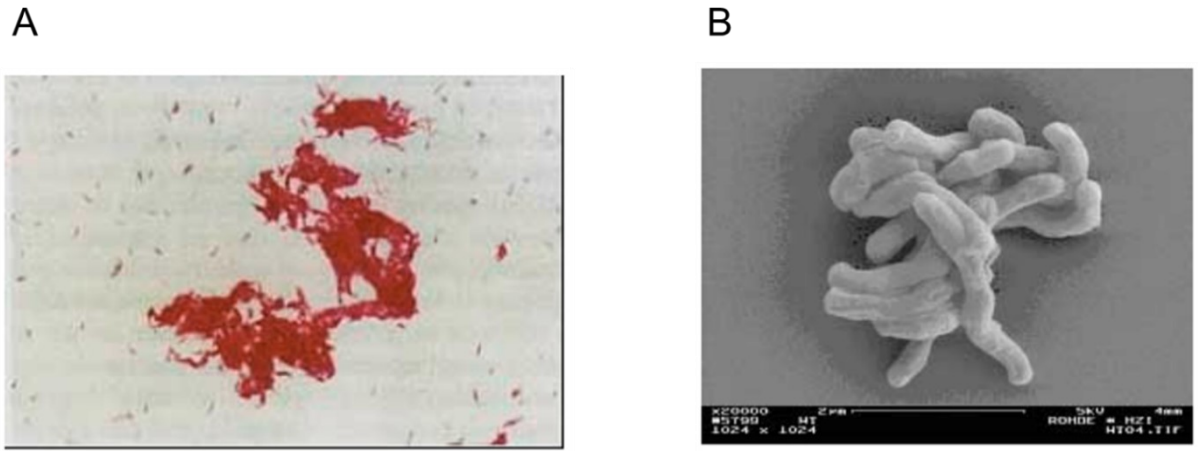

Figure 7. **(A)** Ziehl-Neelsen staining of *Mycobacterium tuberculosis* growing in culture at 1.000x magnification. (B) Electron microscopy of *M. tuberculosis* growing in culture (Courtesy of M. Rohde -M. Singh)[9].

1.2.3. Cellular characteristics

The cell wall structure of mycobacteria is of particular research interest because of its complexity in structural components, which is directly related to their pathogenicity[11]. Interestingly, the cell walls in mycobacteria have no additional membrane in the outer layer as gram-negative bacteria neither rich in protein and polysaccharide as gram-positive bacteria [11,12]. Therefore, *Mycobacterium tuberculosis* is not classified as either Gram-negative or Gram-positive but is considered as acid-fast bacteria resistant to decolonization with acid-alcohol solutions after staining with arylmethane dyes such as carbon fuchsin. There is an critical feature employed in identifying tubercule bacillus in pathological specimens using the Ziehl-Neelsen staining method [11, 12].

The cell wall of *M. tuberculosis* include many layers and has a high lipid content which accounts for more than 60% [11,13]. Outside the cell membrane is a covalently bound peptidoglycan layer with arabinogalactan that establishes rigidity and shapes the bacterial cell structure. This peptidoglycan structure has many cross-links, which accounts for 70-80% in *M. tuberculosis* but only 20-30% in *E.coli* [11]. The next layer is mainly constructed by mycolic acid and other complex lipids that make up the lipid envelope surrounding the bacterial cell [11,12]. Mycolic acid is the unique long alpha-branched lipid that is strongly hydrophobic. In *M. tuberculosis*'s cell wall, mycolic acid accounts for up to 50% of the dry weight. There is considered a significant determinant of *M. tuberculosis*'s virulence, and its molecular structure is species-specific [11,13]. Outside the cell membrane is a covalently bound peptidoglycan layer with arabinogalactan that has the function of establishing rigidity and shaping the bacterial cell structure. [14,15].

Specific mycolic acids in *M. tuberculosis* is alpha, keto, and methoxymycolates which the branched chains contain from 76 to 90 carbon atoms. These mycolic acid molecules contribute to a wax-like coating on the surface of the bacteria, making it impermeable to the dyes of the normal gram staining technique [15]. The

outer layer of TB bacteria is also a peptidoglycolipid which contains a distinctive lipid molecule called mycobacterial glycolipid trehalose 6, 6'-dimycolate. (also called the cord factor) [11,12,15].

This molecule affects the morphology of virulent colonies *M. tuberculosis* which is round colonies in solid media, expanded gummy veils on the surface of liquid media and serpentines on microscopic smears. In contrast, the attenuated tubercle bacilli and non-virulent mycobacteria develop smooth colonies, form discrete mats and distribute randomly on solid media, liquid media and microscopic smears respectively [9,11].

The outermost layer of the TB cell wall also contains many other lipid components such as phthiocerol dimycoserosates (PDIM), phenolic glycolipids (PGL), trehalose-containing glycolipids and sulfolipids (SL) [11].

The cell wall structure of mycobacteria is a dynamic structure, exhibits an adaptive response to ambient conditions such as nutrition, pH or oxygen concentration [12].The cell wall of TB bacilli thickened under hypoxic conditions. Under acidic conditions or inside the macrophages vacuoles, the expression of genes encoding porins, protein channels that transport substances across the membrane, increased [11].

The mycolic acids together with the other protein and carbohydrate composition in the other layers form a very effective physical barrier with exceptional impermeability [11,13,14].

This impermeability limits the diffusion of molecules across the membrane and contributes to protecting bacterial survival within the wall against external agents [11,13,14]. Although large molecules can be transported via porins, *Mycobacterium tuberculosis* expresses significantly fewer porins than other bacteria[12,16]. That intrinsic impermeability contributes to the slow growth and naturally high drug resistance. A study by Chamber et al showed, compared to *Escherichia Coli*, the penetration of β-lactam antibiotics which is the first line of anti-tuberculous drugs into *M. tuberculosis* is 100 times lower [17]. Therefore,

the TB cell wall is responsible for the high resistance to physical and chemical challenges, which must be overcome to have an effective TB treatment.

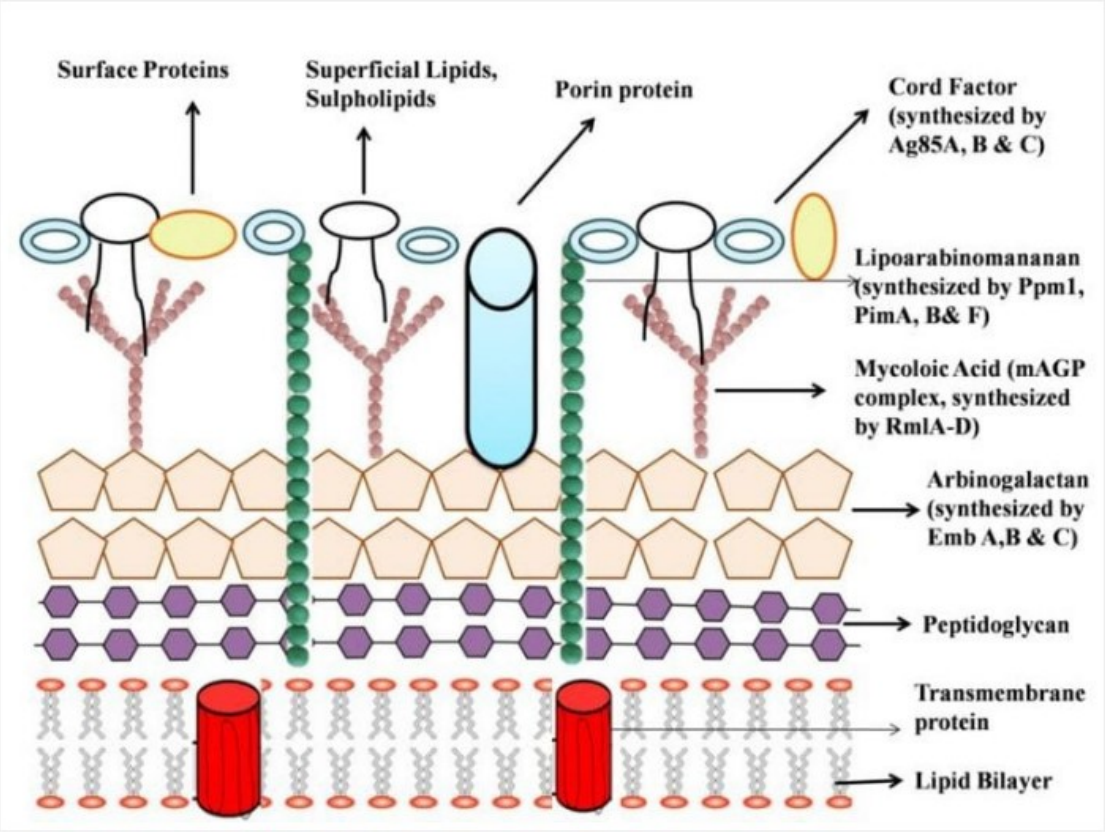

Figure 8. ***Mycobacterium tuberculosis*** **cell membrane**[18]

1.2.4. Classification and genomic organization

The *Mycobacterium tuberculosis* strain H37Rv was the first pathogenic strain to have a whole-genome sequenced and this information is still used today to study *Mycobacterium tuberculosis* and tuberculosis[19]. Genetic information can be used to elucidate many questions about the biology, physiology and treatment resistance of pathogenic TB bacteria. The genome of *Mycobacterium tuberculosis* H37Rv contains 4411529pb with a high GC content which accounts for more than 65% of the whole genome [19]. In the *Mycobacterium tuberculosis* H37Rv strain, 91% of the coding capacity of the genome was previously described to encode for 3924 ORFs, and a re-annotation of *Mycobacterium tuberculosis* H37Rv incorporated 82 additional genes were published in 2002[19,20].

The presence and distribution of insertion sequence (IS) have received much attention, especially IS6110, a member of the IS3 family. The copy number variation, and insertion site of this IS were used for phylogenetic analysis and considered a principal epidemiological marker for *M. tuberculosis*.

The predicted ORFs were represented for unique characteristics and classified into 11 classes functional groups as in table 2. Because of the high GC content in the genome, the initial codon GTC is used in 35% of those ORFs in H37Rv genome compared to 9% in *Escherichia Coli* [19,20]. Account for almost 10% of the genome capacity in H37Rv strain is a distinct group of genes encoding for PE and PPE protein family (named for the homologous proline-glutamate (PE) or proline-proline-glutamate (PPE) repeated regions in their N terminus). Those PE/PPE genes present a very high GC content of 80% and have an essential role in guaranteeing and sustaining the survival of bacteria under different environmental conditions. A PE member called the polymorphic GC-rich sequence (PE-PGRS) is found to be exclusive to the *M. tuberculosis* complex which may function as an inhibitor of antigen presentation by MHC-I [19,20].

It is also clear from the genetic information that *M. tuberculosis* has high flexibility in metabolic pathways, which have the potential to switch from aerobic to anaerobic respiration. This feature allows *M. tuberculosis* to adapt to different oxygen concentrations in the host organism, such as high oxygen concentrations such as in the lungs or low oxygen concentrations in the spleen and stomach [21].

Another notable genetic property is the presence of 250 distinct enzymes involved in lipid metabolism, compared to only 50 enzymes in *E.Coli*. Despite the lack of the MutS-used mismatch repair system in the genome, *Mycobacterium tuberculosis* possesses replication machinery of exceptionally high accuracy which the presence of nearly 45 proteins involved in DNA repair mechanisms [19,22].

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

Table 2. Functional classification of *Mycobacterium tuberculosis* H37Rv, a the functional groups numbers were taken from the Tuberculist database, publically available at. b. Data taken from Fleischman 2002; c. Data taken from Målen 2010 [20,23].

Many previous studies have been conducted to compare the genomes of different pathogenic *Mycobacterium tuberculosis* families. *Mycobacterium tuberculosis* clinical isolates were compared for single nucleotide polymorphism (SNPs), large sequence polymorphism (LSPs), and region of differences (SDs) which originated from mutations, deletion, genome rearrangement, frame-shift mutation and multi-cop genes to find the diversity, frequency and phenotypic effects of polymorphisms in the population [20,21,24,25]. This genetic variation among pathogenic strains indicates selective pressure and plays an essential role in pathogenesis. Gagneux et al. used comparative genomics and molecular genotyping tools to describe up to 6 *Mycobacterium tuberculosis* lineages that adapted to the human population, which are the Indo-Oceanic lineage, East-Asian lineage, East-African-Indian lineage, Euro-American lineage, and two West-African lineages[26]. Using the spoligotyping database, Brudey *et al*. analysed and classified *M. tuberculosis* into 62 clades/lineages [27]. These strains have a corresponding accumulation of genetic traits adapted to their

geographical and racial distribution. Among them, strains 2,3 and 4 are leading cause of TB today [20,21,25,27]. This difference in gene trait also represents an adaptation between the *Mycobacterium tuberculosis* and the host throughout evolution.

Shabbeer *et al*. proposed a web tool that allows the classification of *Mycobacterium tuberculosis* strains into lineages or genetic groups that will be automatic, fast and efficient base on the available large-scale database [28].

Together with many other studies, the rule of this approach is based on the characteristics of specific spoligotype signature of seven modern lineages of *Mycobacterium tuberculosis*. The characteristics of modern lineages are described below.

East-Asia lineage (Beijing)

The *Mycobacterium tuberculosis* strains have the Beijing and Bejing-like genotype clinically manifest a high virulence and frequent association with anti-TB drug resistance, which might be evolved during the Genghis Khan reign or before. From 2006, Gagneux renamed the Beijing lineage as the East Asian Lineage, but both terms have been used widely. The Beijing specific spoligotype signature was introduced in 1995 by Van Soolingen with the presence of contiguous deletion of spacers 1-34[28]. Due to its high virulence, which may be a severe threat to TB control, the genetic properties of IS, SNP and LSP were also described in more detail in subsequent studies of this lineage. The genome in these strains contains an inverted IS6110 copy in the DR region, an IS6110 element at a particular insertion site close to the origin of replication and one or two IS6110 in the NTL region [28]. The SNP G81A in Rv3815c in Beijing genotype was reported [29]. More recently, Tsolaki and colleagues sub-divided the Beijing lineage into four monophyletic subgroups [30]. Nowadays, the East-Asian lineage is prevalent in China, Japan, South East Asia and Russia.

East-African Indian

The East-African Indian lineage is also called the Central-Asian (CAS) of Delhi lineage, which was shown to be endemic in Sudan, Sub-Saharan countries and Pakistan and has been frequent in South-East Asia, India and East Africa. This CAS genotype was first described in Guinea-Bissau in 1999, characterized by a low number of IS6110 copies [27,28,31,32]. The spoligotyping characteristic of those strains presence a contiguous deletion of spacers 4-7, contiguous deletion of spacers 23-24 and contiguous deletion in loci 29-32 or contiguous deletion of spacers 4-7, contiguous deletion of spacers 23-24, at least one spacer in loci 29-32 absent and at least one spacer in loci 33-36. The spoligotype signature of these strains shows variation in their genome which could be implemented for sub-classified them into subgroup as CAS1- Kili for the strain found in Kilimanjaro, CAS-1 Dar for Dar-es-Salaam [27,28]. The result from a multiple-approaches genetic analysis suggest that this East-African Indian genotype family could be the ancestor of the Beijing lineage

The Euro- American

The Euro- American lineage is highly diverse which include the Haarlem, Latin American and Mediterranean (LAM), X and T families which their spoligotypes present contiguous deletion in loci 33–36 and contiguous deletion in loci 29–32, or contiguous deletion from loci 33 to 36 and at least one spacer presents in loci 29–32. The Haarlem family was found and first described in the Netherlands in 1999 and is prevalent in Northern Europe. The Latin American and Mediterranean (LAM) clade is frequent in Mediterranean countries and has high heterogeneity in genotype, which can be further sub-classified into LAM01-LAM-12 subgroups [27,28]. Understanding genotype and evolutional processes based on genetic patterns may explain phenomena such as transmission and drug resistance of high viral strains. Through it, we understand the TB epidemics and help analyse and predict TB outbreaks in regions and develop and suggest strategies to deal with TB.

1.3. Drugs in tuberculosis treatment and the drug resistance

1.3.1. Common drugs in tuberculosis treatment

Drug-resistant tuberculosis is a major obstacle to the global TB control programme. The rate of drug-resistant and multidrug-resistant TB is increasing, along with the spread of drug-resistant strains in the community, posing a significant challenge for the medical workforce and researchers. The first antibiotic used to treat tuberculosis was streptomycin, but after a short time, a decrease in effectiveness was recorded. New antibiotics and anti-tuberculosis drugs are continuously being investigated, but they only provide effective treatment for a certain period of time. With common primary TB, the chance of successful treatment is very high, up to 95% [9]. However, for patients with drug-resistant and multidrug-resistant TB, the treatment success rate is less than 30% despite the use of multiple antibiotics [9].

The pharmacological approach for TB treatment started with streptomycin (SM) and para-aminosalicylic acid (PAS). Those drugs were discovered in 1944 and the regimen use of a combination of SM and PAS suggested a better treatment efficacy in 1950. Isoniazid (INH) and Ethambutol (EMB) were developed later in 1952 and 1960 and were included in the regimen. They reduced the duration of treatment from 18 to 24 months. In the 1970s, Rifampicin (RIF) was introduced and reduced the treatment course to 9 months. Pyrazinamide (PZA) was first introduced in 1980 and reduced the duration of TB treatment to 6 months. Traditionally, TB drugs are divided into first-line which includes INH, RIF, PZA, EMB and SM and second-line including aminoglycosides kanamycin and amikacin, the polypeptide capreomycin, PAS, cycloserine, the thioamides ethionamide and prothionamide and several fluoroquinolones such as moxifloxacin, levofloxacin and gatifloxacin [9]. Currently, the usage of anti-TB drugs has to follow the guideline which has four leading principles. One is to use a combination of anti-tuberculosis drugs. Because each drug will have a different effect on TB bacteria, such as killing bacteria and inhibiting bacteria's

growth, it is necessary to use a combination of drugs to have the best treatment effect of eliminating bacteria. For instance, INH has a strong killing effect on bacterial cells extracellular in the lung cavity when dividing, while RIF and PZA can act on non-dividing cells. The second principle is to use the drug in the correct dose. If the dose is too high, it will easily cause serious side effects and harms patients, while the low dose will be ineffective and quickly induce drug resistance. Dosages of first-line anti-tuberculosis drugs are presented in table 3. Third, the drug must be taken regularly at certain times for maximum absorption and to ensure drug concentration in the body. Fourth, the drug must be used for a sufficient time to ensure the complete eradication of TB bacteria and avoid recurrence. Following the current guideline, the treatment schedule for complete elimination of *Mycobacterium tuberculosis* bacilli involves two phases which are (i) the initial phase which rapidly kills the actively dividing bacteria, resulting in the negativization of sputum and (ii) continuous phases which target to kill any remaining or dormant bacilli and prevent the recurrence[9].

