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**Rapid detection of drug resistance  
and genetic characterisation of  
*Mycobacterium tuberculosis*  
isolates in Honduras**

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*To my family.*



## ABSTRACT

**Background:** New challenges for tuberculosis (TB) control are emerging in the 21<sup>st</sup> century. The deadly combination TB/HIV as well as the development and spread of new forms of drug resistance are some examples. In this context, the timely detection of TB cases, especially of those with resistant TB is important. Improved laboratory methods can contribute to the early detection of such patients. Therefore, this thesis focuses on the evaluation of new diagnostic tools and the genetic characterisation of the disease in a low-middle income setting.

**Aims:** The purpose of this work was to increase the knowledge about effectiveness of new laboratory assays for early detection of drug-resistant TB. In addition, this thesis aimed to increase our understanding of the *M. tuberculosis* biodiversity and transmission patterns in Honduras.

**Methods:** The diagnostic accuracy of two low-cost techniques for rapid detection of drug-resistant TB was assessed. The microscopic-observation drug susceptibility assay (MODS) and the nitrate reductase assay (NRA) were compared to the proportion method on Lowenstein Jensen medium for detection of multidrug-resistant TB (MDR-TB) directly from sputum samples. Furthermore, the indirect NRA was evaluated for detection of resistance to selected second-line drugs using as reference standard the BACTEC-460TB. Additionally, the frequency and distribution of resistance-related mutations *M. tuberculosis* was explored. This characterisation was conducted in clinical MDR-TB isolates from different geographical settings. Finally, we described the genetic variability of clinical *M. tuberculosis* isolates from Honduran TB patients, using spoligotyping and restriction fragment length polymorphism (RFLP).

**Results:** Both NRA and MODS were highly sensitive and specific for direct detection of resistance against isoniazid and rifampicin. The indirect NRA had an acceptable performance for detection of resistance to ofloxacin, with less good results with kanamycin. The genotypic detection of drug-resistance in MDR-TB isolates confirmed the prevalence of the most common mutations associated with this phenotype. It also showed the differences in distribution depending on the geographic origin of the strains. In Honduras, the Latin American Mediterranean spoligotype is the most frequent in a *M. tuberculosis* population with high biodiversity.

**Conclusions:** MODS could be used for screening of suspected MDR-TB patients. However, NRA is the most reliable option for early detection of MDR-TB in resource-limited settings. NRA also has the potential to be further optimised for detection of extensively drug resistant TB. We also confirmed that knowledge about the frequency of geographic-specific mutations provides useful information for development and/or assessment of new genotypic tools for detection of drug resistance. The high level of genetic variability in the Honduran *M. tuberculosis* isolates suggests that the transmission of the disease in this setting is not caused by clonal spread of a specific strain. Further studies are needed to determine whether or not there is an association with TB clinical manifestations or HIV status with the genotypes prevalent in the country.

## LIST OF PUBLICATIONS

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## LIST OF ABBREVIATIONS

AFB	Acid fast bacilli
AMI	Amikacin
DOTS	Directly-observed, short course treatment strategy
DST	Drug susceptibility testing
EMB	Ethambutol
FQ	Fluoroquinilones
HIV	Human Immunodeficiency Virus
INH	Isoniazid
KAN	Kanamycin
LPAs	Line probe assays
LTBI	Latent TB infection
MDR-TB	Multidrug-resistant TB
MODS	Microscopic Observation Drug Susceptibility
MTC	<i>Mycobacterium tuberculosis</i> complex
NRA	Nitrate reductase assay
NRL	National TB reference laboratory
NTP	National TB control programme
OFX	Ofloxacin
PCR	Polymerase chain reaction
PZA	Pyrazinamide
RFLP	Restriction Fragment Length Polymorphism
RIF	Rifampicin
SM	Streptomycin
TAT	Turnaround time
TB	Tuberculosis
WHO	World Health Organization
XDR-TB	Extensively drug-resistant TB



# 1 BACKGROUND

## 1.1 THE NATURAL HISTORY OF TUBERCULOSIS

### 1.1.1 The tubercle bacillus

Tuberculosis (TB) is an infectious disease caused by bacteria belonging to the *Mycobacterium tuberculosis* complex (MTC). The MTC consists of the following species: *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. canetti*[81], *M. microti*[82], *M. caprae*[9] and *M. pinnipedii*[26]. These pathogens can cause TB both in humans and animals. *M. tuberculosis*, *M. africanum*, and *M. canetti* primarily cause disease in the human host whereas *M. bovis*[37], *M. microti*[31], *M. caprae*[27, 56] and *M. pinnipedii* [54] infect cattle, domestic animals, goats, seals and can also be transmitted to humans .

The MTC members belong to the genus *Mycobacterium* from the *Mycobacteriaceae* family. More than 100 species have been described as part of this genus, most of which are non-pathogenic and free-living organisms. The MTC mycobacteria are able to survive for several months on surfaces or in soil. They are sensitive to heat, sunlight and ultraviolet radiation, but highly resistant to cold, freezing and desiccation[13, 82]

Mycobacteria are aerobic, non-motile, non-sporulated slightly curve rods with a slow proliferation rate (up to 20 hours). The mycobacterial cell wall is constituted by peptidoglycolipids, mycolic acids, free lipids and interspersed proteins. The high content of lipids in the cell wall confers one of their most predominant features: acid fastness. This property implies that mycobacteria are not decoloured with acid-alcohol solutions after being stained with aniline dyes[82].

The tubercle bacillus requires a mesophilic environment with neutral pH and adequate nutritional sources. Although it is capable to adapt to different conditions, exposure to harsh surroundings triggers its latency state. Based on its growth and metabolic characteristics, MTC identification and speciation can be performed *in vitro*. Utilization of carbon and nitrogen sources, production of metabolic intermediates and activity of some enzymes can be used for phenotypic speciation and also for detection of drug resistance. For instance, typical growth on solid media (non-pigmented, rough, crumbly colonies) as well as the microscopic observation of serpentine cords characterizes *M. tuberculosis*[13].

From the public health perspective, the most common and clinically relevant etiologic agent of TB is *M. tuberculosis*. Therefore, and considering the focus of this thesis, from now on only *M. tuberculosis* will be referred to in the text.

### 1.1.2 The chain of transmission

*M. tuberculosis* spreads person-to-person though inhalation of infectious droplets. Although airborne transmission is the main pathway for infection, it is not the only one. *M. tuberculosis* can use less conventional infection routes (digestive, mucocutaneous, placental, inoculation) and is also capable to infect any organ in the body [20].

The primary site of infection is usually the lung. After inhalation of virulent bacilli, there are three possible scenarios: 1) the immune system is able to control and clear-out the infection, 2) the bacilli migrate to another location and start a latency phase that can last years, or 3)

the immune response is not able to control the infection and the active disease develops. If the second scenario occurs, the human host becomes a reservoir of the pathogen without being infectious to others. This is also known as latent TB infection (LTBI). On the other hand, if there is a progression to disease, the human host becomes an infection source when bacilli are released to the air by coughing, talking or sneezing[20].

There are several factors conditioning the exposure, infection as well as disease development. These factors include environmental conditions, social determinants, age and immune status of the susceptible individual, among others. Only 10% of those infected with *M. tuberculosis* will develop the disease at some point of their life; half of them (5%) will present symptoms during the first two years after initial infection. For persons co-infected with HIV this risk increases to 10% annually [20, 88].

TB is preventable and curable, but it can lead to death if no actions are taken. In order to prevent transmission, it is necessary to identify infectious TB patients in a timely manner. Suspected TB patients, that is, persons with persistent cough and expectoration lasting more than two weeks, need to be diagnosed promptly. Appropriate chemotherapy must be available for treatment of confirmed TB cases and for prophylaxis of those who were in closed contact with them. Additionally, identification of subjects with LTBI is also necessary since dormant bacilli can be reactivated and cause disease, especially in HIV patients. Another measure to prevent infection is the vaccination of newborn, unexposed children.

## **1.2 TUBERCULOSIS TREATMENT AND DRUG RESISTANCE**

### **1.2.1 TB treatment**

The chemotherapy era for TB treatment began in the 1940's, with the discovery of streptomycin(SM) and the p-amino salicylic acid (PAS). It was until 1950 that the combined therapy with both drugs was introduced, after an increase in cure rates was demonstrated. Since then other drugs have been discovered and used for treatment of TB patients [2, 49]. Currently, standard treatment regimens recommended by WHO using first-and second-line drugs are used[126]. First-line treatment is indicated to new TB cases, while the second-line treatment is used for drug-resistant TB patients. The first-line drugs are rifampicin (RIF), isoniazid (INH), ethambutol (EMB) and pyrazinamide (PZA). Second-line treatment includes the following drugs: SM, aminoglycosides, such as amikacin (AMI) and kanamycin (KAN); capreomycin, fluoroquinolones (FQ) like levofloxacin and ofloxacin (OFX);PAS, ethionamide (ETO) and thioacetazone.[126]

There are two bacteriological principles behind TB chemotherapy:

1. To rapidly kill metabolically active bacillary populations, living extra-cellularly
2. To sterilize and eliminate less-active bacilli living intra-cellularly in acidic and anoxic conditions [20].

The currently available drugs are active to the above mentioned bacillary populations (table 1) and need to be used in combination in order to achieve TB chemotherapy's goal [38]. Up to date, there is no drug available that fully successful against dormant bacilli populations [20, 72].

## 1.2.2 Molecular mechanisms of drug resistance

**Box1.** Frequency of resistance- conferring mutations:

INH	$1 \times 10^5$ - $10^6$ bacilli
RIF	$1 \times 10^7$ - $10^8$ bacilli
SM	$1 \times 10^5$ - $10^6$ bacilli
EMB	$1 \times 10^5$ - $10^6$ bacilli
PZA	$1 \times 10^2$ - $10^4$ bacilli
FLQ	$1 \times 10^5$ - $10^6$ bacilli

*M. tuberculosis* characterizes for developing natural resistance to anti-TB drugs. This natural resistance is due to random, spontaneous mutations and occurs as independent events for each anti-TB drug (box 1)[20, 129]. Thus, the probability that simultaneous resistance to two drugs develops is equal to the product of their respective mutation rates. It has been estimated that  $1 \times 10^4$ - $10^9$  bacilli are present within a TB lesion; this amount is below the population size needed to observed natural resistance to two or more drugs. Therefore, TB drug resistance is result of the treatment regimen used. If only one drug is used for TB treatment, the resistant mutants will be selected and will become the dominant bacillary population [20].

Several studies have identified different genetic targets associated to drug resistance development, as shown in Table 1.

### 1.2.1 Definitions

The following are important definitions related to drug-resistant TB according WHO [122, 125, 126]:

**Primary resistance:** theoretical concept that refers to transmission of a drug-resistant strain to TB patients who have not been previously treated.

**Resistance among new cases:** drug-resistant TB cases with no or less than one month of treatment. This is a proxy for primary or initial resistance.

**Acquired resistance:** theoretical concept that refers to the development of resistance among patients diagnosed with TB who start anti-TB treatment. In the past, resistance among previously treated cases was used as a proxy of acquired resistance. Nowadays this category also includes patients re-infected with a resistant strain, those with treatment failure and relapse. Therefore, the only way to truly defined acquired resistance cases is by determining the DST pattern before and after the start of treatment. Furthermore, molecular fingerprinting is recommended to avoid misclassification of re-infection with a resistant strain.

**Multidrug-resistant TB (MDR TB):** is defined as resistance to INH and RIF, the most effective first-line drugs.

**Extensively drug-resistant TB (XDR-TB):** is defined as MDR-TB also resistant to FQ and any of the second-line injectable anti-TB drugs (AMI, KAN and/or capreomycin)

**Table 1.** Mechanisms of action and genetic markers of anti-TB drugs [84, 85, 94, 95, 105, 129, 130]

Drug	Mechanism of action	Activity/ bacillary population	Genetic marker of drug resistance		Frequency of mutations (%)
			gene	Protein encoded	
INH	Inhibits mycolic acids synthesis in the cell wall	Bactericidal. Extra and intracellular replicating bacilli	<i>katG</i>	Catalase peroxidase	42-58
			<i>inhA</i>	Enoyl ACP reductase	21-34
			<i>ahpC</i>	Akyl hydroperoxide reductase	10-15
			<i>kasA</i>	B-ketoacyl ACP synthase	--
			<i>ndh</i>	NADH dehydrogenase	
RIF	Inhibits transcription of DNA to RNA	Bactericidal. Replicating and low-metabolic bacilli	<i>rpoB</i>	$\beta$ -subunit of RNA polymerase	96-100
PZA	Affects membrane energy metabolism	Bactericidal. Intracellular, semi-dormant bacilli in acidic environment	<i>pncA</i>	pyrazinamidase	72-97
SM	Decreases ribosomal protein synthesis	Bactericidal. Extracellular, actively replicating bacilli	<i>rpsL</i>	S12 ribosomal protein	52-59
			<i>rrs</i>	16S rRNA	
EMB	Inhibits cell wall synthesis	Bacteriostatic. Actively replicating bacilli	<i>embCAB</i>	Arabinosyl transferase	47-65
AMI /KAN	Reduces ribosomal protein synthesis	Bactericidal. Replicating bacilli	<i>rrs</i>	16srRNA	76
ETO	Inhibits cell wall synthesis	Bacteriostatic. Extra and intracellular bacilli	<i>inhA</i>	Enoyl ACP reductase	--
FQ	Inhibit DNA gyrase	Bactericidal	<i>gyrA</i>	DNA gyrase	75-94

### 1.3 METHODS FOR DRUG SUSCEPTIBILITY TESTING

Since the discovery of the tubercle bacillus by Robert Koch in 1882, the diagnosis of TB has relied on the microscopic observation of the pathogen. The smear microscopy uses a staining procedure based on the acid fastness of *M. tuberculosis* to determine if acid-fast bacilli (AFB) are present in a clinical specimen. This technique, although simple and inexpensive, has a low sensitivity since it requires 5000-10000 AFB/ml to be positive. Additionally, it cannot differentiate between MTC and non-tuberculous mycobacteria.[2, 82]

Therefore, culture of concentrated and decontaminated clinical samples is necessary to isolate and identify *M. tuberculosis*. The culture has the advantage of being very sensitive (it detects 10-100 AFB/ml) but it needs up to eight weeks to observe visible growth. The identification process takes into account the slow growth and the colony morphology in solid culture medium. The species identification is also based on the metabolic profile of mycobacteria, determined by using biochemical tests. *M. tuberculosis* characterizes for having slow-growing, non-pigmented, rough colonies capable to produce niacin, reduce nitrate and use a thermo labile catalase [82].

Determination of drug-susceptibility profiles generally requires primary isolation of *M. tuberculosis* in order to standardize the inoculum to be seeded on culture medium with and without the drugs of interest. This type of DST is known as indirect method. It is also possible to directly inoculate decontaminated clinical specimens using direct DST methods. Most of the conventional and widely used DST methods are indirect techniques. DST methods can also be categorised as phenotypic or genotypic, as it will be described below.

#### 1.3.1 Phenotypic Methods

Phenotypic methods assess inhibition or detection of *M. tuberculosis* growth in the presence of anti-TB drugs to distinguish between susceptible and resistant strains. These are the phenotypic techniques considered as reference methods for DST:

- Proportion method: It is a widely used method, especially in resource-limited settings (RLS). It uses solid media, either Lowenstein-Jensen or Middlebrook agar, to determine the proportion of resistant mutants to a given drug. Its turnaround time (TAT) is between 4-6 weeks and can be used both as direct or indirect method [22, 23].
- Absolute concentration method: The resistance of a strain is expressed in terms of the lowest concentration of a certain drug that inhibits all or almost all the growth of the strain. This is an indirect method on solid media with a TAT of 4 weeks[23, 53]
- Resistance ratio method: In this method the unknown clinical isolate is compared with a susceptible standard laboratory strain. It is also performed as an indirect method on solid media, with a TAT of 4 weeks[22].
- BACTEC 460 TB: it is a commercial semi-automated liquid system (Becton Dickinson, Sparks, MD) that uses radiometric detection of  $^{14}\text{CO}_2$  as growth indicator. The principle behind BACTEC 460TB is the same used in proportion method. Its TAT is 5-7 days[91].
- Mycobacterial Growth Indicator Tube (MGIT): The MGIT 960 system (Becton Dickinson, Sparks, MD), uses a modified Middlebrook 7H9 broth combined with fluorescence quenching-based oxygen sensor to detect growth. The indicator fluoresces under UV light if there is viable growth. The method can be used as a manual or automated technique. [87]

These conventional phenotypic methods although reliable and accurate, are time-consuming, cumbersome and have prolonged TATs. The BACTEC 460TB, due to the fact that uses liquid media has the advantage of a reduced TAT. However, it is an expensive method that requires especial conditions for the management of the radioactive waste generated. New automated broth-based methods such as the BACTEC-MGIT960 and the BacT/Alert 3D system (bioMeriux, Durham, NC, USA)[8] have been developed to replace the BACTEC460TB. Then again, the high cost related to the acquisition of the equipment and supplies is an important barrier for their implementation in RLS.

The need of rapid and affordable DST techniques for implementation in RLS has lead to the development of alternative, low-cost, non-commercial methods. Based on their principle for detection of growth, the non-commercial DST methods can be categorised as follows:

- Colorimetric methods: these methods use either an oxidation-reduction indicator or nitrate reduction detection. For instance, the alamar blue, the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and resazurin have been used as redox-indicators that change colour in presence of viable mycobacteria in the medium[74, 79]. The oxidation-reduction assays use liquid media in a microplate format, which allows easy determination of minimum inhibitory concentration. They have been evaluated for detection of resistance to first- and second-line drugs, demonstrating a high sensitivity and specificity (>95%) in different settings. Furthermore, their TAT is comparable to the one obtained with automated broth-based systems[68].

On the other hand, the nitrate reductase assay (NRA) is based on the ability of *M. tuberculosis* to reduce nitrate to nitrite, which is detected as a colour change after adding commonly available chemical reagents[7]. NRA has demonstrated its high reproducibility as well as its good sensitivity for detection of resistance to RIF and INH (> 94% and 92% respectively)[66]. It has the advantage of using Lowenstein-Jensen (LJ) medium and the same breakpoint concentrations as the proportion method (PM), which can facilitate its implementation in RLS where PM is already used. NRA was originally developed as an indirect DST on solid media, but it has also being used as direct DST using liquid media [4, 5, 103]. The TAT for the indirect NRA is 10 days, when solid media is used, and 5 days using liquid media. Recently it also has being adapted for detection of second-line drugs and PZA [63, 65, 102].