Drug	Adults or Children[a]	Daily dose (max. dose)	Three times per week (max. dose)	Twice per week (max. dose)
INH[b]	Adults	5 mg/kg (300 mg)	10-15 mg/kg (900 mg)	15 mg/kg (900 mg)
	Children	10-15 mg/kg (300 mg)		20-30 mg/kg (900 mg)
RIF	Adults	10 mg/kg (600 mg)	10 mg/kg[c] (600 mg)	10 mg/kg[c] (600 mg)
	Children	10-20 mg/kg (600 mg)		10-20 mg/kg (600 mg)
PZA[d]	Adults	18.2-26.3 mg/kg (1-2 g)	27.3-39.5 mg/kg (1.5-3 g)	36.4-52.6 mg/kg (2-4 g)
	Children	15-30 mg/kg (2 g)		50 mg/kg (2 g)
EMB[d]	Adults	14.5-21.1 mg/kg (800-1,600 mg)	21.8-31.6 mg/kg (1.2-2.4 g)	36.4-52.6 mg/kg (1.2-2.4 g)
	Children	15-20 mg/kg (1 g)		50 mg/kg (2.5 g)
SM	Adults	15 mg/kg[e] (1 g)		
	Children	20-40 mg/kg (1 g)		20 mg/kg (1 g)

Table 3. Recommended doses for first-line anti-tuberculosis drugs. (a) Patients under 15 years of age; (b) INH can also be given once per week, on a 15 mg/kg

basis, up to a maximal dose of 900 mg; (c) For RIF, some manuals also recommend higher doses (10-15 mg/kg) intermittently (three days per week) having a maximum of 900 mg (Martindale 2004); (d) For PZA and EMB, doses have to be calculated precisely depending on the weight range (for details, see CDC 2003a); (e) SM: doses should be reduced to 10 mg/kg in people over 59 years old [9].

1.3.2. The definition and classification of drug resistance

Drug resistance in TB is a public health crisis, especially in high 30 TB burden countries. Drug-resistant tuberculosis is defined as the decreased susceptibility of *M. tuberculosis* to antibiotics [13]. In nature, because of the complex molecular and cellular structure, several tuberculosis bacteria strains have intrinsic resistance to certain antibiotics. For example, *M. bovis* are resistant to pyrazinamide, *M. tuberculosis* is naturally resistant to penicillin [13]. In humans, primary drug resistance occurs when a patient has not previously received any TB therapy and secondary drug resistance develops during or after drug therapy [33,34]. There are *M. tuberculosis* strains resistant to at least one of the first-line anti-TB drugs of RIF and INH. Those strains are defined as multidrug-resistant TB (MDR-TB). Besides that, the extensive drug-resistant (XDR) strains have been currently introduced and defined as "resistance to at least rifampicin and isoniazid, which is the definition of MDR-TB, in addition to any fluoroquinolone, and to at least 1 of the 3 following injectable drugs used in anti-TB treatment: capreomycin, kanamycin and amikacin.", as in the report of WHO Global Task Force. WHO estimated 465.000 incident cases of rifampicin-resistant TB and 182,000 deaths from MDR/RR-TB in 2019 globally [1]. Of which India, China and the Russian Federation account for nearly half of the global burden in MDR-TB which had 27%, 14% and 8%, respectively. In Vietnam, the rate of drug-resistant TB is among the highest in the world. In 2019, there were an estimated 8.400 cases to develop DR-TB among notified TB cases, 5.386 cases missed detection DR-TB. The proportion of TB cases with

MDR-TB among new and retreatment cases is estimated to be 3.6 and 17 %, respectively, with around 6 % among TB cases co-infected with HIV. There were 11.400 people died because of TB [1].

1.3.3. Molecular mechanism of drug resistance to RIF and INH.

Drug resistance of TB bacteria is associated with the accumulation of random mutations in certain genes during DNA replication during the bacteria proliferation [35,36,37]. Each TB cluster usually contains about 10^7 or more TB bacilli and the frequency of mutations associated with isoniazid resistance is 10^{-7} – 10^{-9}, rifampicin resistance is 10^{-10} and resistance to both drugs has a frequency of 10^{-14}. [34,38] During treatment, antibiotics exert selective pressure on bacilli that have resistance mutations. Depending on the mechanism of action of antibiotics, drug-susceptible TB bacilli are either inhibited or destroyed, creating conditions for mutant-carrying bacilli to develop and dominate in the TB population, especially in patients with high TB bacteria levels [35,38]. The spread of drug-resistant TB bacteria to others causes primary resistance even though they have never been treated with anti-TB drugs.

Rifampicin-resistance

Rifampicin (RIF) is an antibiotic that is active against both dividing and non-dividing bacilli that began use in 1966. RIF inhibits transcription by binding to DNA-dependent ribonucleic acid (RNA) polymerase enzyme [37,39]. This enzyme is assembled of four different subunits and is encoded by the four genes *rpoA, rpoB, rpoC* and *rpoD*. RIF binds to the beta subunit, around the poker for double-stranded DNA entry where nearby the catalytic centre. Thus, mutations in the *rpoB* are closely related to rifampicin resistance usually identified around an 81bp core region called rifampicin resistance determining region (RRDR) containing codons 507 to 533 and encoding the 27 amino acids of the subunit beta in RNA polymerase [39]. Research data have shown that approximately 95% of clinical *Mycobacterium tuberculosis* strains resistant to rifampicin carry mutations in this RRDR core region [33,38-40]. Most strains resistant to RIF have

one mutation in *rpoB*, but there are rare strains with 2-4 mutations in this gene. The mutations in codon 516/526/531 resulted in strong RIF resistance while mutations in codon 514 or 533 resulted in weaker resistance[33,38-40]. The frequency of mutations in codons in the core region of *rpoB* depends on ethnic communities and different geographical regions. However, many studies show that codons 531 and 526 account for a large proportion of RIF-resistant TB samples [38,39]. Because of the high frequency of mutations is identified in RRDR in *rpoB,* this hotspot sequence analysis allows the development of methods to detect RIF resistant strains and estimate the resistant level. Currently, there are several well-known commercial testing systems to detect RIF resistance by analyzing the sequence of the RRDR in the *rpoB* such as Xpert*MTB*/RIF assay or The GenoType *MTB*DRplus test (Hain).

Isoniazid resistance

Isoniazid has been considered as one of the most powerful anti-TB drugs which are actually a pro-drug that required a further process to be activated by the catalase-peroxidase in the bacterial cytosol. Although INH has simply made up of a pyridine ring and a hydrazide group, INH functions in the most complex fashion among antibiotic molecules. INH is able to inhibit the biosynthesis of mycolic acid, which then alter the cell wall production and against both active and resting bacilli. Furthermore, the inhibition of mycolic acid synthesis by NIH leads to the release of many toxic free radicals. 85-90% of *Mycobacterium tuberculosis* strains resistant to INH are reported with mutations in the genes *katG, inhA, kasA or ahpC* which encodes for catalase-peroxidase, enoyl acyl carrier protein (ACP) reductase, b-ketoacyl A C18 P synthase and alkylhydroperoxide reductase, respectively [35,40-41]. In clinical practice, high INH resistance is mainly associated with mutations in the *katG*, most commonly in Ser315Thr mutations occurring in 30-60% of INH-resistant strains. One reason for this mutation prevalence is that it can decrease INH activation but not overall

catalase-peroxidase activity [42,43]. Mutations in the *ahpC* promoter that lead to overexpression of this gene also lead to INH resistance [42]. Meanwhile, mutations in the *inhA* gene cause resistance to INH and ethionamide (ETH), typical mutations in Ile16Thr, Ile21Thr, Ile21Val, Ile47Thr, Val78Ala and Ile95Pro [44]. Mutations at codons 66, 269, 312, 413 of *kasA* also confer weak resistance to INH [42]. With these molecular studies results, the GenoType *MTB*DRplus test (Hain) is a recently marketed test that detects INH resistance by targeting *katG* and *inhA* mutations.

1.4.Tuberculosis and tuberculosis's drug resistance diagnosis methodologies

1.4.1. Tuberculosis diagnosis

1.4.1.1. Traditional methods

The previous TB detection methods were generally based on the sputum smear microscopy using the Ziehl-Neelsen (ZN) stain, which is the primary diagnostic strategy recommended by World Health Organization (WHO)[45]. The mechanism of ZN stain is explained by the carbon fuchsin retaining ability of acid-fast organisms such as TB. ZN stain method can detect the presence of TB within 3 to 4 hours by light microscopy, and are considered a low-cost and straightforward method. However, the accuracy of this method also relies on human skill and time-intensive nature, which can bring disadvantages to developing countries where are limited in skilled technicians and microscopy facilities[46]. Additionally, due to the high requirement in initial bacteria concentration in the patient sample (10^4bacteria/ml), the sensitivity of ZN stain is lower than other traditional culture methods. Recently, ZN stain has been improved by advanced techniques in centrifugation and concentration. Cytosine slides with Triton processing were applied to enhance the sensitivity of ZN stain and also provide technicians more convenient tool for rapid detection of TB [47]. Applying fluorescence microscopy (FM) has brought an improvement to the traditional ZN stain method using light microscopy and has been used in high-income countries. The mechanism of the FM-ZN detection method has been

based on the combination of an acid-fast fluorochrome dye and a light source of a high-pressure or halogen vapour lamp (MVP). FM-ZN stain is known as a simple, sensitive and rapid method in TB detection but is only popular in developed countries where the laboratory resource can meet requirements for implementation [48]. In order to bring down the cost, MVP has been replaced by light-emitting diodes (LEDs) in FM. LEB-FM was suggested to have higher specificity than MVP and was recommended for TB diagnostics by WHO in 2009 [49]. Recently, Shah's group has proposed the use of a camera-enabled Smartphone microscope for imaging ZN-stained smear slide6. This technique has become a promising method where it is not only achieved high sensitivity and specificity of 93.3% and 87% respectively on detecting TB positive and negative. However, it is also proper for low and middle-income countries due to its availability and accessibility [50]. Solid media culture to test for TB is the classic golden standard for TB diagnosis. In this method, the samples of TB-suspected patients are collected and treated on different substances, usually, Lowenstein-Jensen, which can facilitate the bacteria to grow. However, this method can also be used for testing the drug sensitivity of isolates. After *Mycobacterium tuberculosis* is present on the solid media culture, it can be subculture with media containing antibiotics to find out which antibiotics can be used to inhibit the growth of this strain or which antibiotics can be resisted by it [51]. Compared to sputum smear microscopy, TB culture test is more complex and expensive because it requires specific laboratory facilities and equipment. Besides that, the slow growth rate of TB bacilli can extend the testing time, which is normally between 4 to 6 weeks [52].

1.4.1.2. Molecular methods

Following the rapid development of molecular biology, polymerase chain reaction (PCR) has recently become an important method in TB diagnosis. By PCR, a specific blueprint of any known gene can be amplified to millions of copies and easily detected within a couple of hours. PCR has overcome the

difficulty in other traditional because it provides the massive advantages of low time-consuming, high specificity and potentially high sensitivity. Based on the classic PCR, several TB diagnostic methods such as multiplex PCR or real-time PCR have been developed to simplify the technique as well as shorten the testing time. Several internal controls are also included to enhance the accuracy and specificity of the PCR assay. The remaining barrier of this method is the requirement of skilled technicians/specialists and expensive laboratory infrastructure.

In 1985, based on the mechanism of in vitro DNA replication of DNA polymerase, Kary Mullis proposed PCR, a molecular method in which a small amount of DNA can be amplified into million copies of the same DNA sequence. A standard PCR include main components: template DNA, two primers, DNA polymerase, deoxynucleotide (dNTP) and other chemicals playing the role of reaction buffer. After mixing the reagents together, the reactions are happened by following a thermal cycle where the mixture is incubated in 3 main steps with different temperatures and periods of time. The first step is denaturation of the template at 94-96^{0}C, in which the double-stranded DNAs become single-stranded. After that, the temperature is cooling down (40- 70^{0}C) to facilitate the primers binding to the desired target. The last step is the extension of new DNA strands from the primers by DNA polymerase and normally happens at 68-70^{0}C depending on the type of used polymerase. These three core steps are repeated 30-35 cycles and in each cycle, the amount of desired DNA is duplicated. The final product of PCR is million to billion copies of DNA which have the same sequence as the target template.

PCR products can be visualized by the DNA electrophoresis method and identify the accuracy of amplification based on the size of the amplicon. The main barrier of traditional PCR is only targeting one DNA sequence at a time. Therefore, multiplex PCR techniques were described and developed in 1988 by Chamberlain and colleagues to overcome the limitation of single PCR.

Combining two or more different primer pairs can increase the number of target sites in one PCR. Multiplex PCR plays an important role in detecting *M. tuberculosis* from clinical samples and distinguish the false and true negatives.

1.4.2. Tuberculosis's drug resistance diagnosis

TB and TB's drug resistance diagnosis have a virtual part in choosing the suitable treatment for each patient. Detection of TB is world widely based on phenotype-based methodology and molecular methodology. Although each method has its advantages and limitations, they are still necessary and supportive for each other to identify the drug resistance TB stain correctly [53,54].

1.4.2.1. Phenotype-based methodology

The phenotype-based methodology (PBM) is an empiric observation of bacteria growth in the culture plate containing antibiotics. The most basic assay of PBM is the "Proportion method" developed by Canetti in the 1960s [51]. In this method, TB bacteria are culture on media without and with the antibacterial drugs in different concentrations. If the proportion of bacteria grown on media with and without antibiotics is higher than 1% (for INH, RIF and para-aminosalicylic acid) or 10% (other drugs), this bacterial strain is considered drug resistance. The main disadvantage of the Proportion method is the long incubating time, which is at least 28 days for bacteria appearing.[51,53]

1.4.2.2. Molecular methodology

Currently, molecular methodology for TB resistance diagnostic is intensively invested and developed into many different branches and methods, including MAS-PCR, PCR-RFLP, PCR-SSCP, DNA microarrays, INNO-LIPA, Whole Genome Sequencing, Western Blot, real-time PCR, etc. These methods can identify the mutation corresponding to the drug resistance phenotype. Limitations of the molecular assay require expensive equipment and high-skilled specialists. However, these methods can shorten the testing time and offer specific and sensitive results directly from the patient samples without culturing.

Among other molecular methods, real-time PCR is one of the most popular techniques in detecting TB and TB's drug resistance [55,56].

Real-time PCR was developed based on the mechanism of PCR, where a target DNA is amplified to million copies. However, real-time PCR result is not reflected through the final PCR products but the observation of detected signal during the thermal cycles. The critical component of real-time PCR techniques is fluorescence dye tagged reagents. The signal of fluorescence dyes will be detected following the PCR. Two main types of real-time PCR are SYBR Green-based, and TaqMan-based detection, mainly focused on and described in Figure 9.

In this study, the fluorescence dye tagged reagent is the TaqMan Probe. The body part of the probe is the single-stranded oligonucleotide which is complimentary with the specific DNA sequence (target site). The 5' end of the oligonucleotide probe is attached with a fluorescent reporter, while the 3' end is linked with a quencher. The emitted fluorescence signal is reduced when the quencher stays close to the reporter. In the presence of target DNA, the probe binds downstream from one of the primer sites. During the extension step, DNA polymerase synthesis the complementary DNA and cleave the probe. This cleavage distributes the reporter dye from the quencher, therefore, enhance the reporter dye signal [57]. On the other hand, DNA extension continues to the end of the DNA strand and creates a new copy ready for the next thermal cycle. Each thermal cycle doubles the number copy of amplicon and increases fluorescence intensity proportional to the number of DNA copies [57,58].

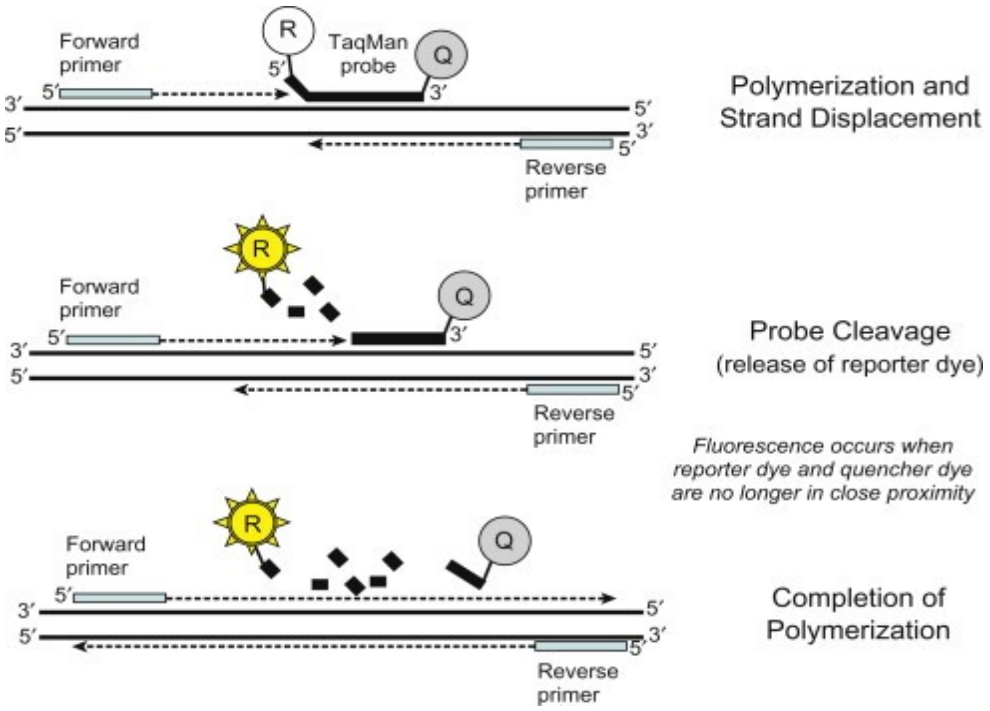

Figure 9. Schematic of TaqMan (5′ nuclease) assay [59].

Compared to traditional PCR, real-time PCR Taqman does not require Post-PCR processing, reducing material cost and assay labour. Secondly, multiple mutations or variants within the amplicon can be detected by real-time PCR Taqman by using distinguishable reporter dyes. Lastly, a combination of probe and primers to generate fluorescent signals ensures the specificity of methodology. On the other hand, the primary limitation is the requirement of different probes for different target sequences. To overcome this disadvantage, real-time multiplex PCR include multiple primers for different amplification and probes containing different dye colours in one single PCR reaction. In that way, real-time multiplex PCR can save testing time and cost to allow specialists to detect directly multiple mutations from clinical samples. [60].

2. RESEARCH OBJECTIVE

Tuberculosis is an old infectious disease caused by *M. tuberculosis*, but nowadays, it still remains a burden on the global health system by the uncontrolled rise of drug-resistant TB. In 2020, according to WHO estimated 10.4 million patients with TB and 1.5 million deaths were attributed to this disease[1]. Currently, Vietnam is in the 16th position among the 30 countries with a high burden country for Tuberculosis and ranked 13th out of 30 countries with the most cases of drug-resistant TB prevalence in the world[1]. Vietnam National Tuberculosis Control Program is well-established, has nationwide coverage but the diagnosis, treatment, and management of TB and DR-TB are the challenges of public health problems. The commercial DST as liquid medium BACTEC MGIT 960 systems, molecular DST methods as the GeneXpert MTB/RIF are rapid results, reduced the turn-around time. However, these methods require costly reagents, modern equipment, detect some drugs, and are only performed in a few Vietnam laboratories. Therefore, the development of phenotypic DST of *M. tuberculosis,* with determining the MIC values of many TB drugs, considering the level of resistance of each drug, helps clinical doctors provide better treatment decisions. In addition, their simplicity, reliability, and low cost are necessary for high DR-TB prevalence countries.