- Micro-colony-detection assays: These methods rely on the visual detection of *M. tuberculosis* micro-colonies either on solid or liquid medium. More specifically, the cord-formation capacity of *M. tuberculosis* is used in the microscopic-observation drug-susceptibility assay (MODS). MODS has been primarily used for TB diagnosis, and it has been adapted for direct detection of resistance to first-line drugs in liquid medium. MODS requires the use of an inverted microscope to examine the microplates containing specimen-inoculated medium. It has been demonstrated MODS capacity for rapid detection of resistance (7-14 days) with acceptable performance parameters (sensitivity >86%).[10, 24, 69, 70, 80]  
Another technique within this category is the thin-layer agar (TLA). The principle of detection relies on the microscopic detection of micro-colonies in solid media. And advantage of TLA compared to MODS, is that it uses a standard microscope instead of an inverted one [92].
- Phage-based assays: They are based on the ability of mycobacteriophages to infect and replicate within viable *M. tuberculosis* bacilli. Propagation and replication of these



mycobacteriophages in rapidly-growing mycobacteria will be directly proportional to number of *M. tuberculosis* bacilli[67].

### 1.3.2 Genotypic methods

Genotypic DST methods rely on the detection of genetic markers related to phenotypic drug resistance. Generally, these are polymerase chain reaction (PCR)-based methods targeting well characterised resistance-related mutations. Currently there are two commercially available, solid-phase hybridization techniques, commonly referred as line probe assays (LPAs). The INNO-LiPA Rif TB Assay, (Innogenetics, Ghent, Belgium)[50, 93] and the GenoType® MTBDR<sub>plus</sub> assay (Hain Lifesciences, Nehren, Germany)[43] have been shown to have high sensitivity for detection of RIF-resistance (>95%). Sensitivity for INH-resistance using GenoType MTBDR<sub>plus</sub> has improved, in comparison with the previous version of this test, but still is variable (70-100%)[61].

Among other techniques used for detection of conferring-resistance mutations can be mentioned the real-time PCR[107], the automated DNA sequencing[97] and microarrays[34, 104]. Recently, a new automated one-step system for direct detection of tuberculosis and RIF-resistance in sputum samples has been developed. The Xpert MTB/RIF system (Cepheid, Sunnyvale, CA, USA)[14, 41] has been evaluated in a multicenter study demonstrating its potential for rapid and accurate detection of drug resistance in RLS.

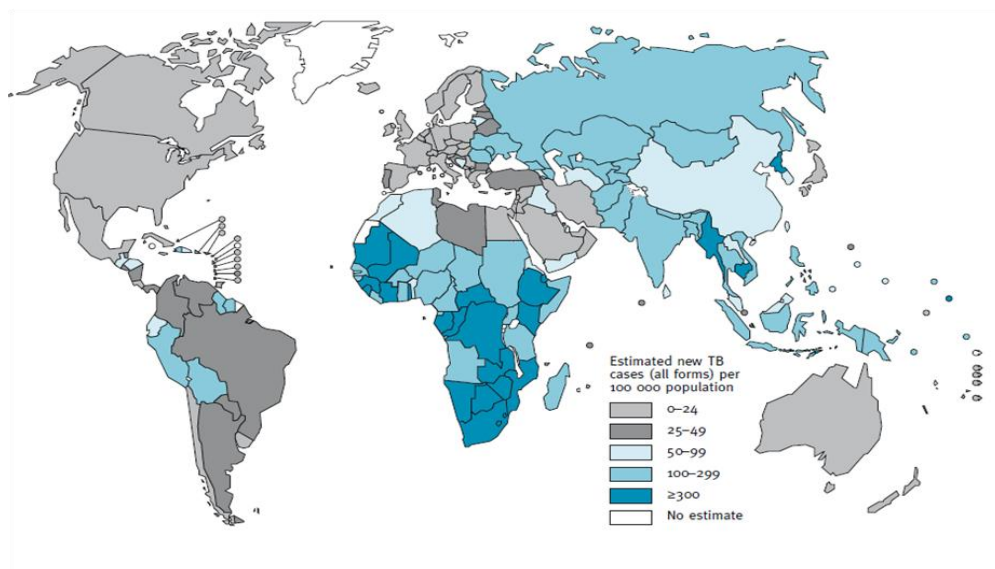
In general, genotypic DST methods offer a shorter TAT than the phenotypic alternatives currently available (1-2 days vs. 1-4 weeks) and can be used directly on clinical specimens.

## 1.4 TUBERCULOSIS EPIDEMIOLOGY

### 1.4.1 Global burden of the disease

TB remains as one of the main public health problems worldwide. In 2009, WHO estimated 9.4 million of new TB cases globally (incidence rate of 137/100000 population). Of those, only 5.8 million cases were notified; that is equivalent to a case detection rate of 63%. The TB burden is concentrated in developing countries (95% of the cases) and more specifically, in 22 high-incidence settings that account for 81% of the cases. Most of the estimated cases occurred in Africa and Asia, as shown in figure 1. The prevalence rate was estimated to be 200/100000 population, which is equivalent to 14 million TB cases [120].

**Figure 1.** Estimated TB incidence, by country. 2009.WHO [120]



### 1.4.2 HIV and TB

The HIV-TB co-infection represents one of the main challenges in TB control especially in sub-Saharan countries [120]. HIV is the strongest risk factor for developing active TB among those with LTBI [88]. On the other hand, TB is one of the main causes of mortality in people living with HIV. In 2009 TB mortality among HIV-positive people was estimated in 1.3 million and 0.38 million, respectively. Only 22% of TB patients knew their HIV status. The magnitude of this deadly association is even more worrisome taking into account the significant association of drug-resistant TB and HIV-infection [33].

### 1.4.3 M/XDR-TB

As previously mention, development and diagnosis of drug resistance is another main topic that needs attention. In 2009 only 12% of the estimated MDR-TB cases were notified. The MDR-TB incidence cases are estimated in 440,000 (2008), 5.4% of which are estimated to have XDR-TB. By March 2010, 58 countries have reported at least one XDR-TB case [120].

### 1.4.4 Strategies for TB control

The main objective of TB control is to reduce mortality, morbidity and disease transmission, while avoiding drug-resistance development.

#### **Box 2.** DOTS components.

1. Political commitment to sustained TB control activities
2. Case detection through direct sputum smear microscopy
3. Standardized short-course treatment with direct observation of patient's drugs-intake
4. An effective drug supply and management system
5. Monitoring and evaluation system and impact measurement

After TB was declared as a global emergency in 1993, WHO proposed a global strategy for control TB. The directly observed treatment, short course (DOTS) strategy was implemented in 1995. DOTS is based on 5 key elements (box 2) [127] to assure access to diagnosis and treatment. Within this approach, two major targets were defined: to achieve a case detection rate of 70% and to cure 85% of the new TB cases detected

Case detection rates and treatment outcomes improved after DOTS introduction. A similar approach was adapted for MDR-TB treatment called DOTS-plus. WHO created the Green Light Committee (GLC) in 2000 as a mechanism to facilitate access to affordable, high-quality, second-line anti-TB drugs for the treatment of MDR-TB in RLS [121].

New challenges and inherent limitations of the DOTS strategy prompted the implementation of an improved strategy lead by WHO's Stop TB Partnership in 2006 [118]. The Stop TB strategy comprises 6 components (box 3) and together with the United Nations Millenium Development Goals (MDG), has proposed the long-term targets for TB control in the Global Plan to Stop TB 2006-2015 (box 4)[118, 120].

Recently, a mid-point update of the Global Plan to Stop TB 2006-2015 has been presented[119]. This updated version for the period 2011-2015 still focuses on the 2015 targets but also takes into account the progress made since 2006 and the changes in TB

policy and epidemiology. Low- and middle-income settings are still prioritized within the global plan for TB control.

**Box 3.** Components of the Stop TB strategy:

1. To pursue high-quality DOTS expansion and enhancement.
2. To address TB/HIV, MDR-TB and the needs of the poor and vulnerable populations.
3. To contribute to health-system strengthening based on primary health care
4. To engage all health providers
5. To empower people with TB and communities through partnerships
6. To enable and promote research.

One of the major components that has been incorporated in the new plan is laboratory strengthening. It has been acknowledged that the TB laboratory capacity available worldwide is insufficient to address the diagnostic challenges related to HIV- associated TB and drug –resistant TB. Consequently, the Global Laboratory Initiative (GLI) was created and subsequently established as a new Working Group of the Stop TB Partnership in 2008. GLI has as main purpose to expand access to quality assured TB diagnostic services within integrated laboratory systems [117].

**Box 4.** Global TB Control targets:

**Millennium development goals (MDG):**

- To have halted and begun to reverse the incidence by 2015, in comparison with 1990

**STOP TB partnership:**

- To have halved deaths by 2015 in comparison to 1990
- To have reduced the global incidence of active TB cases to < 1 case per million per year by 2050

## 1.5 MOLECULAR EPIDEMIOLOGY

Molecular epidemiology has become an important tool for TB transmission surveillance. Through molecular epidemiology techniques it is possible to confirm whether or not a patient has been the source of infection for another TB case. It also allows us to discriminate between reinfections and relapses as well as to detect cross- contamination of specimens in the laboratory [110].

The identification of several genetic markers useful for strain typing of *M. tuberculosis* in the early 1990's led to the development of standardized methods [42, 55]. The restriction fragment length polymorphism (RFLP) is still considered the gold standard for *M. tuberculosis* genotyping due to its high discriminatory power. RFLP uses the insertion sequence (IS) 6110 as genetic marker. IS 6110 is specific for the MTC mycobacteria and the number of copies of this mobile element differs from one strain to the other. The

technique uses chromosomal DNA extracted from the *M. tuberculosis* clinical isolates. After digestion with a restriction enzyme (*PvuII*), the DNA is separated by gel electrophoresis and transferred to a membrane by southern-blot. The individual patterns are detected by chemiluminescence and further analyzed with bioinformatics software. The grade of similarity of the different isolates determines the relatedness of the TB cases [108].

Some *M. tuberculosis* strains have low-copy numbers (<5) of the IS6110, therefore RFLP discriminatory power among this type of strains decreases [99]. Additionally, RFLP requires sufficient bacterial growth to ensure high-quality DNA extraction and is a laborious method.

Another DNA-fingerprinting tool is spoligotyping. This PCR-based technique relies on the detection of 43 short non-repetitive oligonucleotide sequences interspersed within the direct repeat (DR) region of the MTC genome. The number of spacer sequences varies between the different strains, allowing the strain-typing. Spoligotyping is available as a commercial kit, and uses solid-hybridization of the biotin-labelled products obtained after the PCR amplification [51]. The detection and analysis of the spoligopatterns is similar to the procedure used for RFLP.

Besides of being a fast, simple and robust tool, spoligotyping allows easy international comparison of the fingerprints. The SpolDB4 database [17] was developed as an open-access, internet-available, international database that facilitates the identification of major *M. tuberculosis* genetic lineages or families according spoligotyping. The SITVIT2 database is the updated version of SpolDB4 and comprises more the 70,000 spoligopatterns from 160 countries.

The latest molecular epidemiology tool is called MIRU-VNTR typing [101]. The name of the method stands for mycobacterial interspersed repetitive unit (MIRU)-variable number of tandem repeats (VNTR). This is also a PCR-based method which analyzes different MIRU loci (12-24 loci) and determines the VNTR based on the size of the amplicons obtained. The PCR products can be labeled or not and the detection can be done using gel electrophoresis, automated capillary systems or high performance liquid chromatography. MIRU-VNTR is faster than RFLP and, similarly to spoligotyping, its results can be compared easily. However, it still does not reach the discriminatory level offered by RFLP[110].

Neither spoligotyping nor MIRU-VNTR alone reaches the discriminatory power of RFLP. Therefore, complementary strain-typing with different techniques is needed to obtain a better definition of clustering proportion. This approach combined with conventional epidemiology methods can also provide an improved overview of TB transmission dynamics in a given setting.

## 1.6 HONDURAS

### 1.6.1 General information



Figure 2. Map of Honduras

Honduras is located in the heart of Central America. The country shares boundaries with Guatemala in the West, El Salvador in the South-West, Nicaragua in the South-East, the Pacific Ocean in the South and the Caribbean Sea in the North. Figure 2 is the map of Honduras and box 4 presents general information of the country [1, 44, 45]. With an area of 112,492 Km<sup>2</sup> and a population of 8 millions (estimate 2010), Honduras is administratively divided into 18 provinces (“departamentos”), comprising 298 municipalities. Most part of the territory is mountainous; the climate varies from sub-tropical in the lowlands to temperate in the

mountains. The country is extremely vulnerable to natural hazards such as hurricanes, flooding and mild earthquakes [6].

### 1.6.2 Health care system

The Ministry of Health (MoH) is the regulatory agency responsible to implement the national health policies, in order to guarantee sufficient supply of medicines and access to health care services. The health care system comprises the MoH, the Honduran Social Security Institute (IHSS) and the private sector. The MoH and IHSS constitute the public sector.

The health care system in Honduras is fragmented; the communication and coordination between the health care providers is weak. Therefore, there is a duplication of efforts and concentration of resources, leaving some areas unprotected [77]. The MoH provides health care services to approximately 60% of the population; 18% have access to the IHSS and 5% go to the private sector. The remaining 17% do not have access to any type of health care services [48]

#### Box 5. General information about Honduras

**Capital:** Tegucigalpa

**Languages:** Spanish (official), Amerindian dialects

**Economic activities:** Agriculture, Industry, Services

**Export products:** coffee, bananas, African palm, timber, shrimp, lobster

**GDP (PPP) /per capita:** \$4000 (est. 2009)

**Income level:** lower-middle income.

**Population below poverty line:** 59% (2008)

**Literacy rate** (adults >15years): 84%

**Rural population:** 49%

**Sex ratio:** 1.05 male/female

**Age distribution:**

- ✓ 0-14 years: 38%
- ✓ 15-64 years: 58.4%
- ✓ ≥65 years : 3.6%

The health-care-facilities of the MoH are organized in three levels, as shown in figure 3. Although it is the main provider within the health care system, the MoH has limitations in terms of infrastructure, quality and coverage of the services offered to the population.

The IHSS is a decentralized institution that provides services to workers and their families, if they are affiliated and employed in the formal sector. The IHSS beneficiaries have access to

primary and emergency health care, ambulatory clinics and specialty hospitals[48, 77]. Regarding the private sector, it is estimated that 33 hospitals and 300 clinics belong to this category of health services [48].

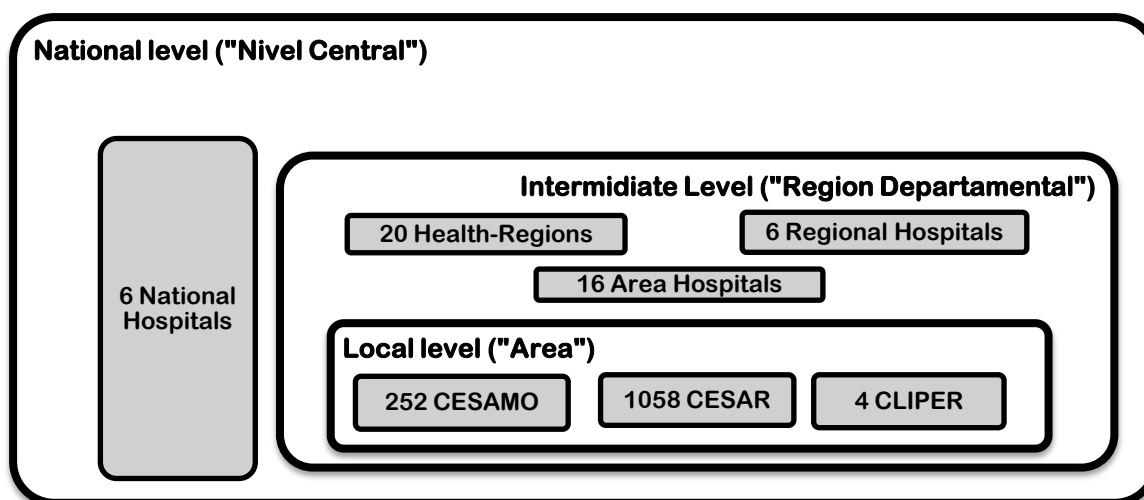
#### 1.6.2.1 Health indicators

Life expectancy in Honduras is 73.3 years; 69.9 for men and 76.9 for women. The infant mortality rate is 26/1000 live births and the fertility rate is 3.4 children born/woman (2009) [45]. Although the fertility rate has decreased during the last decades, the incidence of teenage pregnancies has increased, especially among poor, uneducated women living in rural areas. Among children under 5 years, the health problems are often related to acute respiratory infections, bacterial diarrhoea and malnutrition. This situation is due to environmental factors such as air contamination, lack of access to potable water and sanitation and is also associated to the mother's low education-level [48].

Communicable diseases remain as major public health problems in Honduras, extremely related to poverty conditions in the country. The most common infectious diseases are intestinal parasitic infections, dengue fever, malaria, Chagas disease and TB. Sexually-transmitted diseases are also an important health issue and may be associated to the increase of HIV/ AIDS incidence as well. It is estimated that around 28,000 people are living with HIV/AIDS, with a prevalence of the infection among adult population of 0.8% [48]

**Figure 3** Structure of the MoH attention levels [48].

CESAMO: health center with a medical doctor. CESAR: rural health center. CLIPER: peripheral clinic



### 1.6.3 National TB Control Programme

The organized fight against tuberculosis in Honduras started in the early 1940's with the opening of the first TB dispensary. TB patients were clinically evaluated and diagnosed at the dispensary, after active case-finding was done by nurses. Towards the end of that decade a TB Sanatorium was inaugurated to provide health care services to the many TB patients in need of specialized treatment. The National TB Control Program (NTP) was established in 1957. Since then the NTP has been responsible to coordinate and implement activities to improve prevention, diagnosis and treatment of TB patients[111].

The short-course chemotherapy regimen was introduced in 1985. The short-course regimen consists of two phases[111]. The first phase is a 2-months combined treatment with INH, RIF, SM and PZA. In the second phase only INH and RIF are given during a period of 4 months. TB treatment is only available in the public sector[46].

In 1998, the DOTS strategy was implemented in the country. The NTP was able to expand DOTS to 100% of the health-care units countrywide by 2003. The NTP has also reported the achievement of detection and treatment-success goals (>70% and 85% respectively), with a slow but steady decline of the reported TB incidence. The reported incidence rate has decreased from 77/100000 in 1990, to 37/100000 in 2008. However, the NTP also faces challenges and limitations. For instance, the case-detection among symptomatic respiratory patients has decreased, the default rate and death rates still range between 6 to 9%[112].

Pulmonary TB is the most common clinical manifestation of the disease in Honduras. The extra-pulmonary forms of TB have been increasing since 2001, probably as a consequence of the HIV/TB co-infection. However, data about the co-infection prevalence is limited; only 60% of the TB patients are HIV tested. Of those who were tested in 2007, 10% were HIV positive[112].