Moreover, there are limited genetic studies that characterize genotype of *M. tuberculosis* isolates in central Vietnam. The insights that emphasize and thorough understanding of the genotypic DR-TB isolates are assisted in focusing on infection control and surveillance to prevent new cases of DR-TB in this region. In order to observe the evaluation of molecular assay has implemented for detecting a wide variety of resistant mutations of *M. tuberculosis* in clinical isolates and clinical samples is very important to reduce the morbidity and mortality of DR-TB because of an accurate and rapid diagnosis in the early stages.

It seems necessary that a combination of phenotypic and molecular methods until more information regarding the clinical relevance of phenotypic susceptibility isolates with drug resistance mutations mechanisms is thoroughly understood and assist decreasing DR-TB cases in Vietnam.

In that scenario, this study aimed to evaluate the frequency of DR-TB to Rifampicin and Isoniazid- the most effective first-line drugs in TB treatment. We first collected the clinical specimens in the central of Vietnam and performed a phenotypic test to establish the proportion of Rifampicin resistance (RIF) and Isoniazid- resistance (INH). REMA- a culture-based phenotypic drug susceptibility testing (DST) was applied in this task. Then we designed a PCR TaqMan probes panel to identify the DR-TB- associated mutations in *rpoB* and *katG* in cultured TB strains from patient's samples and mapped the genotyping results. Toward the clinical application, we also tried to develop our PCR panel to detect Rifampicin- and Isoniazid- resistance-associated mutation directly from clinical specimens.

The specific objectives include:

- *Objective 1:* To determine the rate of Rifampicin- and Isoniazid-resistant tuberculosis in patients in Central Vietnam based on the phenotype drug susceptibility testing.

- *Objective 2*: To develop the method using Real-time PCR TaqMan allelic discrimination for rapid detection of Rifampicin- and/or Isoniazid-resistant *M. tuberculosis* from clinical isolates and clinical samples.

- *Objective 3:* To analyse genotypic characteristics in *rpo*B and *kat*G of resistant isolates.

3. MATERIALS AND METHODS

3.1. Study design

This research was a cross-sectional study that was conducted at following settings:

Collecting, culturing TB from patient's sample and performing the drug susceptibility testing by MGIT and REMA from June 2019 to June 2020 at:

- Da Nang Lung Hospital, Da Nang, Vietnam.
- Central Hospital 71, Thanh Hoa, Vietnam.
- Department of Microbiology, Hue Central Hospital, Hue city, Vietnam

Performing the drug susceptibility by Real-time PCR Taqman allelic discrimination assay from July 2020 to September 2021 at:

- The Carlo Urbani Centre, Microbiology department, Hue University of Medicine and Pharmacy, Hue University, Hue city, Vietnam.
- Department of Microbiology and clinical Microbiology, Hospital University Sassari, Italy.

3.1.1. Inclusion criteria

- This study includes patients suspected of having tuberculosis at three hospitals (as stated above) in Central Vietnam was enrolled into.
- Data on socio-economic and demographic features, TB history, HIV status and presenting clinical features was collected prospectively on a case report form.
- Ethical approval for this study was granted by The Ethical Committee in Biomedical Research of University of Medicine and Pharmacy-Hue University.

3.1.2. Exclusion criteria

- Patients have already received TB therapy for more than two weeks was excluded from the study
- Patients who refuse to participate in this research.

3.1.3. Sample size

According to guidelines for surveillance of drug resistance in TB of WHO [61,62]. Applying the equation for the percentage of MDR-TB in Vietnam is 3.6% new cases and 17% previously treated cases, 500 *M. tuberculosis* isolates needed for our study based on this equation:

$$n = Z^2_{(1-\alpha/2)} P (1-P) / d^2$$

P : expected proportion of MDR-TB from available data

d : desired absolute precision.

$Z_{(1-\alpha/2)}$: Confidence level (if CI 95%, 1.96)

n : the minimum sample size

3.2. Specimens' preparation, examination, and store

Specimen decontamination

All specimens were digested and decontaminated from the presence of other bacteria using the standard N-acetyl-L-cysteine-sodium method (except for cerebral spinal fluid).

Processing sputum specimens:

All sputum samples were transferred into sterile 50ml tubes, and 4% sodium hydroxide-N-acetyl-L-cysteine (NALC-NaOH) solution was added to each specimen in a ratio of 1:1. After 15 min, the solution was neutralised by an equal volume of phosphate buffer saline (PBS); and centrifuged at 3000 rpm at 40C for 15 minutes. The supernatant was removed, and the pellet was resuspended with PBS. All samples of sterile origin (headache fluid, pleural fluid, etc.) were centrifuged, and the pellets were resuspended.

Ziehl-Neelsen staining

All samples were examined by microscopic examination.

The smear stained with a concentrated solution of carbon fuchsin was heated with a lamp until smoke appeared; after 5 minutes the slide was washed

with water and decolourized with a 3% acid-Alcohol solution, for 3 minutes and rinsed.

The slide was then coated with a methylene blue solution for 60 seconds, rinsed and finally the stained smears must be view using a 100X oil-immersion objective.

Reading:

In a positive sputum sample, red bacilli could be observed on a blue background; the bacteria could be bacillary, slightly curved or straight, sometimes branched, beaded or bird - winged.

Reporting

Table 4. Grading AFB scale (WHO-IUATLD) [63, 64,65].

Number of acid-fast bacilli (AFB)	Report
No AFB	0
1-9 AFB per 100 field	Scanty (report number of AFB)* or +
10-99 AFB per 100 field	1+
1-10 AFB per field	2+
More than 10 AFB per field	3+

* 1-9 AFB per 100 field signals Scanty or (+)

3.3. Culture *M. tuberculosis* isolates by BACTEC MGIT 960 system

Processing clinical sample culture:

The system BACTEC MGIT 960 (Becton, Dickinson, Sparks, MD) was used for the isolation, growth and continuous monitoring of mycobacteria. The reading takes place in MGIT (Mycobacteria Growth Index Tube) bottles; the fluorescence sensor is embedded in a solid matrix at the bottom. The sensor is inhibited by the oxygen present in the culture medium, but when the

microorganisms' metabolism reduces the oxygen tension, the degree of fluorescence emitted increases, up to the value detectable as a positive signal. The bottle contains the liquid growth medium, Middlebrook 7H9 modified, which was added before inoculating the sample, the mixture PANTA-OADC. This mixture allows the enrichment with the OADC (oleic acid, albumin, dextrose, catalase; BBL) while the addition of PANTA, a mix of antibiotics serves to make the growth broth more selective towards mycobacteria.

The system takes a reading of the bottle at a 1-hour interval and constructs the interpretation curve by extrapolating each point of the curve through the average value of 5 successive readings.

Indicator lights and an audible alarm indicate positivity. All positive samples were removed, and a slide was prepared from them, then stained with ZN, and a drop was placed on a blood agar plate to check for the presence of contaminating or non-contaminating bacteria.[66]

3.4. Drug susceptibility testing

3.4.1. Mycobacterial isolates

1547 clinical samples were inoculated into BACTEC MGIT 960 and of these 500 were positively, subsequently all were identified as *M. tuberculosis.*

M. tuberculosis strain H37Rv (ATCC 27294) was used as the susceptible control strain.

Multidrug-resistant TB strain identified previously from the clinical microbiological department at Da Nang Lung hospital was used as the resistance control strain.

By storing all MGIT instrument-positive tubes in 37^0C MTB isolates continued to grow and were used for DST and real-time PCR testing.

3.4.2. Drug susceptibility testing from MGIT cultures

The first-line drug-susceptibility testing was performed using the automated BACTEC MGIT 960 as instructed in the manufacturer's protocol. The commercial kit contains the freeze-dried first-line drugs: Isoniazid,

Rifampicin, Ethambutol, and Streptomycin, which are then reconstituted with sterile distilled water to obtain critical concentrations. Finally, 0.1 ml of each drug thus diluted was inoculated MGIT tube containing into a new the bacterial suspension according to the manufacturer's instructions. In this study, two antibiotics INH and RIF were considered at concentrations of 0.1µg/ml, 1.0µg/ml, respectively.

The DST can be performed 24-48 hours (day 1 to 2) after the BACTEC MGIT 960 positivity (day 0) or from day 3 to 5.

On day 1 or 2, the MGIT positive isolate must be diluted 1:100 and for this purpose 0.1 ml of isolate was inoculated into a tube containing 9.9ml sterile water, and from there 0.5ml was inoculated into a new MGIT tube without drug as control; 0.5ml was also taken from the MGIT positive tube and inoculated directed into 4 MGIT tubes each containing the specific drug.

When DST was performed on day 3-5, 1ml from the MGIT positive culture was transferred to another tube containing 9.9ml of sterile water then 0.5ml of this tube was taken and inoculated into a new MGIT tube, without drug that also in this case acts as a control. Finally, 0.5ml from the dilution 1:4 was always transferred into 4 MGIT tubes containing the drugs [67,68].

3.4.3. Drug susceptibility testing from REMA

Bacteria

From all *M. tuberculosis* isolates grown in MGIT, sub-cultures were made until the 1.0 McFarland grow was reached and finally diluted 1:20 in the 7H9-S medium.

Drugs (Antibiotics)

All drugs (HiMedia Laboratories Pvt. Ltd – India) in lyophilised form were rehydrated and filtered to form the stock solution.

Isoniazid was sterile diluted water to a concentration of 1 mg/ml while Rifampicin was diluted with methanol to a concentration of 10 mg/ml. The drugs were then stored at -20^{0}C until use.

Resazurin reagent

Resazurin powder (resazurin sodium salt - Acros Organic NV) was diluted in sterile water to a concentration of 0.02%, then sterilised, filtered and stored at 4^0C for 1-2 weeks protected from light.

Culture medium

The resazurin microtiter assay plate was performed in 7H9-S medium containing Middlebrook 7H9 broth with 0.1% Casitone, 0.5% glycerol, and 10% Oleic acid, Albumin, Dextrose, and Catalase (OADC) supplement (Becton Dickinson, USA).

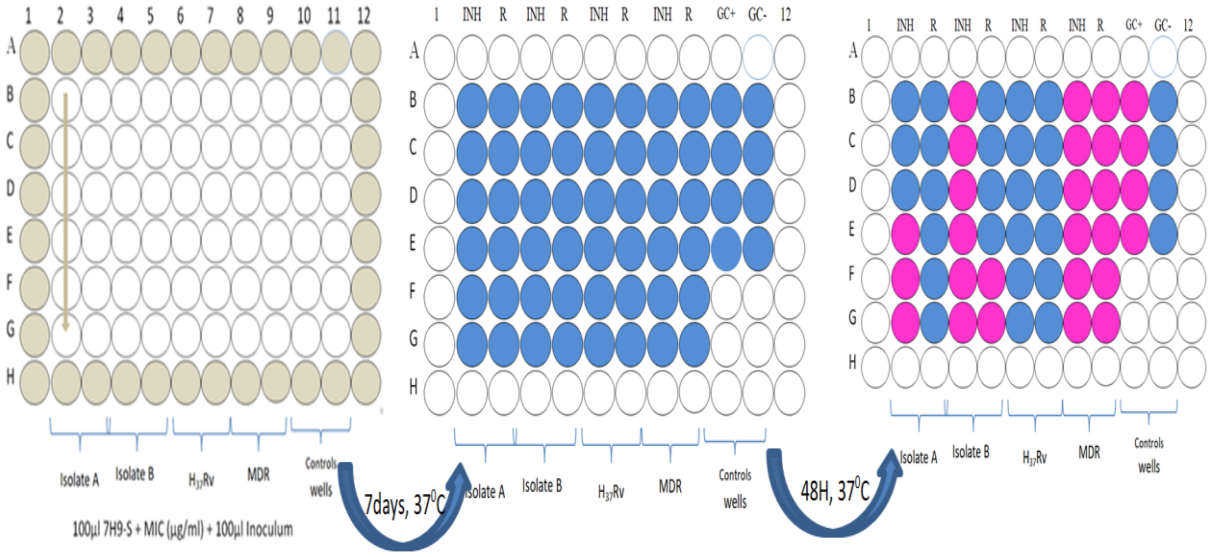

Figure 10. REMA plate method flowchart

Palomino et al.'s protocol, the Resazurin Microtiter Assay (REMA), was used for drug sensitivity testing:

100 µl of the 7H9-S medium was dispensed into each well of a 96-well plate (Corning), 100 µl of the drug was inoculated from the stock solution into the first well (dilution 1: 2 of the concentration) and from here 100 µl containing medium and drug were transferred to the next well, to halve the initial concentration of the drug; this operation was carried out on the remaining wells of the plate to halve the drug each time while maintaining the same volume (100

µl final volume). The concentration range of drugs was for INH between 1.00 and 0.031 µg/ml, these of RIF was between 2.00 and 0.061 µg/ml.

The positive control consisted of the isolate, and the negative control consisted only of the medium, both free of drug, were added to the test. Finally, 200µl of sterile water was placed in each well of the outer perimeter to prevent evaporation during the incubation. At this point, the plate was covered, sealed with a plastic bag and incubated at 37°C for 7 days. After this period, 30µl of resazurin solution was added to each well, and the plate was incubated for 48 hours at 37°C. The colour change from blue to pink highlight in the wells indicates the level of resazurin reduction was correlated with bacterial growth [69].

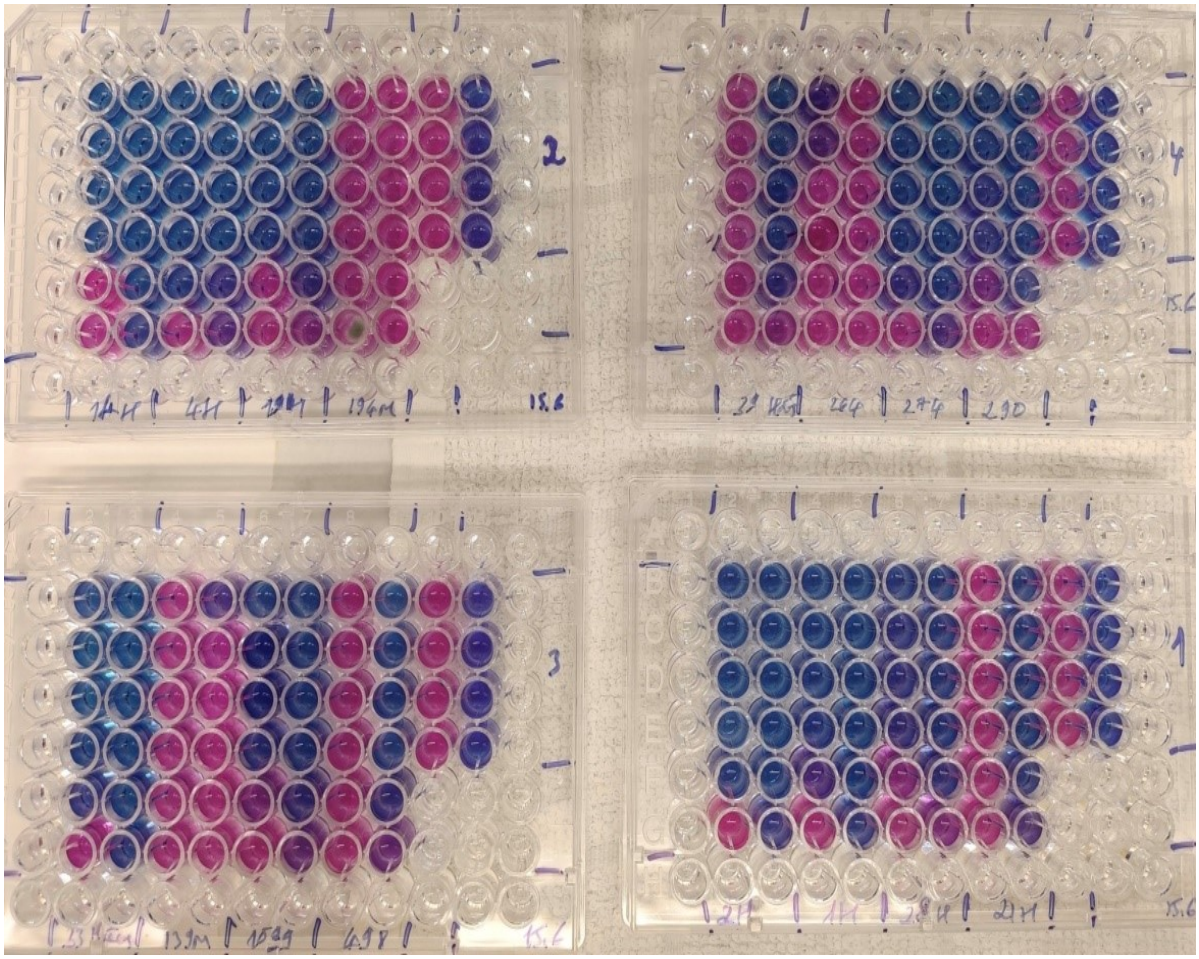

Figure 11. The REMA results

MIC is defined as the lowest concentration of drug that inhibits bacterial growth and in the Palomino test was evidenced by the lowest concentration of drug present in the well where resazurin was not reduced and therefore retained the blue colour; in contrast, the pink coloured wells indicate the reduction of the indicator suggesting cell viability and therefore drug resistance. In the case of *Mycobacterium tuberculosis*, the criterion of resistance or susceptibility was defined as follows: a strain was considered resistant to isoniazid if the MIC was ≥ 0.25µg/ml, for RIF, a strain was considered resistant if the MIC was ≥ 0.5µg/ml.

3.5. DNA extraction

DNA extraction from M. tuberculosis isolates and M. tuberculosis clinical samples:

Fifty two clinical specimens and 52 corresponding isolate samples which were identified as *M. tuberculosis* complex were used extracted for genomic DNA. 200µl of sample was placed in an Eppendorf tube, then 400µL InstaGene™ Matrix (Biorad, CA, USA) was added, following by vortex and incubation at 100^0C for 10 minutes. Then, the mixture was centrifuged at 14.000rpm for 2 minutes and the DNA in the supernatant was collected and stored at -20^0C.

3.6. Real-time PCR assay for identifying *M. tuberculosis* complex

A qualitative real-time PCR assay (LightPower iVAMTB rPCR Kit from VietA Company, Vietnam), which identify the members of the *M. tuberculosis* complex for the 52 isolates cultured positive.