The NTP has a vertical structure, which is integrated to the MoH attention levels, mentioned previously. The primary level is responsible of the case detection, sample collection and smear microscopy (if the health unit has a laboratory)[46]. In coordination with the National TB Reference Laboratory (NRL), the NTP offers TB diagnosis in 150 laboratories of the public network. The bacteriological confirmation of cases relies on smear microscopy. Only five laboratories have capacity to perform solid culture on LJ and DST in centralized at the NRL. The national laboratory network needs to improve its overall infrastructure, resources availability and quality performance in order to provide an optimized and timely diagnosis of TB[112].

Regarding the occurrence of drug resistance among TB patients, according to the national TB drug resistance survey carried out in 2002, 12% of the new cases and 34% of the previously-treated patients developed resistance to any of the anti-TB drugs[116]. Primary MDR-TB prevalence was 1.8% whereas the acquired MDR-TB was prevalent in 12% of the TB patients. The drug-resistant cases are concentrated in the regions with high population density, like Tegucigalpa and San Pedro Sula [47].

Second-line drugs treatment for MDR-TB cases was available in 2005 after the Green Light Committee approved the Honduran programme. A total of 54 patients received second-line drugs.. Among this cohort, the cure rate was very low (9%), a high proportion of defaulters (56%) were observed, 9% of the patients had treatment failure and the mortality rate was 15%[112].

In 2009 the NPT launched the National Strategic Plan for TB control 2009-2015. As part of this plan, the NTP proposes to raise the goals for case-detection and treatment-success to 90%. Following the MDG, the aim is to achieve a prevalence < 47/100000 population, an incidence rate < 38/100000 population and a mortality rate < 5% [47]. Besides these challenging goals, the NTP also is giving priority to the strengthening of the national laboratory network, to expand laboratory services and to increase MDR-TB case detection rate.

## 2 ABOUT THIS THESIS

### 2.1 GENERAL AIM

The overall aim of this thesis is to increase knowledge about the effectiveness of laboratory assays for early detection of drug-resistant tuberculosis as well as the understanding of *M. tuberculosis* biodiversity and transmission patterns in Honduras.

### 2.2 SPECIFIC OBJECTIVES

- To evaluate the microscopic observation drug susceptibility (MODS) assay for the detection of *M. tuberculosis* drug-resistance to isoniazid and rifampicin among symptomatic respiratory patients at risk for multidrug-resistant tuberculosis (MDR-TB). (Study I)
- To evaluate the indirect and direct nitrate reductase assay (NRA) for rapid low-cost detection of patients with MDR and XDR- TB. (Study II and III)
- To explore the frequency and type of mutations in genes associated to rifampicin, isoniazid and fluorquinolones resistance in *M. tuberculosis* isolates collected from MDR-TB patients from Honduras (Study IV)
- To describe the genetic diversity and the transmission patterns of *M. tuberculosis* strains in Honduras, using molecular fingerprinting. (Study V)



### 3 MATERIALS AND METHODS

This chapter summarizes the materials and methods used in the studies included in this thesis. A more detailed description of the methods can be found in the corresponding articles and manuscripts (I-V). The thesis research framework is shown in figure 4.

#### 3.1 STUDY POPULATION

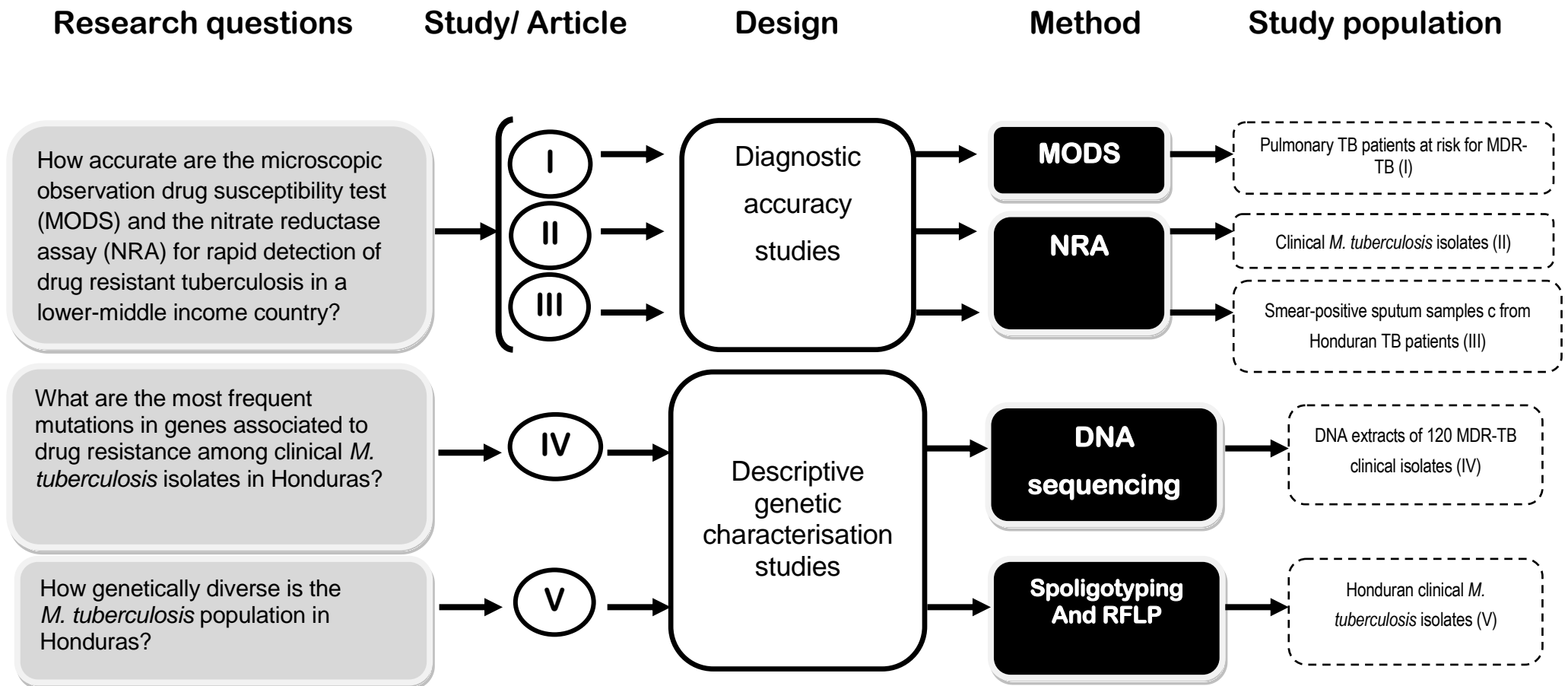
In study I, Honduran and Brazilian patients at risk for drug resistant tuberculosis were enrolled between 2002 and 2005. Symptomatic respiratory patients undergoing evaluation for pulmonary tuberculosis at the Instituto Nacional Cardiopulmonar (INCP), previously known as Instituto Nacional del Torax, and the Federal University of Rio de Janeiro were included in the study according to the following criteria: 1) Age  $\geq 12$  years, 2) Cough  $\geq 3$  weeks, 3) At least one of the following risk factors: a) Suspected TB treatment failure, b) Suspected TB relapse, c) Treatment default and d) Contact with a confirmed MDR-TB case. Then, the study subjects' respiratory specimens submitted for routine-care laboratory testing were analyzed. Their clinical data was also recorded for subsequent analysis.

For the evaluation of the indirect NRA carried out in study II, a panel of 192 clinical *M. tuberculosis* isolates was used. The strains were chosen based on their susceptibility profiles to selected first- and second-line drugs, as defined by either proportion method or BACTEC 460 TB. The purpose of using this panel was to ensure a reasonable proportion of resistant and susceptible strains, and thus mimic the spectrum of drug-resistant TB patients intended to be diagnosed with the indirect NRA. Among the 89 resistant strains, 40 were MDR, 21 were XDR and the remaining 28 had resistance to at least one of the drugs tested. The majority of the susceptible strains belonged to the NRL strain collection, whereas most of the resistant isolates belonged to the Swedish Institute for Infectious Disease Control (SMI) archive.

In study III, consecutive smear-positive sputum samples from Honduran pulmonary TB patients were used for the field evaluation of the direct NRA. Only one specimen per patient was analyzed and no personal identifiers were registered for study purposes. The samples were collected in purposely selected health-care centres located in the main metropolitan areas of Tegucigalpa and San Pedro Sula between March 2008 and June 2009. The four health-care units included were selected based on their annual average of reported TB cases and the basic logistics requirements to conduct the study.

In the multicenter study IV, DNA samples obtained from MDR *M. tuberculosis* isolates were used to explore the frequency and distribution of mutations conferring resistance to selected first- and second-line drugs. The convenience sample of 120 clinical isolates was selected based on its phenotypic categorisation as MDR-TB by reference drug-susceptibility tests. The geographic origins of the isolates were Belarus, China, Honduras, Iran, Iraq, Romania and Uganda. The time period of the strains isolation was 1994-2009.

A total of 206 *M. tuberculosis* strains isolated from the same number of Honduran TB patients were used for the molecular epidemiology characterization described in study V. The clinical isolates were collected at two different time points. The strains isolated between 1994 and 1998 were collected at the INCP, whereas the strains isolated in 2002 were collected countrywide and characterized at the NRL. General epidemiological data was retrieved from laboratory records and/or medical files.



**Figure 4.** Research framework of the thesis

## **3.2 COLLECTION AND STORAGE OF STRAINS**

### **3.2.1 Primary isolation of *M. tuberculosis***

Respiratory specimens (expectorated sputum samples, induced sputum samples, bronchoalveolar lavage), provided by TB patients, were decontaminated using the *N*-acetyl-L-cysteine-sodium hydroxide method or the modified Petroff's method [53, 128]. Smear microscopy of the decontaminated sediments was performed using Ziehl-Neelsen acid-fast staining[53].

Lowenstein-Jensen slants were inoculated, incubated at 37°C and examined at least once a week for 8 weeks. Bacterial growth with characteristic mycobacterial colony-morphology and AFB positive results was further characterised with standard biochemical tests (niacin production, nitrate reduction and catalase activity) [53] for designation as MTC isolates. The clinical isolates were kept in Middlebrook 7H9 or skim milk at -20 or -70°C.

### **3.2.2 Clinical isolates**

The clinical *M. tuberculosis* isolates stored at SMI were kept at -70°C in Middlebrook 7H9 supplemented with glycerol. The Honduran clinical isolates stored at the NRL were kept in skim milk at -20 or -70°C.

All isolates were sub-cultured on Lowenstein Jensen medium before testing in studies II, IV and V.

## **3.3 DRUG SUSCEPTIBILITY TESTING**

### **3.3.1 Non-commercial methods**

Two rapid, non-commercial and low-cost techniques were evaluated for detection of drug-resistant tuberculosis. In study I, the MODS assay was tested using the following critical drug concentrations: INH 0.1µg/ml and RIF 2.0 µg/ml. Both, undiluted and diluted specimens were used for media inoculation. MODS DST results were interpreted 14 after initial detection of *M. tuberculosis* growth in the controls.

In study II, the indirect NRA on LJ was evaluated for detection of resistance to INH, RIF, OFX and KAN, using as critical concentrations 0.2µg/ml, 40.0µg/ml, 2.0µg/ml and 30.0µg/ml, respectively. In this study two interpretation criteria were used: a) a strain was considered resistant if the colour change in the drug-containing tube was greater or equal to the one observed in the 1:10 diluted growth control (original model); b) a strain was considered resistant if any colour change was observed in the drug-containing drug after the control tube was positive (alternative model).

In study III, the diagnostic accuracy of NRA for direct detection of multidrug-resistance on smear-positive sputum samples was assessed, using the same drug concentrations for INH and RIF used in the indirect NRA.

### **3.3.2 Conventional reference methods**

The proportion method (PM) on LJ was used for first-line DST of the Honduran isolates in studies I-V. For routine DST of the isolates belonging to the SMI's collection as well as for the determination of resistance to the selected second-line drugs, the BACTEC 460 TB was used. In study IV, depending on the geographic origin of the clinical isolates included, either

the absolute concentration method or BACTEC MGIT 960 was used, besides the already mentioned reference tests.

### 3.3.3 Determination of Minimum Inhibitory Concentration

In study II, minimum inhibitory concentration (MIC) for OFX and KAN were determined. Two-fold dilutions series of the drugs tested (drug concentration range of 0.062-512 µg/ml for OFX and 0.031-64 µg/ml for KAN) were incorporated on Middlebrook 7H10 agar supplemented with OADC. The MIC was defined as the lowest concentration at which no bacterial growth was observed.

## 3.4 GENETIC CHARACTERISATION

### 3.4.1 DNA isolation

DNA was extracted from the clinical *M. tuberculosis* isolates using the cetyl-trimethyl ammonium bromide method [109] or by lysis. The lysates were obtained after heat-killing the bacteria (80°C, 30-60 minutes), followed by a treatment with Tris-HCl [50] or NaCl.

### 3.4.2 DNA sequencing

In study IV, Sanger DNA sequencing [97] was used to identify resistance-related mutations in the *rpoB*, *kat G* and *gyrA* genes as well as in the promoter region of the *mabA-inhA* gene. Specific primers were synthesized by Invitrogen (Life Technologies, USA) and PCR products were obtained after amplification following standard procedures at SMI. The purified PCR products were sequenced and analysed in an automated genetic platform. The DNA sequences were aligned using the clustal W algorithm [106].

### 3.4.3 Genotyping techniques

The DNA fingerprinting strategy used in the study V consisted in the initial characterisation with spoligotyping of all the isolates; thereafter, only those strains belonging to the main spoligotyping cluster were further characterised using IS6110-RFLP.

Spoligotyping was performed according standard protocol [51] and the binary patterns obtained were converted to octal numbers. This conversion allowed the comparison of our results with the SITVIT2 proprietary database of the Institute Pasteur of Guadeloupe. Spoligotyping international types (SIT) numbers as well as the major *M. tuberculosis* genetic lineages were assigned to the Honduran isolates according previously defined criteria [17].

RFLP was also performed using international standard procedures [108]. All the genotyping results were analyzed using the BioNumerics software version 5.1 (Applied Maths, Sint-Martens, Latem, Belgium) and correlated to the general epidemiological data of the patients

### 3.4.4 Quality assurance

The reference strains *M. tuberculosis* H37Rv (studies I-V), *M. tuberculosis* 14323 and *M. bovis* BCG (study V) were used as controls. All culture media lots were checked for contamination prior being used.

### 3.4.5 Statistical analysis

Data was compiled and analyzed using EpiInfo™ version 3.5.1 (Centers for Disease Control, Atlanta, USA) in studies I, III and V. Additionally, Microsoft Office Excel 2007® (studies II, III, IV) and the open-access software OpenEpi version 2.3 [29] (study III) were used.

Major outcome measures for the studies of diagnostic accuracy were:

- sensitivity (ability to detect truly resistance)
- specificity (ability to detect truly susceptible strains)
- positive likelihood ratio (ratio of true resistance rate to the false resistance rate)
- negative likelihood ratio (ratio of true susceptibility rate to the false susceptibility rate)
- positive and negative predictive values (proportion of resistant and susceptible strains correctly diagnosed).[30, 89]

The agreement between the evaluated test and the reference method was assessed using the kappa coefficient and /or McNemar's  $\chi^2$  test.

In study V, clustering proportions were calculated and association between demographic characteristics and clustering by spoligotyping was assessed using Yates-corrected  $\chi^2$  or Fisher exact test. For point estimates, 95% confidence intervals were calculated

### **3.4.6 Ethical considerations**

Study I was approved by the Institutional Review Boards at Johns Hopkins University School of Medicine (Baltimore, MD, USA), the University of Alabama at Birmingham (Birmingham, AL, USA), the Federal University of Rio de Janeiro, and the INCP (Tegucigalpa, Honduras)

For studies III, IV and V, involving samples collected and/or analysed in Honduras, ethical approval was granted by the Ethical Committee in Biomedical Research [ *Comite de Etica en Investigaciones Biomedicas (CEIB)*] of the Universidad Nacional Autonoma de Honduras (UNAH). Additionally, the Ethical Committee of INCP also granted its approval for collection of smear-positive sputum samples from patients attending this facility, for inclusion in the study III.

Studies II and IV were regarded as *in vitro* evaluations according to local guidelines of the countries from which the isolates used originated.

## 4 RESULTS

### 4.1 NON-COMMERCIAL DST METHODS

#### 4.1.1 Detection of resistance to first-line drugs

##### 4.1.1.1 *Direct susceptibility testing*

In study I, MODS results from 180 confirmed pulmonary TB patients with at least one risk factor for drug-resistant TB were available for the final analysis. Sensitivity for both INH and RIF using undiluted samples was  $\approx 96\%$ , whereas INH specificity (78%) was slightly lower than the one observed for RIF (83%). The missclassification of resistance (false-resistant results) was somewhat more evident when undiluted specimens were used. Overall, the MODS assay was able to correctly categorised 66 of the 69 MDR-TB cases (96%) identified by proportion method on LJ.

In study III, the direct NRA performance indicators were estimated based on 108 comparable results with proportion method on LJ. We observed 100% sensitivity for INH-resistance and a relatively low sensitivity for RIF-resistance (80%). The specificity for both drugs was  $\geq 99\%$ . Regarding detection of MDR-TB, the direct NRA was able to detect 4 out of 5 MDR-TB cases. One of the MDR-TB cases correctly identified by NRA was confirmed by DNA sequencing analysis. The proportion method results of this isolate could not be interpreted due to insufficient growth.

##### 4.1.1.2 *Indirect susceptibility testing*

As expected, in study II we confirmed the indirect NRA excellent agreement for INH and RIF, with sensitivities between 95 to 98% and a specificity of 100% for both drugs. These results were consistent, independently of the interpretation criteria used to read the NRA results.

Of the 39 MDR strains, 36 (92%) were correctly identified by NRA. The major discrepancies observed were two false RIF-susceptible and one false INH-susceptible result. In addition, four cases, although correctly identified as INH- and RIF-resistant, were missclassified in their susceptibility profile to second-line drugs. More details of these discrepancies are given below.

Tables 2 and 3 show the performance of all the non-commercial DST for detection of resistance to INH and RIF, respectively.

#### 4.1.1 Detection of resistance to second-line drugs

In contrast to the high sensitivity for detection of resistance to first-line drugs, we observed a lower sensitivity to detect OFX and KAN resistance. Sensitivities for both second-line drugs ranged between 56% to 86%, using the original interpretation criteria. These figures improved using the alternative interpretation of the NRA results. On the other hand, the specificity was high, between 96% and 100% for these drugs.

As previously mentioned, 4 of the MDR cases were misclassified, either as false pre-XDR isolates or were not detected as such. Additionally, 11 of the 21 XDR cases were not correctly detected by NRA, mainly due to false KAN-susceptible results. The MIC results suggested that some of the missclassified strains had MIC values borderline to the breakpoint concentration used in the reference test, BACTEC 460.