The kit is developed to increase the sensitivity of the assay by using the target 16S rRNA gene in association with the insertion sequence IS6110. A *Mtb* specific TaqMan probe with a FAM dye label on the 5' end and non-fluorescent a quencher dye (TAMRA) on the 3' end, and a pair of unlabelled *Mycobacteria* specific PCR primers for the 16S rRNA gene target with the 95 base-pair amplicon are designed. Add 5µl of template DNA to 20µl of a ready-to-use master mixture and perform PCR amplifications in 25µl final volume.

The target DNAs were amplified as follows: initial denaturation at 95°C for 5 minutes, 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, in Mx3000P qPCR System (Agilent Technologies Inc., CA, USA).

3.7. Real-time PCR TaqMan allelic discrimination assay

52 clinical isolates of *M. tuberculosis* (31 MDR-TB, 01RIF mono-resistant, 10 INH mono-resistant, 10 drug-sensitive isolates) and 52 clinical samples correlatively, were performed the real-time PCR TaqMan allelic discrimination assay.

3.7.1. Primers and probes

The primers used for the allelic discrimination test were designed to amplify a sequence of 208 bp of the *rpoB* which cover the RRDR of 81bp and 110 bp of the *katG*.

In this study, four TaqMan wild-type probes, which discriminate one-base mismatches, were used to detect the genetic variation in the *rpoB* and *katG* in the wild-type *M. tuberculosis* strain (H37Rv). Two fluorescence dyes (FAM, HEX) were labelled to probes at the 5′- end and a BHQ at the 3′- end to simultaneously detected in a single PCR reaction to identify mutations in the hot spots 81bp region of the *rpoB* (rpoB1 probe 510 - 516 and rpoB2 probe 526-531). To detect the mutation of *katG*, the *katG* probe was designed located at position 311-316. The TB control probe was designed outside the hot spot 81-bp located at position 498-505 of the *rpoB* as a control probe to determine the amount of DNA and identify *M. tuberculosis* [64,70,71]. All primers and probes in our study were produced by Integrated DNA Technologies, Inc., USA.

Table 5. Primers and probes used in the real-time PCR TaqMan allelic discrimination assay.

Primer or Probe	Target-region	Conc (µM)	Oligonucleotide	Product size (bp)	Design
Real-time PCR					
Primers	*rpoB*	1.0	F: 5'-TCACACCGCAGACGTTGATC-3' R: 5'-CGTAGTGCGACGGGTGC-3'	208	[71]
	KatG	1.0	F: 5'-GGGCTTGGGCTGGAAGA-3' R: 5'-GGAAACTGTTGTCCCATTTCG-3'	110	
Probes	*rpoB* TB control	0.5	5'-HEX-CGATCAAGGAGTTCTTCGGCACCA-BHQ-3'		[71]
	*rpoB*1 510-516	0.5	5'-FAM-CAGCTGAGCCAATTCATGGACCAGA-BHQ-1-3'		
	*rpoB*2 526-531	0.5	5'-HEX-CACAAGCGCCGACTGTCGGC-BHQ-1-3'		
	katG 311-316	0.5	5'-FAM-ACGCGATCACCAGCGGCA-BHQ-1-3'		
Nested PCR	*rpoB*	1.0 1.0	F: 5'- GTCAGACCACGATGACCGTT-3' R: 5'- GAGCCGATCAGACCGATGTT-3'	445	This study
	KatG	1.0 1.0	F: 5'- CCCATGTCTCGGTGGATCAG-3' R:5'-GGCGGTCACACTTTCGGTAA-3'	475	
IPC Primer	*MecA*	1.0 1.0	F :5'-GACCGAAACAATGTGGAATTGG-3' R: 5'-AGTGGAACGAAGGTATCATCTTG-3'	176	This study
	rpoB/MecA	1.0	F:5'**TCACACCGCAGACGTTGATCGACCGAAACAATGTGGAATTGG-3'** R:5'**CGTAGTGCGACGGGTGCAGTGGAACGAAGGTATCATCTTG-3'**	213	
IPC Prober	*S.aureus(MecA)*	0.5	5'-CY5-ACAGCATATGAGATAGGCATCGTTCC-BHQ-2-3'		
Sequencing Primers	*rpoB*	1.0 1.0	F: 5'- GTCAGACCACGATGACCGTT-3' R: 5'- GAGCCGATCAGACCGATGTT-3'	445	This Study
	KatG	1.0 1.0	F: 5'- CCCATGTCTCGGTGGATCAG-3' R:5'- GGCGGTCACACTTTCGGTAA-3'	475	

3.7.2. Procedure

For detecting the mutation in selected regions in the *rpoB* and *katG*, 2 separate reactions were performed in tube A (for detecting mutation in the *rpoB*) and tube B *(*for detecting mutation in the *katG*). The final reaction volume of 25µl was used in each tube. Master Mix produced by Integrated DNA, USA. Technologies Pte. Ltd, USA.

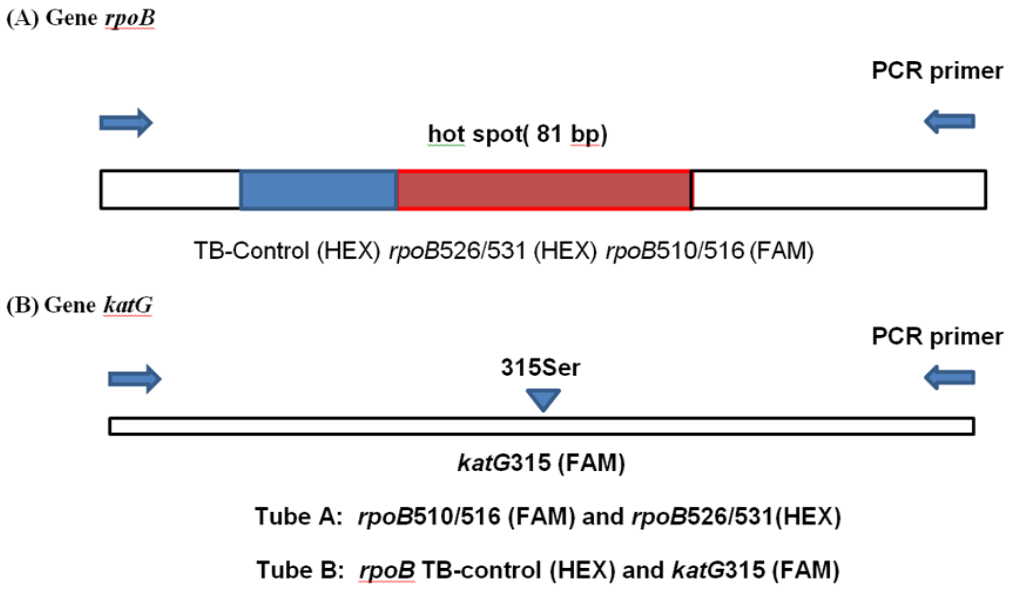

Figure 12. Schematic description of the assay

The optimized multiplex-probe real-time PCR reaction was established in this study as in table 6, the PCR amplification was profiled as follows: initial denaturation at 94ºC for 10 minutes, followed by 40 cycles of 94ºC for 25s, 60ºC for 55 s, in Mx3000P qPCR System (Agilent Technologies Inc., CA, USA).

Table 6: The concentration and volumes of reaction components in the multiplex-probes real-time PCR TaqMan allelic assay.

Tube A			**Tube B**		
Components	**Concentration**	**V(µl)**	**Components**	**Concentration**	**V(µl)**
Master Mix(2X Conc)*	2X	**12.5**	Master Mix(2X conc)	2X	**12.5**
Primer *rpoB*-F(20 pmol)	10µM	**1**	Primer *katG*-F(20pmol)	10µM	**1**
Primer *rpoB*-R(20 pmol)	10µM	**1**	Primer *katG*-R(20pmol)	10µM	**1**
Probe *rpoB* 1(5pmol)	10µM	**0.5**	Primer *rpoB*-F(20pmol)	10µM	**1**
Probe *rpoB* 1(5pmol)	10µM	**0.5**	Primer *rpoB*-R(20pmol)	10µM	**1**
DNA (ng/µl)	Ng	**1**	Probe *katG*(5pmol)	10µM	**0.5**
dH_2O		**8.5**	Probe TB control(5pmol)	10µM	**0.5**
			DNA (ng/µl)	ng	**1**
			dH_2O		**6.5**
Total		**25**	**Total**		**25**

IPC for detecting PCR inhibitors

Internal process control (IPC) plays an essential role in the detection of PCR inhibitors, especially when it is necessary to identify *M. tuberculosis* and DR-TB from clinical samples of patients with suspected TB. In the standard RT-PCR reaction, IPC normally consists of a well-known DNA fragments that have a different sequence from the target gene but can be ligated by the same gene primers. In this study, the IPC was designed based on the *mecA* gene of *Staphylococcus aureus* (ATCC25923). The primers and rpoB/MecA probe were designed using Primer Express 3.0 (Applied Biosystems, Foster City, CA) to target a conserved segment of the *MecA* gene in *S. aureus* (GenBank accession no. L27989). The rpoB/MecA probe consists of a specific Taqman probe labelled with CY5 at the 5' end and BHQ-2 at the 3' end.

Firstly, *MecA* primers were used to amplify *MecA* amplicon in a final volume 25µl reaction volume with Master Mix(2X Conc)x 12,5µl, 20pmol each of primers x 1µl, 5µl of template DNA of *S.aureus*, following thermal cycle: initial denaturation at 95^0C for 5 minutes followed by 40 cycles of 95^0C for 50 seconds and 55^0C for 30 seconds and 72^0C for 30 seconds, followed final extension at 72^0C for 2 minutes in Veriti® Thermal Cycler (Applied Biosystems, CA, USA).

Secondly, PCR products were purified by using the MEGAquick-spin™ plus Fragment DNA purification and used as template to produce IPC. Purified PCR products (5ng) were used as a template for PCR reaction with primers rpoB/MecA following thermal cycle: initial denaturation at 95^0C for 5 minutes followed by 40 cycles of 95^0C for 50 seconds and 55^0C for 30 seconds and 72^0C for 30 seconds, followed final extension at 72^0C for 2 minutes in Veriti® Thermal Cycler (Applied Biosystems, CA, USA). The final product was purified again by MEGAquick-spin™ plus Fragment DNA purification and the concentration were quantified by NanoDrop.

Lastly, serial dilution of PCR amplicon 0.1ng/ µl (1000GE) was used to

perform real-time PCR with rpoB primers to determine the appropriate amount of IPC template. IPC templates were added into the real-time PCR master mix using rpoB primers and worked as a control to detect PCR inhibitors.

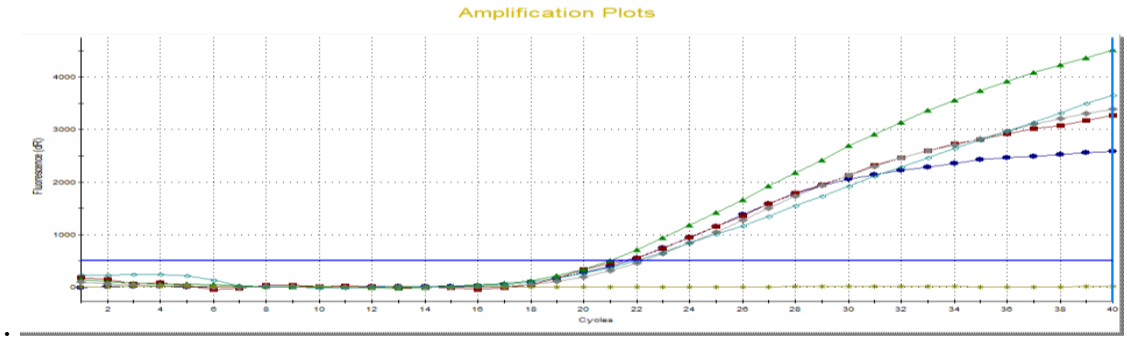

Figure 13: The fluorescence of the four probes in Real Time PCR Taqman allelic assay; one probe IPC positive; and one no-template control. The C_T is expressed as the number of cycles to reach the threshold. The x axis shows cycle numbers of PCR, and the y axis represents the normalized report signal (ΔRn).

Nested PCR

There is a difference in the concentration of clinical samples which showed Ct > 38 in real-time PCR amplification (IPC was positive). The nested PCR was performed for *rpoB* and *katG* with all reactions which Ct value above 38. The PCR reaction was carried out in a total final 25µl reaction volume with Master Mix(2X Conc)x 12,5µl, 20pm each of primers x 1µl, 5µl of template DNA. The PCR conditions were as follows: initial denaturation at 94°C for 5 minutes and then 40 cycles of 94°C for 30 seconds, after that 60°C for 30 seconds, and 72°C for 30 seconds in Veriti® Thermal Cycler (Applied Biosystems, CA, USA).

After the first PCR, there was diluted this amplified product 100-fold with sterilized water, and then the targets were analysed by using the multiplex-probe real-time PCR reaction by using 1µl of this final product, which was established in this study as in the table 6.

Electrophoresis Amplification products were loaded on a 1.5% agarose gel stained with TBE buffer containing 0.5µg/ml of ethidium bromide (Sigma), under 110V in 20 minutes, and observed under the ultraviolet light.

3.7.3. Allelic Discrimination Data analysis

Data were analysed in the qPCR Mx3000P system (Agilent Technologies Inc., CA, USA). The fluorescence of hybridized probes was expressed as a reporter signal. The number of amplification cycles required for emission of a certain luminescence intensity by each probe (ΔRn=0.2), reflected the amount of DNA in the sample. This cycle number was called the threshold cycle (Ct). Therefore, the presence of a mutation would increase Ct (Ct was higher when mutations in the genes were present).

We measured the Ct derived from the control TB probe bound to the outside of the 81 bp hot spots in the *rpoB* and the ΔCt, which expressed the difference between the control and each probe (ΔCt = mutant Ct- control TB Ct). The ΔCt was higher (≥6.65) when there were mutations in the target DNA that had to hybridise with the TaqMan probe.

In these mutant genotypes, the variation of a single base in the target sequence in the *rpoB* or *katG* could prevents matching of the corresponding probe and combination with the target consequently, dropout of the probe from the sequence occurs; negative fluorescence signals were produced during amplification and the Ct values of the mutant sequences were determined to be negative (CT = 0). Finally, *M. tuberculosis* was identified using the TB control probe.

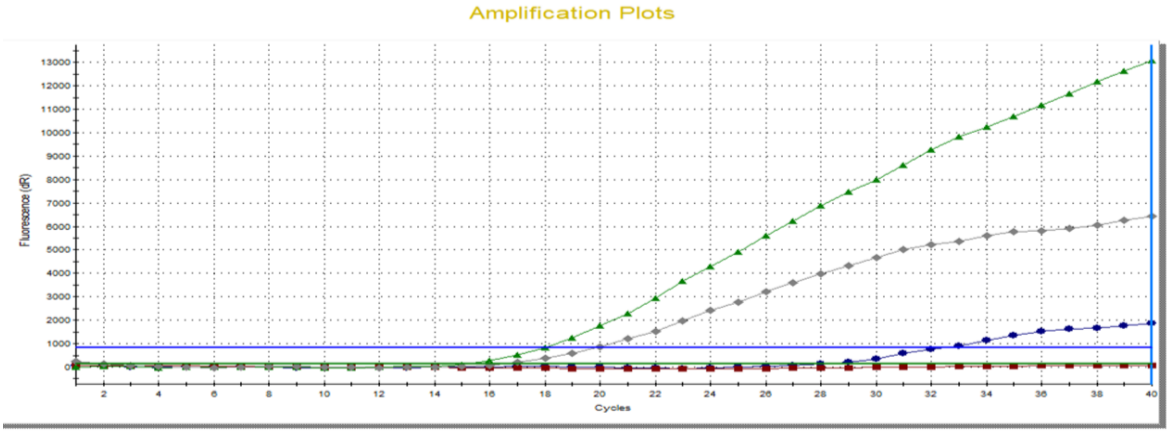

Figure 14. Analysis of DNAs from *M. tuberculosis* with two mutant probes by multi-fluorescence real-time PCR CT = 0 (negative), and other probe Ct =30 (ΔCt =12).

Analytical sensitivity.

This assay was sufficiently specific for *M. tuberculosis*. The evaluation of the sensitivity was performed by diluting genomic equivalent DNAs of H37Rv, The amplification curves of *M. tuberculosis* (10 GE/tube to 10^7 GE/ tube) through real-time PCR with the primers and probes.

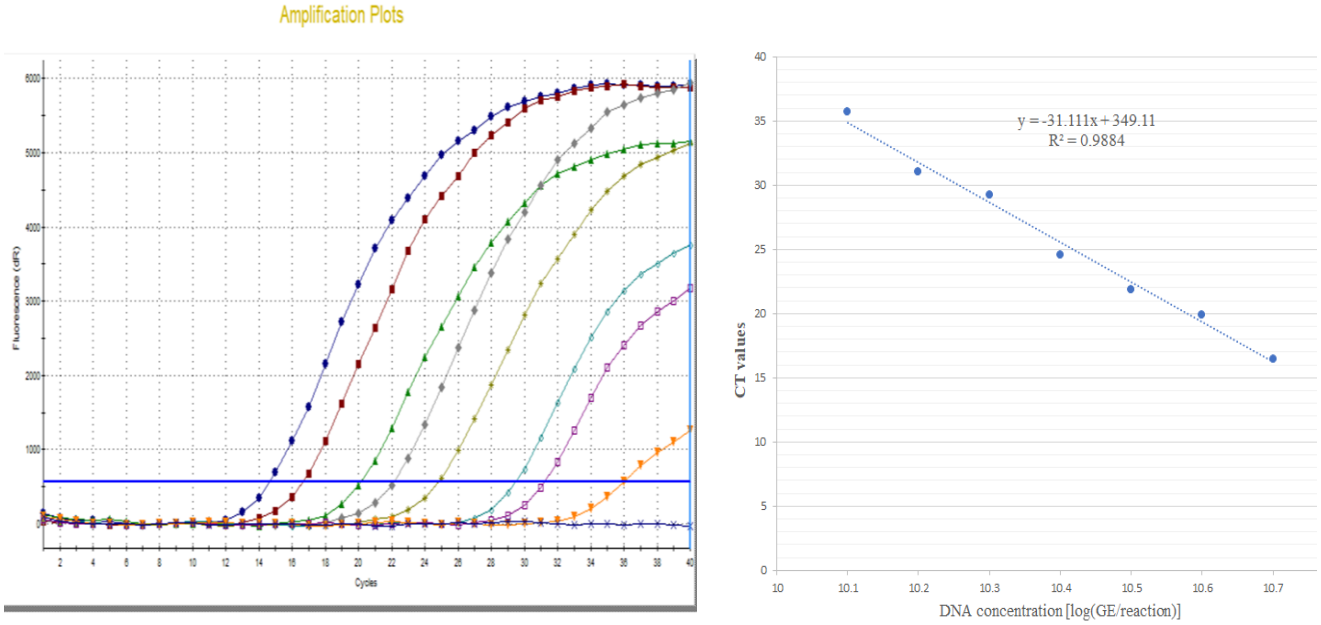

Figure 15. The sensitivity of the probe assay for the detection of *M. tuberculosis* H37Rv through real-time PCR with the *rpoB* primers and TB control probe.

3.8. Sanger sequencing

3.8.1. Sample preparation

DNA samples extracted from the 52 isolates identified by RT-PCR as M. tuberculosis were used to amplify the *rpoB* and *katG* by conventional PCR. A 445 bp fragment of the rpoB primer (include the 81 bp hot-spot region) and a 264 bp fragment of the *katG* primer (include codon 315) were used.

Reaction components included: DNA template (2 µl), 0.4 µM for each primer, 0.2 mM for each dNTPs, 0.5 units of Platinum®Taq DNA Polymerase (Invitrogen, Thermo Fisher Scientific Inc.), were combined in a 50 µL total

volume reaction.

PCR amplification was performed as follows: initial denaturation at 95°C for 10 min, followed by 36 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s; then a final extension at 72°C for 5 minutes in Veriti® Thermal Cycler (Applied Biosystems, CA, USA).

PCR products were sent to Sanger sequencing at Apical Scientific Sdn. Bhd (Malaysia).

3.8.2. Gene profile analysis

Sequencing data were initially analysed by Sequencing Analysis Software v6.0 (ThermoFisher Scientific) and then, quality control checked by the Sequence Scanner software. Multiple sequence alignment of the *katG* and *rpoB* genes to the reference sequences (H37Rv strain) was performed by Bioedit Alignment Sequence Editor.

3.9. Statistical analysis

-Meta-analysis was performed by using Excel 2010 and MedCalc statistical software 2020 (MedCalc Software, Ostend, Belgium).

-A p-value less than 0.05 were regarded statistically significant.

-Our results were analysed using Fleiss' kappa and agreement

The agreement between the two methods was determined by the kappa (κ) statistic. The κ value, a measure of test reliability, was interpreted as follows:

- Kappa<0: No agreement;
- Kappa between 0.00 and 0.20: Slight agreement
- Kappa between 0.21 and 0.40: Fair agreement
- Kappa between 0.41 and 0.60: Moderate agreement
- Kappa between 0.61 and 0.80: Substantial agreement
- Kappa between 0.81 and 1.00: Almost perfect agreement

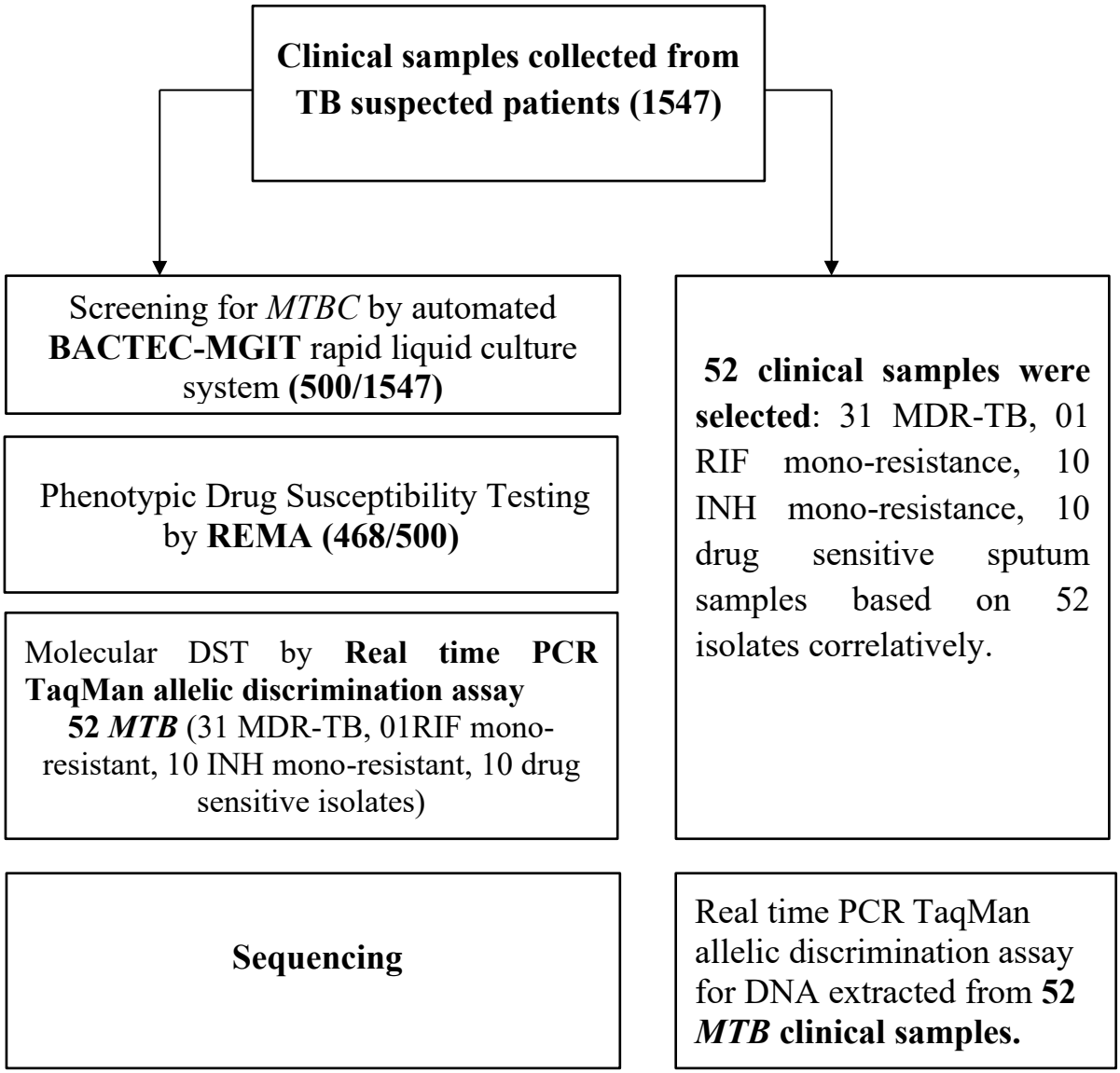

Figure 16. The workflow of study

4. RESULT

In this study, 1547 clinical specimens from patients with suspected TB were examined, of which only 32.32% (500/1547 samples) were isolated positive by using the BACTEC MGIT 960 system and real- time PCR identified all *M. tuberculosis* isolates. A total of the 500 isolates, 6.4% (32/500 samples) were excluded from the DST because they were highly contamination.

The DST was then performed on 468 samples with the average age was 47.59 ± 15.82 (min 1, max 90). In addition, microscopic examination showed a positive result in 56.2% (263) of all the samples considered.

Table 7. The proportion of specimens and AFB smear

Specimens		AFB smear	
		AFB(+)	**AFB(-)**
Total	**468**	**263 (56.2)**	**205 (43.8)**
Sputum	395(84.5)	245 (52.4)	150 (32.1)
Pleural fluid	51(10.9)	9 (1.9)	42 (9.0)
Bronchial lavage	10(2.2)	5 (1.1)	5 (1.1)
CSF	4(0.8)	0 (0.0)	4 (0.8)
Others	8(1.6)	4 (0.8)	4 (0.8)

Among the 468 samples, of these 84.5% were sputum, and pleural fluid, bronchial lavage, cerebral spinal fluid (CSF), and others (gastric fluid, joint fluid, abscess) were 10.9%, 2.2%, 0.8%, and 1.6% respectively. No CSF specimen was AFB positive.

4.1. Phenotypic drug susceptibility testing by REMA

In this study, REMA testing was performed on all 468 *M. tuberculosis* isolates; strains were considered resistant when the MIC (Minimum Inhibitory Concentration) found was > 0.25 μg/ml for INH and 0.5μg/ml for RIF at the cut-off point. Of all isolates, 22.6 % (106) were drug resistant isolates. Specifically, it was observed that of these, 69% (74) were resistant to INH, while 0.94% (1) was resistant to RIF and 29.24% (31) were resistant to both antibiotics (MDR-TB strains). (Table 8)

Table 8. Proportion of TB isolates resistant to TB drugs

Resistant	No. of Isolates	(%)	95% CI
INH	74	69.8	60.1-78.4
RIF	01	0.9	0.02-5.1
MDR-TB	31	29.3	20.8-38.9
Total	**106**	**100**	

Table 9: Proportion of drug resistant *M. tuberculosis* according to patient groups.

History	Overall	INH	RIF	MDR-TB
New TB cases	413(88.2%)	89 (21.6%)	0	23 (5.6%)
Previous TB case	55(11.8%)	16 (29.1%)	1	8 (14.5%)
p	*0.006*	*0.208*	*N/A*	*0.012*

The MDR-TB percentage of *M. tuberculosis* isolates in the group of patients with suspected new tuberculosis was 5.6% while in the group of patients with previous tuberculosis it was 14.5% with statistical significance for a p-value of < 0.05. This finding reflects the epidemiological situation regarding TB drug resistance in Vietnam described in the WHO report 2020, in which the MDR-TB percentage in Vietnam is 3.6% of new cases and 17% previously treated.[1]

Table 10: MICs of INH and RIF for *M. tuberculosis* isolates determined by the REMA plate method.

MIC(µg/ml) of INH mono-resistance						
	≤0.031	0.062	0.125	**0.25**	**0.5**	**≥ 1**
Resistance (n=74)				5 (1.06%)	10 (2.14%)	59 (12.6%)
Susceptible (n=363)	197 (42.09%)	124 (26.50%)	42 (8.97%)			
MIC(µg/ml) of RIF mono-resistance						
	≤0.062	0.125	0.25	**0.5**	**1**	**≥ 2**
Resistance (n=01)						01 (0.21%)
Susceptible (n=436)	430 (91.88%)	5 (1.07%)	1 (0.21%)			
MIC(µg/ml) of INH and RIF resistant to 31 MDR-TB isolates						
				0.25	**0.5**	**≥ 1**
INH Resistance				01 (0.21%)		30 (6.41%)
				0.5	**1**	**≥ 2**
RIF Resistance				7 (1.49%)	2 (0.43%)	22 (4.7%)

REMA results showed that the number of isolates found to be sensitive to INH was 77.56% (363 strains) with a MIC ≤ 0.125µg/ml. The remaining 15.82% (74 INH-mono resistance strains) were resistant with MIC ≥ 0.25µg/ml; in particular of these, MICs were higher than 1µg/ml for 12.6% (59 strains).

Regarding RIF, the REMA showed that out of 468 isolates, 93.16% (436 isolates) were susceptible to RIF with a MIC ≤ 0.25µg/ml while the only RIF mono resistant with a MIC ≥ 2µg/ml.

In addition, 31 MDR-TB were found by the REMA. They showed that a MIC ≥ 1µg/ml with 6.41 % (30 isolates) for INH resistant and a MIC ≥ 2µg/ml with 4.7% (22 isolates) for RIF resistant.

Table 11. Comparing the results of REMA with those of reference method.

Drugs	REMA		BACTEC MGIT 960		TESTING SCREERING				
	Resistance	Susceptible	Resistance	Susceptible	Se %	Sp %	PPV %	NPV %	Accuracy %
INH*	74	394	71	397	100	99.19	96.19	99.72	99.35
RIF*	01	467	01	467	100	100	100	100	100
RH	31	437	30	438	100	99.77	96.77	100	99.78

INH*: INH – mono resistance; RIF*: RIF – mono resistance; MDR-TB*: Isolates resistant to INH and RIF; Se*: Sensitivity, Sp*: Specificity, PPV*: Positive predictive value, NPV*: Negative predictive value.

The REMA results from this study were compared with those that were obtained using the BACTEC MGIT 960 system, independently with REMA results, with three isolates were found to be resistant to INH by the REMA; however, they were found to be susceptible by the reference method. One isolate was found to be resistant to INH and RIF by REMA, although found to be susceptible by the reference method.

The sensitivity for INH resistant was 100% and the specificity was 99.19 %. The specificity and the specificity RIF resistant were respectively 100%. The overall concordances were 99.78% for MDR-TB, the PPV and NPV were respectively 96.77 % and 100 % for MDR-TB.

4.2. Genotypic detection of drug resistant isolates by real-time PCR TaqMan allelic discrimination assay.

4.2.1. Real –time PCR TaqMan allelic discrimination results for clinical isolates.

Table 12. Luminescent patterns of real-time PCR TaqmMan allelic discrimination data and DNA sequencing data for detection of mutations conferring RIF- and/or INH resistant *M. tuberculosis*.

Mutant position Sequencing	Nucleotide Substitution	No.of Strains	Control C_T [a] (mean ± SD)	ΔC_T [b](mean±SD)		
				rpoB510/516	**rpoB526/531**	**katG315**
No mutation		21				
*rpoB*511 *rpoB*515 *katG*315	CTG→CCG ATG→GTG AGC→ACC	1[c]	16.80	**N**[d]	1.80	-0.4
*rpoB*516	GAC→ TAC	1	20.10	**N**	-0.95	-1.49
*rpoB*516			23.66	N	-0.18	-2.67
*rpoB*516 *katG*315	GAC→ TAC AGC→ACC	1	24.45	**N**	-0.69	**N**
*rpoB*522[e] *katG*315	TCG→TTG AGC→ACC	1	29.86	-0.96	-0.56	**N**
*rpoB*526	CAC→ CCC	2	21.43±0.63	-0.38±0.02	**N**	-0.37±0.13
*rpoB*526	CAC→ TAC	1	23.66	-2.38	**N**	-3.38
*rpoB*526	CAC→ TGC	1	18.44	1.58	**N**	0.59
*rpoB*526 *katG*315	CAC→AAC AGC→ACC	1	29.12	-0.55	**N**	**N**
*rpoB*526 *katG*315	CAC→TAC AGC→ACC	1	18.64	2.26	**N**	**> 13.45**
*rpoB*531	TCG→ TTG	6	24.49±3.60	0.64±1.82	**> 9.89**	0.27±1.96
*rpoB*531 *katG*315	TCG→ TTG AGC→ACC	6	24.32±3.46	-0.54±0.94	**> 8.71**	**>7.18**
*katG*315	AGC→ACC	8	20.89±1.61	-0.83±1.13	0.30±1.95	**>6.65**
KatG315			**21.37**	**-0.84**	**0.46**	**N**

[a] Cycle number required when luminescence of TB control probe reaches the threshold (Threshold of cycle).
[b] Difference in CT of TB control probe and corresponding probe(ΔC_T = C_T mutant- C_TTB control)
[c] Sequence results
[d] No C_T

Table 12 shows that 12 groups based on their genotypes by nucleotide sequencing of 52 *M. tuberculosis* DNAs. When ΔCt was higher (≥6.65) and probes dropout from the sequence occurs them negative fluorescence signals

(C_T=0), mutations existed in the target DNA that should hybridize with TaqMan probes.

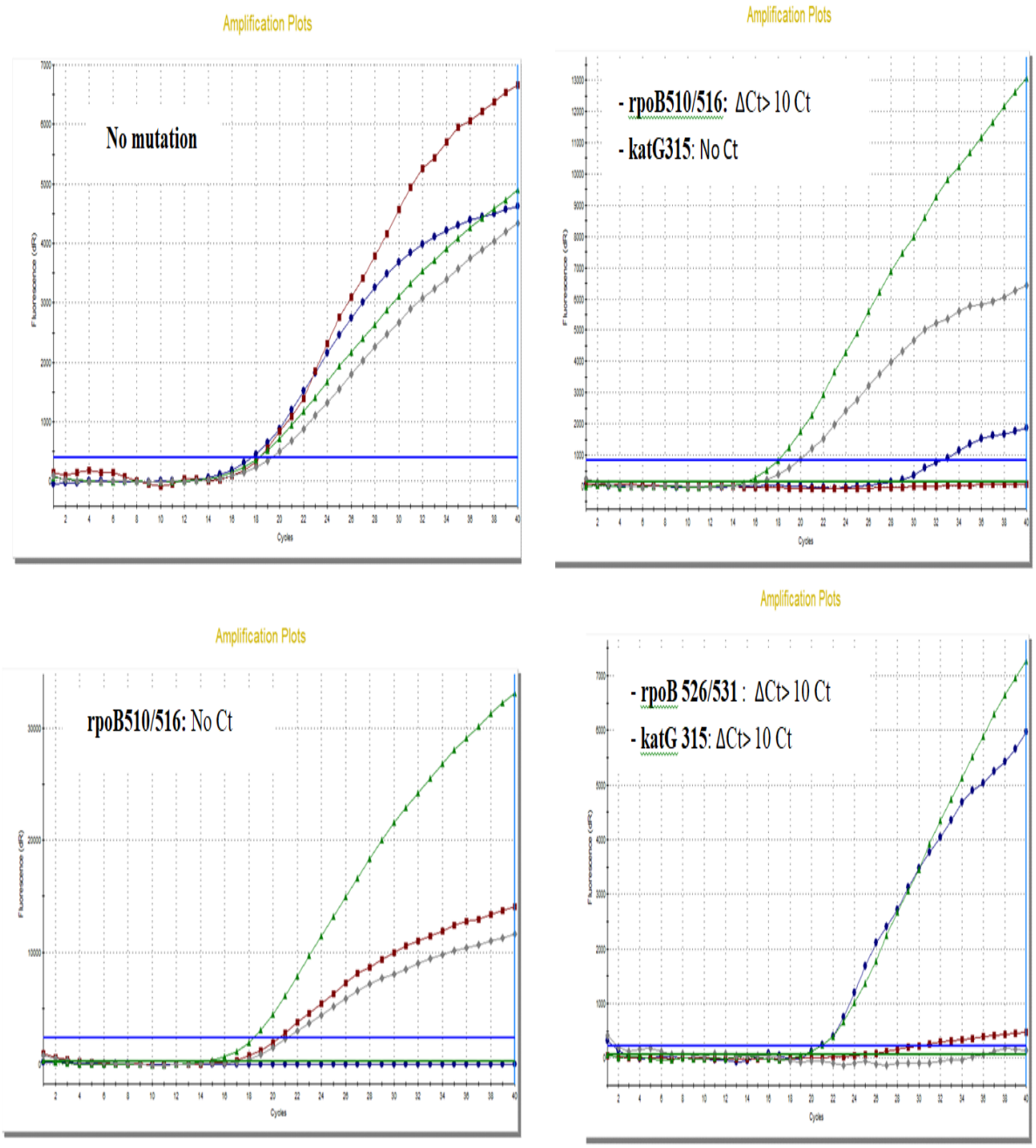

Figure 17. Analysis of DNAs from *M.tuberculosis* isolates with four Taqman probes by multi-fluorescence real-time PCR.

The DNA had mutations in the *rpoB* at position rpoB codon 510/516, codon 526/531 and in the *katG* at position 315, the corresponding probes showed no luminescence signal (Ct=0) or ΔCt increase.

Table 13. INH and RIF resistant – conferring mutations DNA of DR-TB isolates by using sequencing.