**Table 2:** Performance parameters for detection of INH-resistance using MODS and NRA

Performance indicator	MODS		Direct NRA	Indirect NRA	
	Undiluted	Diluted		Original Model	Alternative Model
% Sensitivity (95%CI)	97 (92-99)	97 (92-99)	100 (72-100)	98 (91-100)	100(95-100)
% Specificity (95%CI)	78 (74-81)	83 (78-85)	99 (94-100)	100 (96-100)	100 (96-100)
% PPV (95%CI)	82 (78-84)	86 (82-87)	91 (62-98)	--	--
% NPV (95%CI)	96 (90-99)	96 (91-99)	100 (96-100)	--	--
PLR (95%CI)	4.5 (3.5-5.1)	5.6 (4.3-6.4)	98 (8-1178)	Infinite	Infinite
NLR (95%CI)	0.04 (0.01-0.11)	0.04 (0.01-0.10)	0	0.03 (0.01-0.10)	0
Kappa (95%CI)	0.75 (0.57-0.94)	0.80 (0.62-0.98)	0.95 (0.76-1.14)	0.98 (0.84-1.12)	1 (0.86-1.14)

**Table 3:** Performance parameters for detection of RIF-resistance using MODS and NRA

Performance indicator	MODS		Direct NRA	Indirect NRA	
	Undiluted	Diluted		Original Model	Alternative Model
% Sensitivity (95%CI)	96 (90-99)	96 (90-99)	80 (38-100)	95 (86-99)	97(88-99)
% Specificity (95%CI)	83 (79-85)	85 (81-87)	100 (96-100)	100 (96-100)	100 (96-100)
% PPV (95%CI)	80 (75-82)	82 (77-84)	100 (51-100)	--	--
% NPV (95%CI)	97 (92-99)	97 (92-99)	99 (95-100)	--	--
PLR (95%CI)	5.6 (4.2- 6.4)	6.3 (4.7-7.4)	infinite	Infinite	Infinite
NLR (95%CI)	0.05 (0.02-0.12)	0.05 (0.02-0.12)	0.2 (0.02-1.8)	0.05 (0.02-0.14)	0.04 (0.01-0.12)
Kappa (95%CI)	0.77 (0.58-0.95)	0.79(0.60-0.97)	0.88 (0.69-1.07)	0.96 (0.82-1.11)	0.98 (0.83-1.12)

### 4.1.2 Turnaround time

Results from the direct methods were available within 3 weeks after specimen inoculation. Median time for availability of MODS results was 21 days. For 56% of the samples analyzed in study II, direct NRA results were available at day 14.

The TAT of the indirect NRA was 10 days for most of the isolates tested (79%).

## 4.2 GENETIC CHARACTERISATION

### 4.2.1 Genotypic detection of drug-resistance

In the multicenter study IV, 117 of 120 DNA extracts from clinical MDR-TB isolates were analysed to identify genetic markers of drug resistance. The distribution of mutations varied between the different settings. In this section, special attention will be given to the Honduran isolates.

Mutations in the RIF-resistance determining region (RRDR) in the *rpoB* gene were found in 94% of the isolates. Overall, the most common mutated codons were 531 and 526. The most frequent mutations found were: Ser531Leu (present in all settings), His526Asp (found mainly in Belarus) and His 526Tyr (mostly in Romanian isolates).

The *rpoB* mutation Ser531Phe was only detected in three Honduran isolates which also had the mutation Ser315Thr in *katG* gene. Subsequent genotyping analysis confirmed that these isolates had the same RFLP pattern and that they belong to the spoligotyping T3 family (SIT37). Together with the MDR-TB cluster described in study V (SIT33, LAM3) which harboured the mutations *rpoB* Ser531leu and *katG* Ser315Thr, were the only clusters among the Honduran MDR-TB isolates. The remaining Honduran MDR-TB strains were as diverse in their spoligotyping signatures as the susceptible strains described in study V. [Unpublished data]

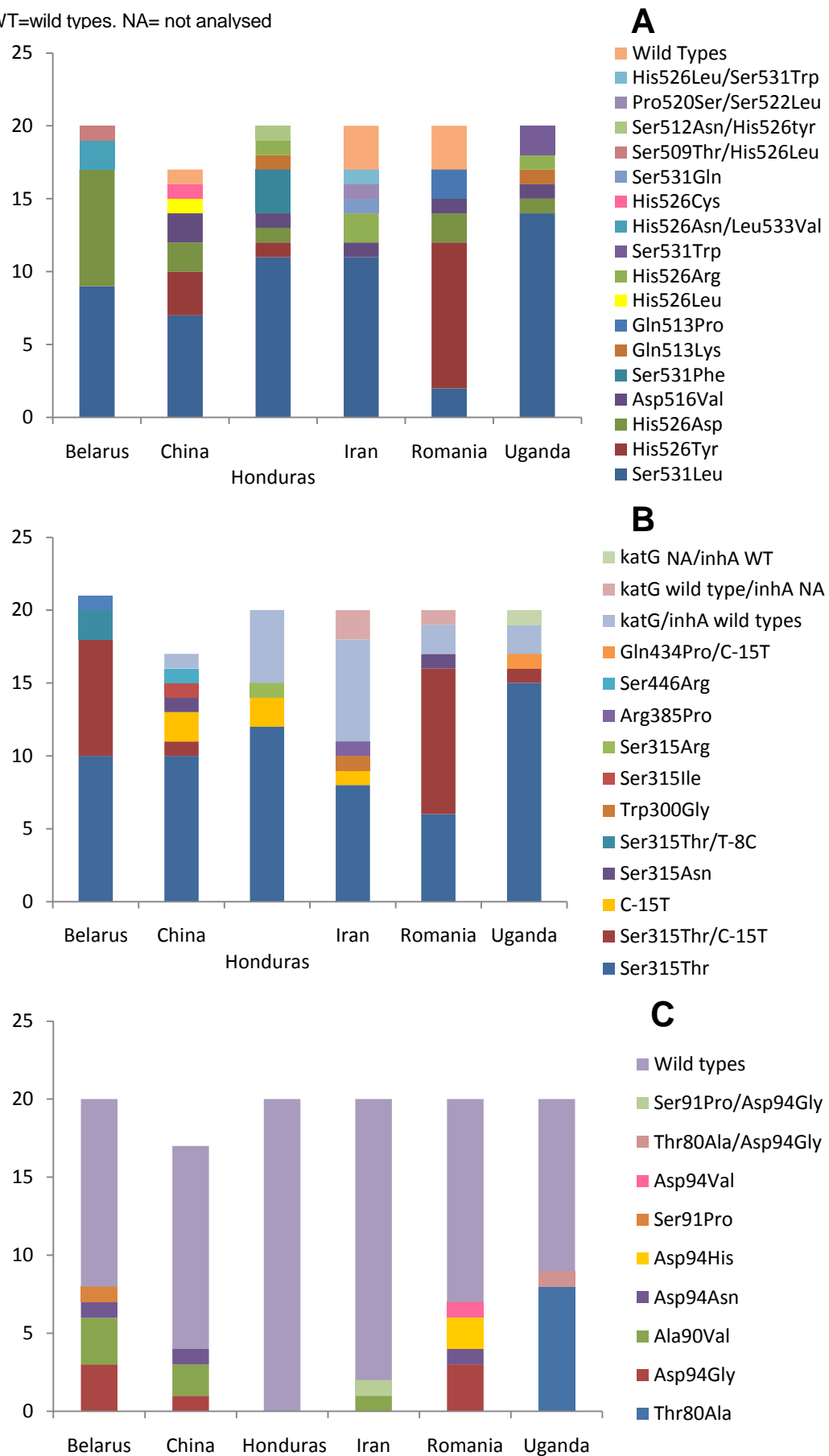
Regarding INH-resistance-conferring mutations, 86% of the strains had mutations in *katG* and/or *mabA-inhA* promoter region. The dually occurring mutations *katG* Ser315Thr + *mabA-inhA* -15 were predominant. This pattern was mainly found in isolates from Romania and Belarus. The majority of strains without mutations in *katG* or *mabA-inhA* promoter region were from Honduras and Iraq.

A total of 22 isolates had OFX-resistance-related mutations in *gyrA* gene. The substitution Asp94Gly was the most common one. No mutations were found in the Honduran isolates, which correlate with the results obtained in the phenotypic DST.

Noteworthy to mention is that the mutation *gyrA* Thr80Ala was only found among Ugandan isolates. One Ugandan strain with Thr80Ala mutation and the resistance-conferring mutation Asp94Gly was susceptible to OFX according to proportion method. Figure x summarizes the distribution of all mutations found.



**Figure 5.** Distribution of mutations found in A) *rpoB*, B) *katG* and *mabA-inhA* promoter region and C) *gyrA*  
WT=wild types. NA= not analysed



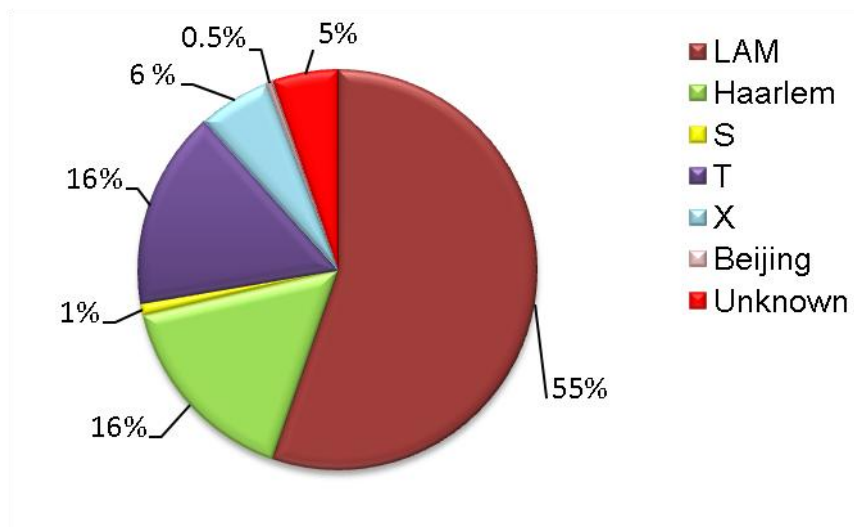
#### 4.2.2 Biodiversity of *M. tuberculosis* in Honduras

In study V, 84% of the 206 clinical isolates characterised were grouped into 27 spoligotyping clusters (2 to 43 strains per cluster). Eighth new international types (SITs) were identified among the Honduran strains, either within the present study or after match with an orphan type previously identified in the SITVIT2 database. In addition, 16 patterns corresponded to orphan (unique pattern), previously unreported isolates.