Drug	Resistance phenotype	Resistance genotype			Total(%)
		Gene	Mutations	No.of strain	
INH	MDR TB (31)	***katG***	Ser315Thr	**15**	15/31(48.4)
			No mutation	**16**	16/31(51.6)
	INH mono resistant(10)		Ser315Thr	**4**	4/10(40)
			No mutation	**6**	
	Susceptible(11)		No mutation	**11**	11/11(100)
RIF	MDR-TB (31)	***rpoB***	Leu511Pro[a]		
			M515Val[a]	**1**	
			Asp516Tyr	**2**	21/31(67.7)
			S522L[b]	**1**	
			His526Asp	**1**	
			His526P	**2**	
			His526Tyr	**2**	
			His526Asn	**1**	
			Ser531Leu	**11**	
			No mutation	**10**	10/31(32.3)
	RIF mono resistant (01)		Ser531Leu	**01**	01/01(100)
	Susceptible (20)		No mutation	**20**	20/20(100)

[a] Double point mutations were observed in one strain
[b] Mutation outside the research probes

Table 13 showed detailed information about the mutations that detected in the *rpoB* 81-bp core region and the *katG* by sequencing results.

A total of 19/41(46.34%) of the phenotypic INH resistant isolates had the mutation at codon Ser315Thr (AGC→ACC). Eight types of the *rpoB* mutations were found in 22/32 (67.7%) from 31 MDR-TB and 01 RIF-resistant isolates. The frequently mutations in *rpoB* at codons 531, 526 and 516 were 37.5% (12/32), 18.75% (6/32) and 6.25% (2/32), respectively. At codon Ser522Leu (TCG→TTG) was detected by sequencing analysis by this point mutation outside observed of two probes determine

mutations in the *rpoB*. Of 11/11 INH Susceptible isolates and 20/20 RIF Susceptible isolates had no mutations in *katG* and *rpoB*.

Table 14. Sensitivities and specificities of Real-Time PCR TaqMan allelic discrimination results compared to DNA-sequencing results.

Real-time PCR TaqMan allelic assay from *M. tuberculosis* isolates	**DNA-Sequencing**			
	rpoB		***katG***	
	Mutation	No mutation	Mutation	No mutation
Mutation	21	1	19	1
No mutation	0	30	0	32
Total	**21**	**31**	**19**	**33**
Se	95.5(77.16-99.88)		95.0(75.1-99.8)	
Sp	100 (88.43-100)		100 (89.11-100)	
PPV	100		100	
NPV	96.8 (81.6- 99.5)		96.9(82.5 to 99.5)	
Accuracy*	98.1(89.7-99.9)		98.08 (89.7-99.9)	

**) depend on the prevalence of disease*

Statistical data for performance are shown in Table 14 with the sensitivity and specificity of INH resistance detection by mutation analysing *katG* codons 311-316 on the real-time PCR allelic and DNA sequencing results were found 95.5 % and 100%, respectively. It yielded 98.08% accuracy in comparison to that of sequencing for the *katG*. The sensitivity and specificity of RIF resistance detection by mutation analysis in the *rpoB* codons 510-531 by the real-time PCR allelic and DNA sequencing results were found 95.00 % and 100%.

4.2.2. Real - time PCR TaqMan allelic discrimination assay results for *M. tuberculosis* from clinical samples.

Table 15. *M. tuberculosis* isolates confirmed by Real-time PCR Taqman Allelic Discrimination assay.

Sample type and analysis		**No.of sample**	**No.(%) detected**	**No.(%) undetected**
DNAs from MTB isolates		52	52(100)	0(0)
DNAs from clinical samples	**Direct analysis**	52	31(59.6)	21(40.4)
	Pre-amplified DNA (after nested PCR)	21	52(100)	0(0)

As showed table 15, all of 52 *M.tuberclosis* isolates from cultured were positive TB control rpoB probe in the *rpoB*.

However, only 59.6% (31 out of 52) *M. tuberculosis* from clinical samples showed strong luminescence in real-time PCR TaqMan allelic assay, and from AFB smear positive mainly, while the pulmonary specimens consisted of sputum with 48 samples, two plural pulmonary, bronchial aspirate is one and one join abscess. 40.4% (21 clinical samples) no luminescence.

Table 16. Sensitivity of real –time PCR TaqMan allelic discrimination assay for clinical sample before and after nested PCR.

Microscopy score[a]	n=52	Ct TB Control (Mean±SD)	Ct TB Control (Mean±SD) after nested PCR
Positive 3+	14	27,34±2,72	-
Positive 2+	10	27,94±1.58	-
Positive 1+	5	29,31±2.03	-
	1*	Undetermined >40	14.05
Positive +	2	30,32±0.56	-
	5*	Undetermined >40	10,66±2,66
Negative	15*	Undetermined >40	11,32±2.33

[a] Microscopy scoring was done according to the WHO standard, with the scale defined as follows: Negative: no acid-fast bacilli (AFB) observed; Positive + (Scanty): 1 to 9 AFB in 100 fields; 1+: 10 to 99 AFB in 100 fields; 2+: 1 to 10 AFB per field in at least 10 fields;3+, >10 AFB per field in at least 10 fields.

A total of 21 (40.8%) samples showed no luminescence this mean undetermined Ct even after 40 cycles of PCR amplification, most of them were AFB smear-negative at 15/15 (100%) samples, 6/37(16.2%) of AFB smear-positive with AFB positive 1+ and AFB positive + .

It seems that the amount DNAs of *M. tuberculosis* in AFB smear negative and AFB positive + are small, less than 10^1 GE DNAs of *M. tuberculosis* in each clinical sample.

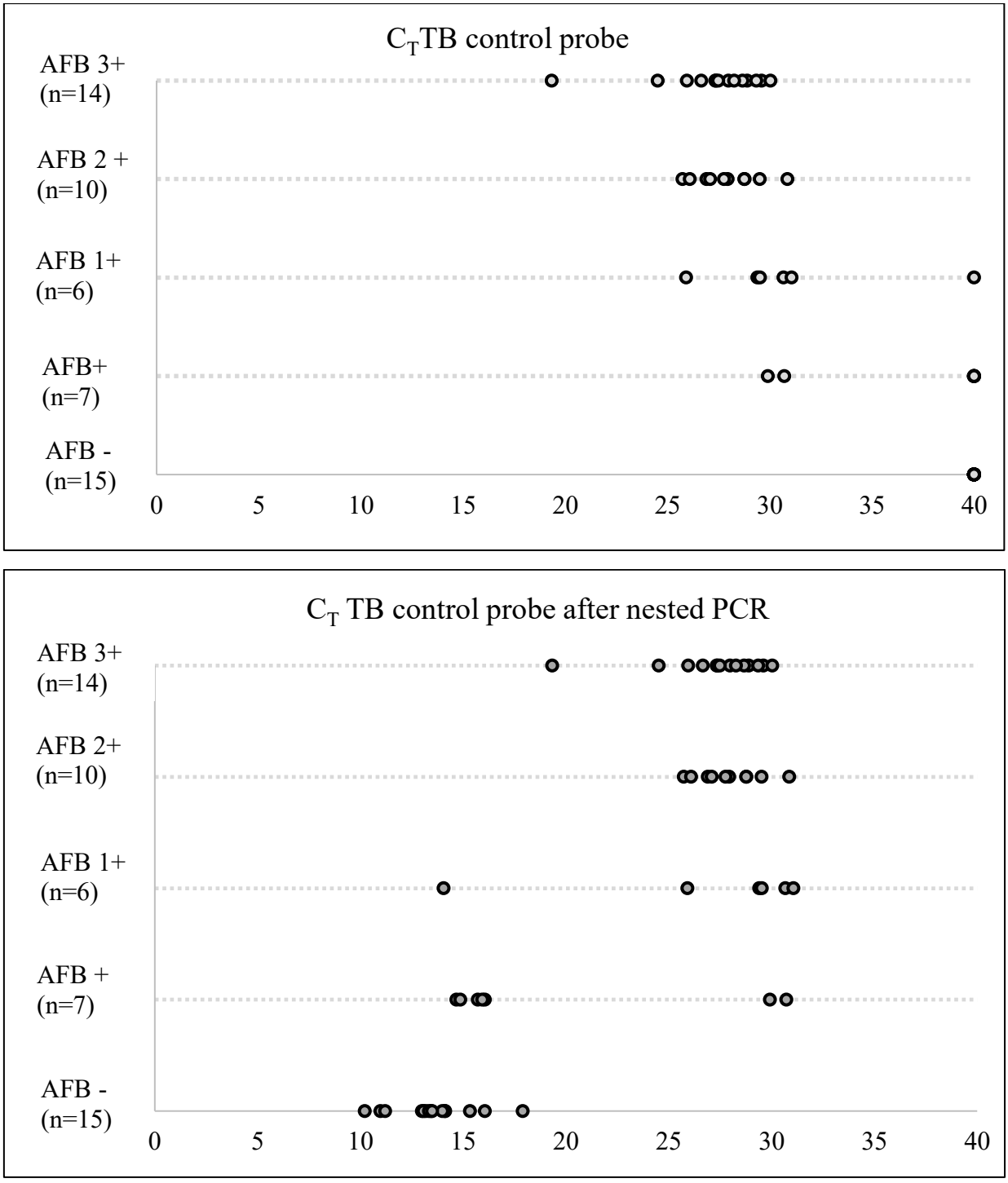

Figure 18. Luminescence of TB control probe reaches the threshold of real-time PCR TaqMan assay for clinical samples before and after nested PCR.

Figure 18 showed that a total of 100% strong luminescence signal and the Ct of the TB control probe were small from cycle 10 to 20. This is corresponding with

the least amount of 10^5 GE DNAs of *M. tuberculosis* in each clinical sample after we performed the nested PCR.

Table 17. Sensitivities and specificities of Real-Time PCR TaqMan allelic discrimination assay from clinical samples results compared to DNA-sequencing results.

Real-time PCR Taqman allelic assay Clinical samples	**DNA-Sequencing**			
	rpoB		***katG***	
	Mutation	No mutation	Mutation	No mutation
Mutation	21	2	19	2
No mutation	0	29	0	31
Total	**21**	**31**	**19**	**33**
Se	100 (83.16-100)		100 (82.35-100)	
Sp	93.55 (78.58-99.21)		93.94(79.77-99.26)	
PPV	99.66(99.55-99.51)		99.68(98.79- 99.94)	
NPV	100		100	
Accuracy*	99.68 (84.05-98.79)		99.70(86.79-99.53)	

(*) depend on the prevalence of disease

Corresponding DNA sequences of the *rpoB* and *katG* were analysed in the four discordant samples. The Table 18 showed the sensitivities 100% for both *katG* and *rpoB* but the specificities were 93.55% (78.58-99.21) for the *rpoB* and 93.94% (79.77-99.26) for the *katG*.

As expert, a high of sensitivities and specificities of Real-Time PCR TaqMan allelic discrimination assay from clinical samples results compared to DNA-sequencing results.

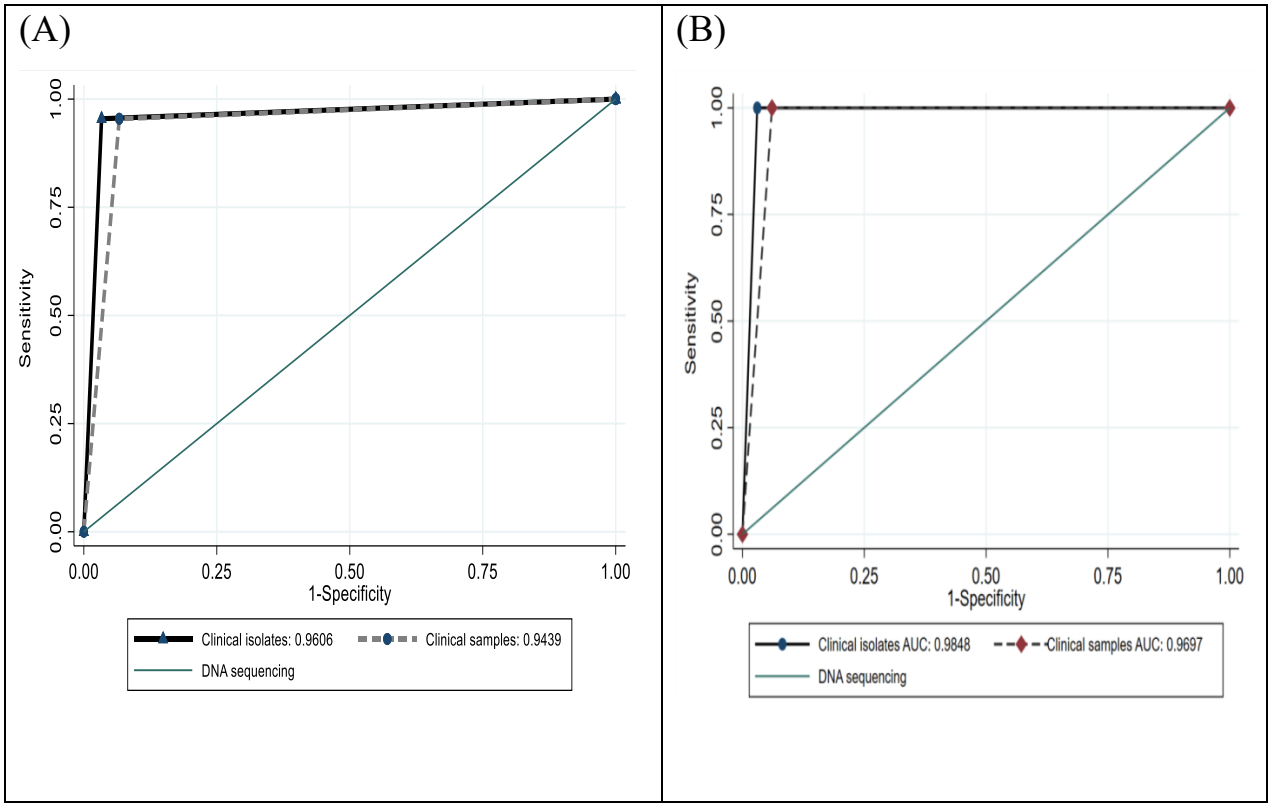

Figure 19. The ROC curve analysis the sensitivities and specificity of real-time PCR TaqMan allelic discrimination to detect drug resistance on paired clinical samples, isolates based on DNA sequencing in *rpoB* (A) and *katG* (B).

There is no statistically significant difference in diagnosis mutations of the *rpoB* and the *katG* by using real-time PCR TaqMan allelic discrimination for *M. tuberculosis* strains from clinical isolates and clinical sample to comparing with reference method.

4.3. Correlation between genetic and phenotypic DST data

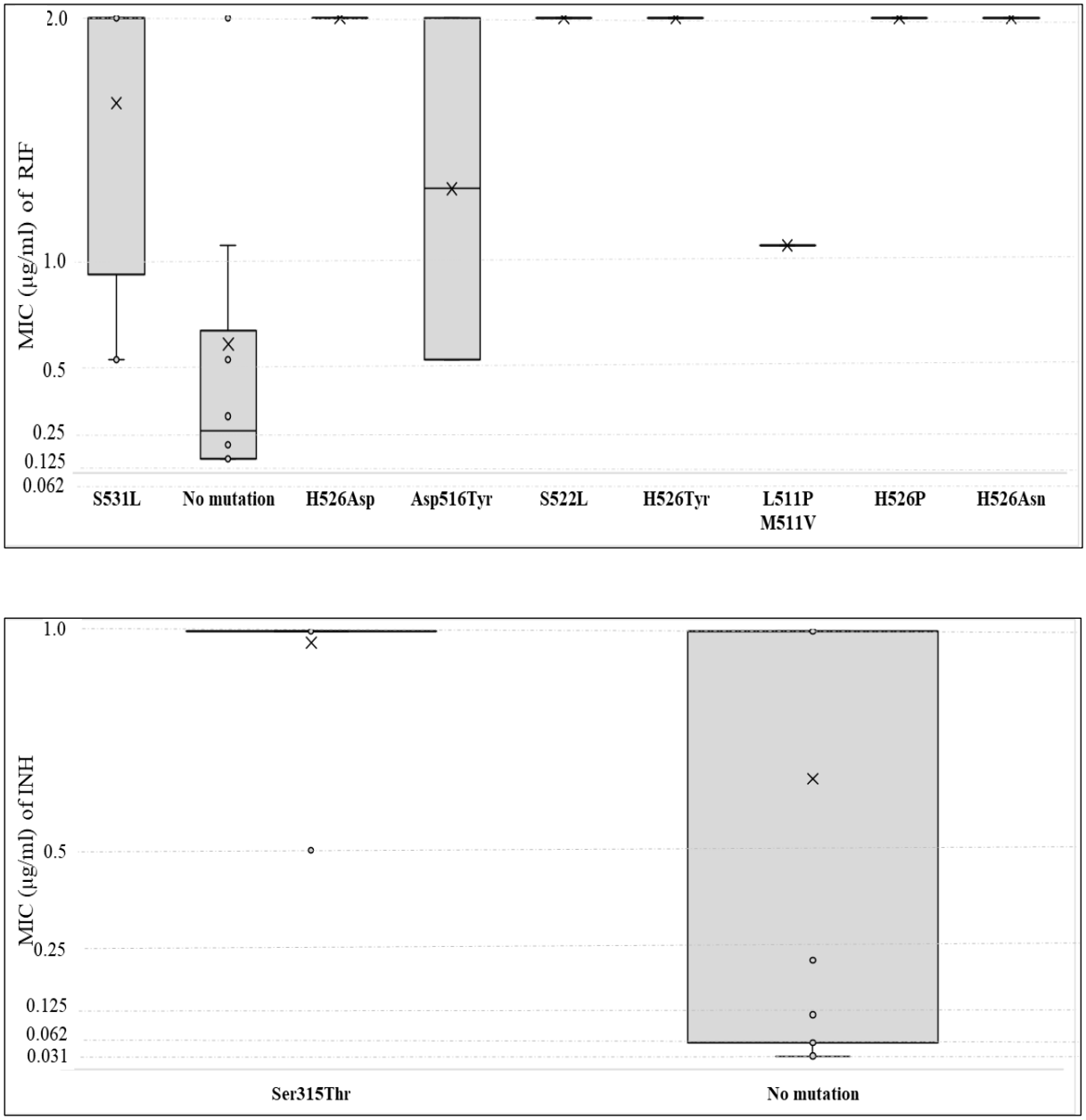

Figure 20. Relationships between genotype and MIC

Figure 20 in the part of results showed that most of the point mutations in either codon 516, 526, or 531 was found in 77.3% (17 out of 22) strains requiring MICs ≥ 2µg/ml in the association between MICs of RIF, genetic alterations in the *rpoB*, and 94.7% (18/19) strains showed point mutations in codon 315 of the *katG*, required MICs of 1µg/ml. In the present study, a double point mutation

was detected in one strain at codon L511P and codon M515V in the *rpoB* requiring MICs of 1µg/ml.

Table 18. Sensitivities and specificities of Real-Time PCR TaqMan compared to REMA results.

REMA	Genes Real-time PCR Taqman allelic assay			
	rpoB		*katG*	
	Mutation	No mutation	Mutation	No mutation
Resistant	22	10	20	21
Susceptible	0	20	0	11
Total	**22**	**30**	**20**	**32**
Kappa (CI95%)	**0.63(0.44-0.82)**		0.29(0.12-0.45)	

In our study, the concordance between the mutations results of the *rpoB* and the *katG* and REMA drug susceptibility testing was slightly lower than expected with 0.63 and 0.29, respectively.

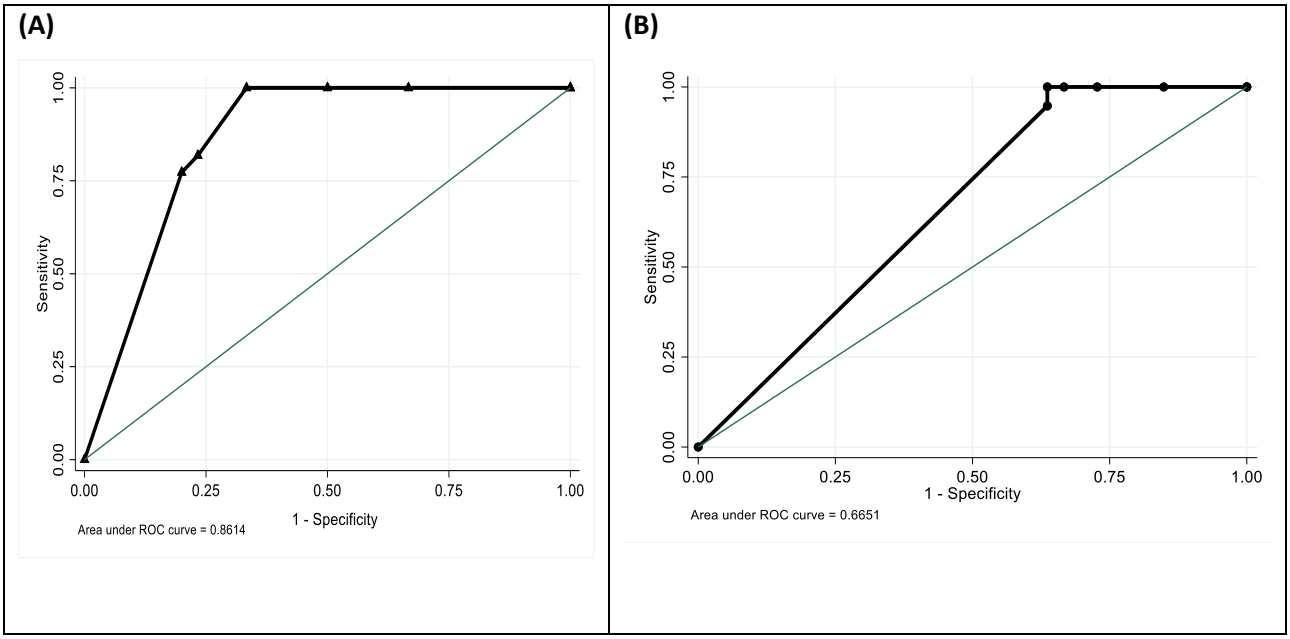

Figure 21. Receiver operating characteristic (ROC) curve analysis and corresponding area under the curve (AUC) analysis of MICs in DST by REMA and DNA sequencing results in the *rpoB* (A) and in the *katG*(B).

There was a significant difference in detection rates between the phenotypic and genotypic methods, which may explain that the agents

responsible for drug-resistant TB involve changes in many genes or multiple possible sites in a gene.

5. DISCUSSION

5.1. Phenotypic drug susceptibility testing by REMA

Thanks to the discovery of anti-tuberculosis drugs, tuberculosis can be cured if diagnosed early and treated correctly[72]. However, the emergence of drug-resistant tuberculosis remains a serious global health problem, especially in underdeveloped and developing countries. Vietnam National Tuberculosis Control Program has many solutions in the diagnosis, treatment, and management of DR-TB. One of the most critical vital points for overcoming this problem is developing reliable, low-cost, and rapid methods, which have been proposed for DST to detect drug-resistant tuberculosis.

The REMA plate method was performed and developed by Martin and Palomino (2002)[73] using the colorimetric indicator resazurin proposed for drug susceptibility testing of *M. tuberculosis*. REMA could only apply as DST to the line-fist TB drugs or second-line TB drugs, determine the resistance level assessed through MIC value, play their role effectively in classifying drug-resistant isolates by helping clinical doctors provide better treatment decisions.

Thus, this study was to apply the Resazurin Microtiter Assay as drug susceptibility testing for detecting the rate of phenotypic isoniazid- (INH) and/or rifampicin- (RIF) resistance of *M. tuberculosis* isolates from three hospitals in central Vietnam.

In the current study, DST of five hundred *M. tuberculosis* isolates test by REMA, 468 isolates gave interpretable results and no colour change in the control well of 32 strains (6.4%), so they gave invalid results. We think there was lousy storage, and these isolates were contaminated. As expected, 106 (22.6%) drug-resistant *M. tuberculosis* isolates were detected with a total of 69.8% were INH resistant; 0.9% was RIF resistant; and 29.2% of them were

MDR-TB. The proportion of MDR-TB were 31(6.62%) strains, (5.6% M. tuberculosis strain from new TB patients and 14.5% *M. tuberculosis* strains from previous TB patients). This result is consistent with the epidemiological situation of drug-resistant tuberculosis in Vietnam. According to the WHO report in 2020, the percentage of MDR-TB in Vietnam is 3.6% new cases and 17% previously treated cases in Vietnam[74].

Some another studies in Vietnam, among 888 new patients in 2001, resistance to isoniazid was observed in 154 (16.6%), resistance to Rifampicin was observed in 22 (2.0%), and MDR-TB was observed in 20 (1.8%). Among 136 previously treated patients in 2001, and MDR was observed in 35 (23.2%)[75]. In addition, according to Caw et al.with 131 isolates resistant to either RIF or INH were collected. 104 (79%) were RIF resistant; 129 isolates (98%) were INH resistant. One of these was RIF mono resistant [76]. In this study, our results alike as the results in study of Caws et al, 2006[76]. Only one *M. tuberculosis* isolate was identified as RIF mono-resistance. The presence of RIF resistance increases the likelihood of multiple drug-resistant tuberculosis because *M. tuberculosis* isolates resistant to RIF are more likely to be resistant to INH drugs (31/32 of which were MDR-TB). In recent studies globally, it seems that a minority of *M tuberculosis* isolates are resistant to only RIF and not INH or other TB anti-drugs, estimated at 1.1% according to WHO [74]. Identifying RIF mono-resistance is critical since these strains have been associated with lower rates of successful treatment outcomes in TB patients and may increase MDR –TB.

Alarmingly, our study showed a high rate of INH resistance at 69.8% of resistant isolates and 21.6% of new cases. According to the fourth national drug resistance survey conducted in Vietnam (2011), among the new cases, the proportion of INH-resistant TB reached 18.9%. In a study of Hang *et al.*, 2013 cohort of 489 newly diagnosed patients in a north city area, INH resistance was observed about 28.2% of the patients [77,78]. Our results are similar to these studies. In the world, previous studies in Ethiopia reported that the proportion of

resistance to isoniazid was within a range of 1.9%–21.4%[79], the study in Belgium reported a higher resistance rate of 27.89% [73], while studies in Algeria reported a lower resistance rate of 12.5% [80].

Moreover, INH resistance of tuberculosis can be considered a signal precedent of the emergence of MDR-TB. INH drug is playing an essential role in tuberculosis treatment TB and treatment of latent TB infection. However, many laboratories of Lung hospitals in Vietnam used the GeneXpert MTB/RIF test to detect the presence of *M. tuberculosis* and identify mutations inside RRDR, where can be detected for RIF resistance. We will also miss the cases of INH mono-resistance, which were not able to be identified at the earliest possible opportunity, and it is difficult to treat TB patients and latent TB infection[81,82,83]. Because in the study of Cohen et al. in South Africa showed that after rapid genetic assessment of RIF resistance such as the GeneXpert MTB/RIF, experimental treatment increases the future risk for developing Multidrug -TB or extensively drug-resistant TB and the overwhelming majority of MDR-TB strains and XDR-TB strains evolved INH resistance before resistance to RIF [84,85]. In addition, patients harbouring isoniazid-resistant isolates have higher rates of treatment failure, relapse and acquisition of multidrug resistance relative to patients with drug-susceptible isolates[82,86]. Similarly, some research demonstrated that *M. tuberculosis* cells grown in vitro have higher spontaneous mutation rates toward INH resistance than RIF. Therefore, INH mono-resistance should be monitored appropriately as an early warning signal to minimize the spread of MDR-TB or XDR-TB strains[87].

The REMA results in this study were compared with those obtained using the BACTEC MGIT 960 system. Three *M. tuberculosis* isolates were found to be resistant to INH by the REMA. However, they were found to be susceptible by the reference method. Two *M. tuberculosis* isolates were found to be resistant to RIF by the REMA despite being found susceptible by the reference method. The results from various studies were compared with those obtained using standard

methods and studies with excellent concordant. Table 20 shows those reported by other authors global, and the results of this study were similar.

Table 19. Sensitivities and specificities of REMA compared to DST results.

Author, publication year	Country	Number clinical isolates	Reference test	Sample size (no. of resistant/no. of susceptible)	Sensitivity (95% CI)	Specificity (95% CI)
INH						
Palomino et al. (2002)[69]	Belgium	80	LJ	54/26	1.00 (0.93–1.00)	0.96 (0.80–1.00)
Banfi et al. (2003)[88]	Italy	13	7H11	5/8	1.00 (0.48–1.00)	1.00 (0.63–1.00)
Montoro et al. (2004)[89]	Cuba	100	LJ	45/55	1.00 (0.92–1.00)	0.96 (0.87–1.00)
Martin et al. (2005)[90]	Belgium	203	LJ	82/212	0.98 (0.91–1.00)	0.98 (0.93–0.99)
Nateche et al. (2006)[80]	Algeria	136	LJ	17/119	1.00 (0.80–1.00)	0.99 (0.95–1.00)
Coban et al. (2015)[91]	Turkey	73	BACTEC	35/38	1.00 (0.85–1.00)	0.95 (0.76–0.99)
Dixit et al(2012) [92]	India	105	LJ	51/54	0.93	0.98
Nour et al(2013)[93]	Epyt	30	PM	20/10	1.00	0.98
This study	Vietnam	468	BACTEC	105/363	100(0.96.-1.00	0.99(0.98-1.00)
RIF						
Palomino et al. (2002)[69]	Belgium	80	LJ	49/31	1.00 (0.93–1.00)	1.00 (0.89–1.00)
Banfi et al. (2003)[88]	Italy	13	7H11	4/9	1.00 (0.40–1.00)	1.00 (0.66–1.00)
Montoro et al. (2004)[89]	Cuba	100	LJ	37/63	1.00 (0.91–1.00)	0.98 (0.91–1.00)
Martin et al. (2005)[90]	Belgium	203	LJ	102/101	0.98 (0.93–1.00)	0.99 (0.95–1.00)
Nateche et al. (2006)[80]	Algeria	136	LJ	12/124	0.92 (0.62–1.00)	0.99 (0.96–1.00)
Coban et al. (2015)[91]	Turkey	73	BACTEC	21/52	1.00 (0.81–1.00)	0.94 (0.89–1.00)
Dixit et al (2012)[92]	India	105	LJ	52/53	0.95	1.00
Nour et al(2013)[93]	Epyt	30	PM	13/17	0.95	0.93
This study	Vietnam	468	BACTEC	32/436	0.99(0.88-1.00)	0.94(0.98-0.99)

WHO recommends using REMA to determine drug resistance, but they are not faster than conventional phenotypic susceptibility tests performed in liquid

media. In particular, the BACTEC MGIT 960 system read at 7-14 days and the DST- Lowenstein-Jensen proportion method read at 21–56 days. The turnaround time of REMA is an average of 7-9 days, and in this study, the results could be read on day 9.

Moreover, REMA is much cheaper than phenotypic commercial culture and drug susceptibility testing, and the cost is estimated at approximately $3 for each strain tested by this method [73]. In contrast, the cost of Culture and DST by BACTEC MGIT 960 for each strain tested against several drugs in Vietnam estimate at approximately $35.

Furthermore, more studies are needed to recommend its wide use thoroughly. Therefore, REMA can detect MDR-TB cases, particularly in TB high prevalence countries and low resource countries, due to its low cost, do not require excessive technical skills and effortless performance. Finally, rapid screening for drug resistance in pulmonary hospital laboratories and rapid availability of results will identify multidrug-resistant strains in these hospitals and allow the establishment of appropriate treatment for the patient. Contribute to reducing the rate of TB and drug-resistant TB. At the same time, we hope to continue DST with REMA for other first-line anti-TB drugs and second-line anti-TB drugs for early detection of XDR-TB.

In contrast, the important disadvantages of performing REMA include:

Firstly, REMA is recommended to perform at the laboratories that already have the compulsory biosafety facilities.

Secondly, we did not perform directly from clinical samples by REMA for detecting *M. tuberculosis* in this study.

5.2. Genotypic drug susceptibility testing by real-time PCR TaqMan allelic discrimination assay

Conventional DST is the current “gold standard” for the assessment of TB - DR. However, the disadvantages are slow turnaround times, high decontamination. Alternatively, molecular assays provide a faster turnover time while maintaining

high sensitivity and specificity. In this study, a total of 52 pairs of *M. tuberculosis* clinical isolates and corresponding clinical samples (31 MDR-TB, 01 RIF mono-resistant, 10 INH mono-resistant, 10 drug-sensitive isolates) were performed by the real-time PCR TaqMan allelic discrimination assay, that MTB drug-resistant strains can be detected by pattern's curve or Ct with three TaqMan probes without MGB in real-time PCR basis on previous researches[70,71]. The proposed method was optimized and evaluated concerning its analytical sensitivity and specificity in clinical isolates and clinical samples. Compared with DNA sequencing results, help the diversity of drug resistance-associated mutations pattern, and insights that emphasize genotypic DR-TB isolates in Vietnam are assisted in focusing on infection control and surveillance to prevent new cases of MDR-TB in this region. In some recent studies in Vietnam, the rate of mutations in genes varies from region to region. The sequencing technique was applied to study the characteristics of mutant genes of tuberculosis bacteria related to resistance anti-TB drugs. The results were variable in sensitivity and specificity in each research and each region of the world.

One strain with rpoB 510/516 probe of the *rpoB* and one with katG315 probe of the *katG* did not determine mutations by using real-time PCR TaqMan allelic discrimination assay for clinical isolates, but their mutations were determined by DNA sequencing. Table 20 gives information about good sensitivity and specificity results comparable to DNA sequencing results in our study and other authors'.

Table 20. Sensitivities and specificities of real-time PCR TaqMan allelic discrimination compared to DNA-sequencing results.

Author, publication year	Gene	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Peng *et al.*, 2016 [94]	*katG*	97.9(95.6–100)	96.4 (92.5–100)	97.9(95.6–100)	96.4 (92.5–100)
	rpoB	97.2(94.5–99.9)	100	100	95.4(91.0–99.8)
Riahi *et al.*, 2015 [95]	*katG*	100	100		
This study	*katG*	95.0(75.1-99.8)	100	100	96.9(82.5 - 99.5)
	rpoB	95.4(77.2-99.9)	100	100	96.8 (81.6- 99.5)

GENOTYPIC CHARACTERIZATION OF INH RESISTANT ISOLATES

In this study, DNA sequencing of the *katG* confirmed the presence of the mutation in table 14 that a total of 19/41(46.34%) INH resistant isolates had the mutation at codon Ser315Thr (AGC→ACC), including 15/31 (48.38%) of MDR-TB isolates and 4/10(40%) of INH mono-resistant isolates. This is considered the only mutation site we have investigated when sequencing the *katG* from codon 256 to codon 420.

The mutation of codon 315 (Ser) in the *katG* is the most frequently encountered mutation that is associated with INH resistance such as in the research of Schwartz *et al* had (53%) the acid amine 315 mutation in the *katG* gene, with 33% mono-drug resistance and 14% multidrug resistance had mutations at codon Ser315Thr [96]. According to a systematic review from 118 publications analysing 11,411 *M. tuberculosis* isolates from 49 countries, it was found that 64% of all observed phenotypic isoniazid resistance was associated with mutation *katG*315 [97]. Only 54.5% harboured the *katG*315 mutation from Japan in research of Chikamatsu. K *et al*.[98] In addition, Van Doorn et al. found that 55 % of INH-resistant strains had a mutation in codon 315 of the *katG*. Riahi *et al.*, reported about 53.55%. In contrast, the *katG*315 mutations of the *katG* in

Belarus, Lithuania, Kazakhstan, Latvia, Moldova, and Russia were identified in 94% [95,98,99].

The question asks which data variables on the genes of INH-resistant bacteria are related to varying in different geographic regions, and the prevalence of genetic mutations results in drug-resistant in *M. tuberculosis* in these regions. INH resistance was shown more complex to detect because the mutations have been associated with multiple genes. In our study, we only investigated the *katG* at codon 315. Further, many studies are needed to gain a complete understanding of the genetic variations in *M. tuberculosis* drug resistance to INH drug and to determine the prevalence of resistant mutations among *M. tuberculosis* clinical isolates in different geographic regions in Vietnam.

GENOTYPIC CHARACTERIZATION OF RIF RESISTANT ISOLATES

The molecular mechanism of *M. tuberculosis* resistance to anti-TB drugs is becoming elucidated. Genetic studies have demonstrated that RIF-resistant isolates have associated mutations in the RIF resistance determination region (RRDR), called 81-bp core region (codon 507–533) of *the rpoB* gene at a relatively high rate of 75-90%, at codon 516, 526, 531 in the *rpoB* gene were most frequency of mutations [79,100,101,102]. In recent research in the South of Vietnam, 56 of which (approximately 76%) had mutations in the RRDR of the *rpoB* gene, at codons 531 (37.8%), 526 (23%), and 516 (9.46%) of the *rpoB* gene [103]. In the difference research, 104 RIF-resistant isolates had mutations in the 81bp RRDR of the *rpoB* gene; the most prevalent mutations were at codons 531 (43%), 526 (31%), and 516 (15%) [76].

Since we used two wild type probes, including these point mutations for determining RIF resistance in this region of the *rpoB*. In this study, eight types of the *rpoB* mutations were found at 22 of 32(68.75%) RIF-resistant isolates, with *rpoB* Ser531Leu (TCG > TTG) was the most frequently at 37.5%. Four different types of amino acid substitution were detected in codon 526 about

18.75% two isolates had His526Asp (CAC>GAC) mutations, and two isolates had His526Tyr (CAC>TAC) mutation; one isolate had a His256Asn (CAC>AAC) mutation, and two isolates had His256Pro (CAC>CCC). At codon Asp516Tyr (GAC>TAC) were only two strains at 6.25%.

A double point mutation was found in one strain with L511Pro (CTG>CCG) and Met515Val (ATG>GTG). Only one RIF mono- resistance (0.21%) and also mutation in *rpoB* S531L. The prevalence of RIF mono-resistance differs considerably by setting, ranging from 0% in some settings to (17%) in New york[102], as high as 21.4% in South Africa[79]. This study showed resistance in strains with mutations within the hot spot 81bp was detected without finding cases with mutations located outside the hot spot of the *rpoB*. It seems that our study can only examine a short segment of the *rpoB* and not observe the *rpoA* and *rpoC* of *M. tuberculosis*.

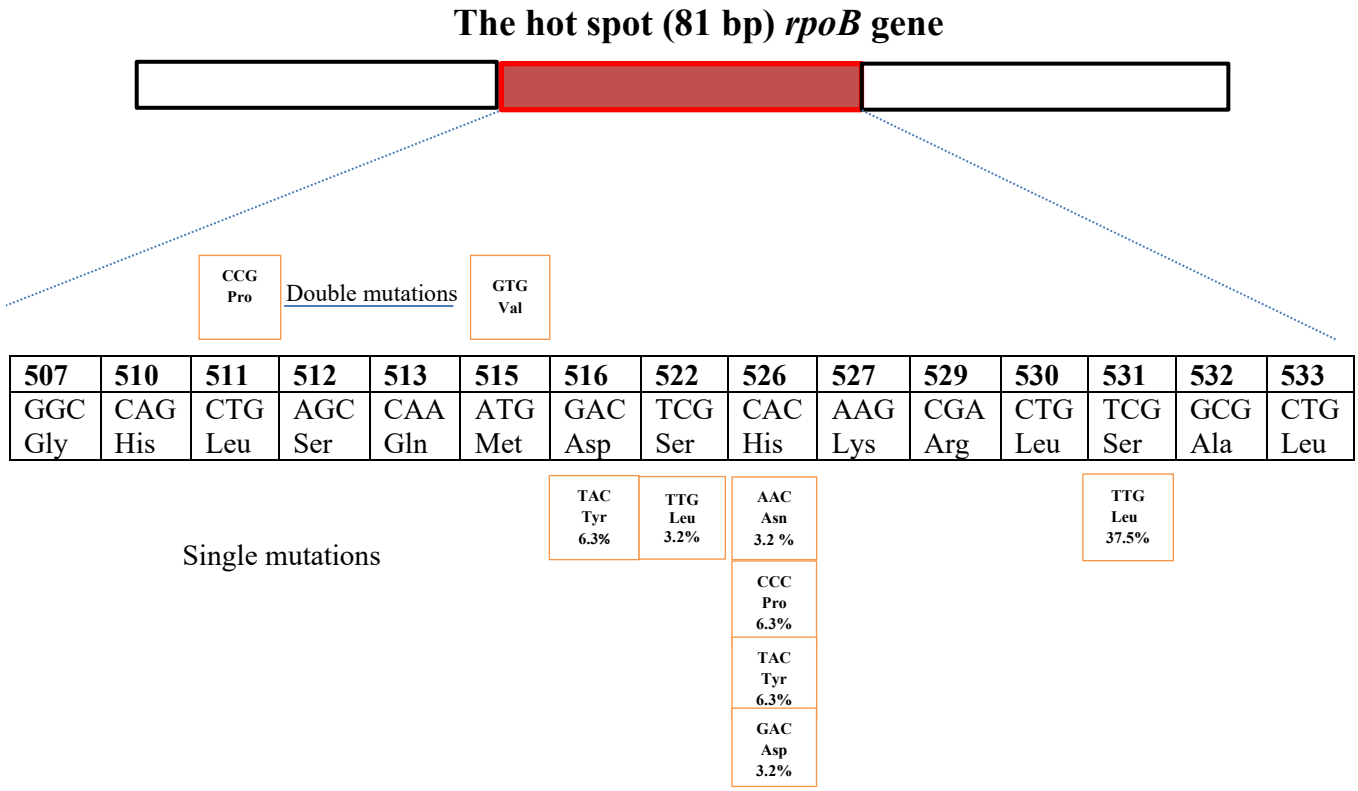

Figure 22: Schematic representations of mutations in the *rpoB* of RIF resistant *M. tuberculosis* isolates.

We could not detect mutations outside the hot spot in the 81-bp region of *rpoB* when surveying by sequencing results. Though, some previous studies found mutations outside the RRDR of the *rpoB* such as Gln490His and Gln490Arg at the North and the South of Vietnam [103,104,105].

5.3. Evaluation of RT-PCR TaqMan allelic discrimination Assay for detecting mutation in *katG* and *rpoB* of *M. tuberculosis* from clinical samples.

The exploration applying this assay directly on sputum samples was helpful to determine if direct testing would be a clinically useful tool. In the last time, most studies using real-time PCR TaqMan probes detected mutations related to INH- and RIF- resistance, focused on direct detection in positive samples.

We further aimed to develop, apply and validate a high-sensitivity real-time PCR TaqMan allelic discrimination assay that may be useful for rapid direct detect DR-TB in various types of specimens.

Table 16 described al 52 (100%) *M. tuberculosis* isolates from cultured were positive TB control rpoB probe in the *rpoB*. However, only 31 out of 52 *M. tuberculosis* from clinical samples (60.78%) showed strong luminescence in real-time PCR TaqMan allelic assay, from AFB smear-positive mainly. Whereas 40.8% (21 samples) showed no luminescence, this means undetermined Ct even after 40 cycles of PCR amplification, most of them were AFB smear-negative at 15/15 (100%) samples and 6/37(16.2%) of AFB smear-positive. The pulmonary specimens consisted of sputum with forty-eight, two plural pulmonary, join abscess.

Some studies in the literature have been published, which direct detection of INH and RIF resistance from *M. tuberculosis* in clinical samples such as Wada et al. performed 27 sputum samples and found a sensitivity of 59.2%, which rose to 100% after using a nested PCR model [70]. Espasa *et al*. used six pairs of fluorogenic 5' exonuclease probes (TaqMan) mutated and wild-type for

detecting DR-TB, with a sensitivity of 30.4 to 35.3% for smear-negative samples and 95.1 to 99.2% for smear-positive samples, a specificity of 100%.[106]. Due to low concentration, the amount of DNA was small because IPC was positive for all 21 samples. As a result, we carried out site-specific nested PCR. The *rpoB* and *katG* were amplified by PCR with their corresponding primer sets. The targets, including *rpoB* and *katG*, were amplified by nested PCR; after that, the nested PCR products used real-time PCR TaqMan allelic assay.

Analysis of all 21 samples showed luminescence. Whereas, in an analysis of the clinical samples, the Ct was more variable, resulting in a smaller Ct than for DNAs extracted from clinical samples with nested PCR and DNAs extracted from clinical isolates. The real-time PCR TaqMan allelic assay required as high as 1000GE of template DNA for successful amplification in our study, showing strong luminescence and cycle threshold 22 to 24. In order to, nested PCR was used for increasing the amount of DNA amplicon for real-time PCR TaqMan allelic discrimination detecting *M.tuberculosis* and mutations in the *rpoB* and *katG* from clinical samples. The results showed that a total of 100% strong luminescence signal and the Ct of the TB control probe were small from cycles 10 to 20. There was corresponding with the least amount of 10^5GE DNAs of *M. tuberculosis* in each clinical sample after performing the nested PCR.

It seems that the amount and/or quality of DNA in the sample had played an important for analysis with real-time PCR using TaqMan probes. So in the present study, we referred to the research of Wada T et al. (2004) to design a TB control probe for identifying M. tuberculosis and confirmation of DNA amount, besides an IPC probe from the *MecA* of *S.aureus* for detection of PCR inhibitors. As a result, confounding factors will be monitored.

Table 18 showed the sensitivities 100% for both *katG* and *rpoB*, but the specificities were 96.88% (83.78-99.92) for *rpoB* and 93.94% (79.77-99.26) for *katG*. Corresponding DNA sequences of the *rpoB* and the *katG* were

analysed in the four strains discordant samples. Previous studies used Real-time PCR TaqMan probes to detect mutation for *M. tuberculosis* from clinical isolates. When comparing the sensitivities and specificities of Real-Time PCR, TaqMan allelic discrimination results for detecting mutations in *rpoB* and *katG* of *M. tuberculosis* from clinical samples compared to DNA-sequencing results. Of the 52 samples, only two RIF-susceptible strains and two INH- susceptible strains were falsely diagnosed as resistant M. tuberculosis by the assay. In a recent study from Korea, Choi. Y *et al*. developed a new susceptible nucleic acid amplification test for detecting M. tuberculosis, combined nested and real-time PCR in a single tube (one-tube nested real-time PCR). This method showed 100% (167/167) for sputum specimens[107].

In summary, the real-time PCR TaqMan allelic discrimination can be used for diagnosing drug resistance Molecular-DST directly from sputum samples, an essential advantage in this study. Until the resulting phenotypic DST is available, the use of real-time TaqMan allele testing as to DNA isolated directly from clinically confirmed samples. Molecular-DST will help physicians select the most appropriate therapy for the patient to initiate treatment with as soon as possible. In future, this method analyses on a larger scale, controlling the purity and quality of DNA of *M. tuberculosis* in clinical samples; it aids to be reliable concludes and easier perform on their applicability.

5.4. Correlation between genetic and phenotypic DST data

Each study has a different way of classifying resistance on each anti TB drug, thereby considering the relationship between drug resistance and each gene mutation. For example, in order to facilitate interpretation, Jamieson *et al*. selected MIC ranges arbitrarily to catalogue the resistance to RIF as high (MIC ≥100 μg/ml), moderate (≥20 to <100 μg/ml), or low (≥1 to <20 μg/ml). As a result, Jamieson et al. showed that high MICs for RIF was associated with specific mutations at codons 531 and 526; at the same time, mutations at other positions were generally associated with low or moderate MICs[108]. In a different

study by Shea *et al*.,2021, including RIF concentration range of 0.12 – 16 µg/mL for MIC testing: high-level resistance strains had RIF MIC ≥16 µg/ml, low-level resistance strains had RIF MIC ranging from 0.25-1.0 µg/ml [102]. In addition, a study in South India, including some *M. tuberculosis* isolates showed mutations in the *rpoB* a level of resistance against RIF at a high MIC (>128 µg/ml), other RIF mono-resistance isolates had resistance at 40 µg/ml [109]. S. Foongladda *et al.* observed 'low-level' resistance to INH MIC < 1 µg/ml, 'median' INH MIC 1.0 vs. 2.0 µg/ml)[110]. High-level INH resistant (0.4 µg/ml) and low-level INH resistant (0.1 µg/ml) according to Somoskovi.A et al [111]. In addition, according to the study of Van Doorn et al., among high-level INH-resistant isolates (MIC > 2), 89% were associated with a mutation at codon 315 of the *katG* [99].

In this experiment, based on a sub-classification of resistant isolates by REMA, the segmentation was made between high and low drug resistance, with cut-off values of 1µg/mL and 2µg/ml for INH and RIF, respectively [69,112], and the critical concentration recommended by WHO[1]. As a result of this sub-classification in this study, the resistant isolates were divided into (50%) INH high-resistance and (50 %) INH low- resistance; (61.54%) RIF high-resistance and (38.46%) RIF low-resistance. These results were similar to those of author Miyata M *et al.,*2013 but lower than those reported by Montoro *et al.*, 2005[90, 113].

However, we could not do MIC concentrations at high resistance levels in two drugs INH and RIF. There are increasing reports of *M. tuberculosis* strains with higher levels of drug resistance worldwide.

Figure 20 in the part of results showed that most of the point mutation in either codon 516, 526, or 531 was found in 17 out of 23 strains (73.9%) requiring MICs of 2µg/ml in the association between MICs of RIF, genetic alterations in the *rpoB*, and 18 out of 19 (94.7%) strains showed point mutations in codon 315 of the *katG*, required MICs of 1µg/ml. In the present study, a double point

mutation was detected in one strain at codon L511P and codon M515V in the *rpoB* requiring MICs of 1µg/ml while Ocheretina *et al.* showed that two strains had double mutations L511P and M515T. A combination of L511P and M515T mutations resulted in RIF MIC between 0.25 and 0.5 mg/ml[112].

Therefore, there was a relationship between the presence of a particular codon mutation and the level of drug resistance in the clinical isolates. On the one hand, some mutations confer low, moderate, and high resistance levels, and not all mutations confer the same resistance level [114]. On the other hand, the other studies had demonstrated that mutations His526Asp, Asp516Tyr, or Ser531Leu had high-level RIF resistance, the mutations in positions of *rpoB* such as 516 (D/Y), 515 (M/I), 510 (Q/ H), or a double mutation in codons 512 (S/I) and 516 (D/G) relate to low level of resistance to RIF. As a result, identifying mutations in a clinical *M. tuberculosis* isolate is not enough to classify the given strain as resistant to RIF because this problem depends on many factors, including the genetic background of the host strain [115].

Hopefully, in future research, we will improve extensive understanding of the relationship between the characteristics genotypic and the drug resistance phenotype of *M. tuberculosis* is needed. A better understanding of the correlation between heteroresistance and MIC level should improve the effective treatment of Tuberculosis. It aids in the development of more accurate molecular diagnostics for drug-resistant Tuberculosis.

Table 21. Sensitivities and specificities of real-time PCR TaqMan allelic discrimination compared to DST results (BACTEC MGIT 960 INH, RIF).

Author, publication year	Gene	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Peng *et al.*, 2016	*katG*	85.9 (80.6–91.2)	95.3 (90.1– 100)	97.9(95.6–100)	72.6 (63.1–82.2)
	rpoB	94.6(91.0–98.2)	100	100	90.8(84.7–96.9)
This study	*katG*	48.8(32.9 - 64.9)	100	100	34.38(27.9- 41.4)
	rpoB	66.7(47.2-82.7)	95.5(77.2-99.9)	95.2(74.4- 99.3)	67.7(55.7 - 77.8)

There was a significant difference in detection rates between the phenotypic and genotypic methods, which may explain that the agents responsible for drug-resistant TB involve changes in many genes or multiple possible sites in a gene. In the present study, also, drug resistance mechanisms in all genes have not been identified, so the molecular methods still cannot be wholly replaced phenotypic susceptibility tests, and it seems necessary that a combination of molecular and phenotypic methods until more information regarding the clinical relevance of phenotypically susceptible isolates with drug resistance mutations mechanisms is thorough understanding.

The advantage of real- time PCR TaqMan allelic discrimination assay in the present study.

Real-time PCR TaqMan allelic had several advantages when we performed this method in Vietnam.

Firstly, RIF is the most potent agents of first-line anti-tuberculosis drugs. 100% of RIF-resistant *M. tuberculosis* isolates possess a point mutation at the hot spot in the 81-bp region of the *rpoB* in this research. For that reason, the detection of mutations in the *rpoB* is a helpful strategy for the diagnosis.

Secondly, the high sensitivity and specificity of real time-PCR TaqMan allelic method were without equivalent to the 3′ -minor groove binder (MGB) probes-based, the cost is about a half of the latter.

Thirdly, the PCR techniques compared with the conventional PCR, which is the rapid speed of the test within 48–72 h after sample collection, and lower risk of contamination if reaction tubes remain unopened after the PCR reaction.

The limitation of real- time PCR TaqMan allelic discrimination assay in the present study.

Firstly, the sample size was insufficient to reach statistical significance for the Real-time PCR TaqMan probe assay.

Secondly, this method needs to calculate the Ct values and ΔCt by threshold line. In the future, this method was analysed on a larger scale, and it aids to be

reliable concludes and easier perform on their applicability to DNA isolated directly from clinical samples.

Thirdly, in this study, only obverse the *katG* in *M. tuberculosis*, while many different genes affect INH resistance.

Finally, we did not survey clinical samples from smears with culture-negative in our study for detecting *M. tuberculosis* and DR-TB.

Although using the molecular method cannot wholly replace the culture-based method and conventional DST, but will allow more rapid detection of drug resistance and focus successfully complement conventional methods

6. CONCLUSION

The drug-resistant isolates, so it helps clinical doctors provide better treatment decisions; it was estimated that 40% of TB patients were not diagnosed and treated each year in Vietnam[1]. Empirical treatment increased DR-TB at hospitals. In central Vietnam, only one Lab of Danang Lung hospital performed DST detecting DR-TB by BACTEC MGIT system, and the cost of the method is too high. The diagnosis, treatment, and management of DR-TB are significant challenges for Vietnam National Tuberculosis Control Program. We developed phenotypic DST by REMA at the Carlo Urbani Institute and the Labs of some hospitals, with its simplicity, reliability, low cost and application for many antibiotics. MIC value plays its role effectively in classifying. In the future, we will apply at many labs of Lung hospital in central Vietnam and continue DST with REMA for other first-line anti-TB drugs and second-line anti-TB drugs, early detection to MDR-TB and XDR-TB. The rate of phenotypic drug-resistant *M. tuberculosis* strains in our study by REMA method includes: a total of 69.8% were INH resistant; 0.9% isolates resistant to RIF, and 29.2% of them were MDR-TB. With the high rate of INH resistance in this study, while many hospitals in Vietnam only used the GeneXpert MTB/RIF for detecting DR-TB, will also miss the cases of INH mono-resistance, which were not able to be recognised at the earliest available opportunity, and it is difficult to treat TB patients and latent TB infection. However, phenotypic DST detected drug resistance time-consuming several weeks.

The development of molecular biology also assists the diagnosis of drug-resistant TB faster, easier, and more effective. In Vietnam, genotypic DST detects DR-TB only using the GeneXpert MTB/RIF and The Genotype MTBDRplus assay, but their cost is unavailable for all TB patients and limited detection sites. In addition, there is not much information about the genetic characteristic of *M. tuberculosis* isolates in central Vietnam. In our research, the molecular DST method by real-time PCR TaqMan allelic discrimination assay

performed clinical isolates and clinical samples. Consequently, eight different type mutations in the RRDR of the *rpoB* were found in 67.7% (21/31) MDR-TB strains and one RIF mono resistance isolate. The mutations at codons 531, 526 and 516 were 37.5% (12/32), 18.75% (6/32) and 6.25% (2/32), respectively. There was detected a double point mutation in one strain at codon L511P and codon M515V. At codon Ser522Leu (TCG→TTG) was detected by sequencing analysis by this point mutation outside observed in the *rpoB*. Most of 46.34% isolates resistant to INH, that had a mutation in the *katG* at codon Ser315Thr (AGC→ACC). All susceptible isolates had no mutations in *katG* and *rpoB*. The real-time PCR TaqMan allelic discrimination also showed the sensitivities 100% for both *katG* and *rpoB*, and the specificities were 96.88% (83.78-99.92) for the *rpoB*, 93.94% (79.77-99.26) for the *katG* from clinical samples.

From these insights, we will perform this method on a larger scale, use to mutant probes with simply assessment, controlling the purity and quality of DNA of *M. tuberculosis*, developing a good kit use for rapid direct detection of DR-TB in various types of specimens, it aids to be reliable concludes and easier perform on their applicability.

In conclusion, the lab of Lung hospitals should use real-time PCR TaqmMan allelic in the first step rapidly detect DR-TB, then determine using REMA to the MIC values of many TB drugs, consider the level of resistance of each drug, assist in deciding the effective drug resistance regimen to the patients. However, there are a lack of equipment, personal resources, and the high cost of molecular DST. REMA is still the most helpful test for detecting DR-TB in these hospitals with its simplicity, accuracy, and low cost.

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