The Latin American Mediterranean (LAM) lineage was the more prevalent in our study (55% (figure 6)). Only one Beijing isolate was identified. The five most common spoligotypes were: SIT33 (21%), SIT42(10%), SIT67 (9%), SIT 53 (8%) and SIT376(6%). The strains belonging to the major spoligotype cluster, (SIT 33 , LAM 3 genotype), were further characterized using RFLP IS6110. A total of 35 different fingerprints were identified, of which 29 (67.4%) were unique patterns. Six clusters with a total of 14 strains (2 to 3 strains per cluster) were identified.

The majority of the TB patients were male (67%). The average age of the study population was 37 years (SD 17; range 11-85 years). There was no significant association between demographic characteristics of the population and the spoligotype distribution found

**Figure 6.** Distribution of spoligotypes among Honduran isolates



## 5 DISCUSSION

### 5.1 RAPID DETECTION OF DRUG RESISTANCE USING NON-COMMERCIAL DST METHODS

Rapid and accurate diagnosis of drug-resistant TB is the first step for the implementation of adequate treatment and decreasing transmission. Therefore, it is important to have access to reliable diagnostics tools for detection of resistance, especially in settings with high-burden of the disease. Conventional DST methods currently available, although sensitive and specific are either too slow or too expensive for a wide implementation in RLS. Ideally, a diagnostic test for detection of drug-resistant TB should be reliable, accurate, fast, easy-to-implement and affordable. The need of low-cost DST methods has motivated the development of new assays that could be used where they are needed the most.

As part of this thesis work, two low-cost phenotypic methods were evaluated: the MODS and the NRA assays. Both techniques have been proposed as feasible options for detection of MDR-TB in low- to middle-income settings. However, there was limited evidence of their performance in different contexts, either as direct or indirect tests, as well as their applicability for second-line DST.

Overall, both NRA and MODS showed excellent performance for detection of resistance to the most important first-line drugs, INH and RIF. Particularly the indirect NRA (study II) confirmed high sensitivity and specificity for these drugs [66].

The indirect NRA offers a relatively shorter TAT (7-12 days) when compared to conventional techniques in solid medium, such as the proportion method (28-42 days). It also has been demonstrated that it might have operational advantages compared to other non-commercial, liquid-based assays. Recently, a head-to-head comparison of seven new DST techniques in a low-income, high-TB-burden setting rendered NRA as the most feasible phenotypic assay for implementation of rapid DST in places with similar limitations[19]. The robustness of the indirect NRA has also been demonstrated in a multicenter evaluation, where a high level of agreement with the proportion method (>96%) for INH and RIF was achieved [64].

The indirect NRA has also shown to be a versatile technique that can be used as a liquid-based assay. This adaptation has allowed to decrease the TAT (5-8 days) and to simultaneously determine MICs [57, 103]. This application of NRA has shown to be comparable with other colorimetric redox-indicator methods in liquid media [58, 74]. However, it is necessary to consider the biosafety issues related to manipulation of liquid-based systems in laboratories with biosafety level-2 prior implementation of such methods.

The inclusion of KAN together with OFX in the drug panel used with NRA for timely detection of XDR-TB strains after initial confirmation of MDR-TB was evaluated for the first time in study II.. In our evaluation, the sensitivity for OFX-resistance detection was good (86-100%) and comparable with the results of a previous study [65]. However, the sensitivity for KAN-resistance detection was considerably lower. Recently, NRA was evaluated for detection of resistance to 5 second-line drugs: OFX (2 $\mu$ g/mL), KAN (40Mg/ml), ethionamide (40 $\mu$ g/ml), cycloserine (40 $\mu$ g/ml) and PAS (1.0 $\mu$ g/ml). The sensitivity for all drugs ranged from 86 to 100%, with specificity >97% for all of them[114]. This finding might imply that the discrepancies for detection of KAN-resistance observed in our study were related to possible fitness-cost in the resistant strains tested. The MIC results obtained from those strain with discrepant results indicate that these isolates had MIC closer

to the breakpoint concentration. Nevertheless, these findings support the capacity of NRA to be used with different drugs; the technique has also been validated for detection of PZA-resistance using solid and liquid media [63, 71, 102].

NRA has also evolved into a direct DST assay, both in solid and liquid media. Special interest has been given to direct testing of clinical specimens because the initial isolation of the strain is not necessary. This factor contributes to improve the TAT and it may have more relevant clinical implications.

The evaluation of the direct NRA conducted in study III is one of the first assessments reported up to date. Previously, direct NRA evaluations have been reported mainly from South American countries [76, 98, 100], one African setting [4, 5] and lately, from India[39].

The results of the field assessment of the direct NRA demonstrated not only the excellent diagnostic performance of the assay but also its capacity for being implemented in “real-world” conditions. The study limitations related to the laboratory-system will be discussed in the next section of this chapter.

The direct NRA showed a high sensitivity and specificity for INH-resistance detection, in agreement with pooled estimates previously reported (sensitivity >96, specificity <99)[66]. Due to the fact that only 5 MDR- TB cases were detected, and considering that one of them was not detected as RIF-resistant, the sensitivity for RIF was lower than expected (80%). However, our findings are similar to those reported from Benin [4], a setting that also has a low-incidence of MDR-TB.

Comparing the results obtained with MODS (study I) and direct NRA (study II), MODS had a high sensitivity for both INH and RIF (> 96%) but a lower specificity for both drugs (range 78-83%). This might have been caused by the reading criteria used for interpretation of the results. Drug-containing wells were reported after 14 days after initial detection of growth. This could have contributed to the misclassification. Another possible reason for the low INH specificity is the breakpoint concentration used. Results of a recent meta-analysis study showed that MODS pooled specificity increased from 95.8% to 98.6%, when the breakpoint concentration was 0.1µg/ml and 0.4µg/ml, respectively [70]. However, the changes in cut-off decreased the pooled sensitivity when a higher concentration was used.

In another meta-analysis [18], MODS was compared with direct NRA, Genotype® MTBDR and Genotype® MTBDR<sub>plus</sub>. This report considered that MODS performance, although with good pooled sensitivity and specificity (86-100%), was slightly less good compared to NRA. Other factors may influence the selection of NRA over MODS as technique of choice for implementation in a RLS. The fact that NRA does not need any additional piece of equipment whereas MODS requires an inverted microscope for its use. In terms of workload, MODS may be more laborious and is dependent on a well-trained technician for correct interpretation of the results.

One common concern when direct phenotypic tests are used is the capacity of the test to accurately identified *M. tuberculosis* isolates from non-tuberculous mycobacteria. It has been proposed that MODS should include one additional well containing para-nitro benzoic acid, a specific inhibitor of *M. tuberculosis* [70]. This approach could also be considered in the NRA assay.

## 5.2 BEYOND ACCURACY: CHALLENGES FOR IMPLEMENTATION OF NEW DST METHODS

In recent years, the TB-diagnostics research field has been changing rapidly. The pipeline of new diagnosis tools has increased fast. Acknowledging the need to facilitate a timely transition from the research laboratories to the programmatic level implementation, WHO has also adapted its processes to be able to speed-up the intake of new technologies based on evidence. This evidence has usually been provided by diagnostic accuracy evaluations, where accuracy performance has been used as a surrogate of patient-important outcomes [78, 124]. However, the proliferation of diagnostic tests and their corresponding accuracy evaluations have raised concerns about the quality of the data generated. Therefore, new guidelines and tools have been developed to assess the quality of the design and report of such evaluations. The Standards for Reporting of Diagnostics Accuracy (STARD) initiative has developed a checklist of the relevant information that must be included in the diagnostic accuracy reports [16]. On the other hand, the Quality Assessment of Diagnostic Accuracy Studies (QUADAS) tool intends to provide a well design instrument for evaluation of the methodological quality of the diagnostic accuracy studies [113].

This shift in paradigms is also reflected on WHO decision to adopt the Grading of Recommendations Assessment, Development and Evaluation (GRADE) approach as framework to translate the evidence into policy [78]. The first time the GRADE approach was applied by WHO for TB diagnostics was for the development of guidance for smear microscopy and non-commercial DST methods, in September 2009.

As result of that evaluation, and even though the evidence was graded as moderate, both NRA (direct and indirect) and MODS were endorsed by WHO for interim used in countries where LPAs or automated liquid-based systems are still not available[123, 124].

More recently, it has been developed an Impact Assessment Framework (IAF), an instrument that aims to summarize the evidence not only in terms of test's accuracy but also taking into account its overall impact. The IAF comprises 5 layers: *layer 1*: effectiveness analysis, *layer 2*: equity analysis, *layer 3*: health systems analysis, *layer 4*: scale-up analysis and *layer 5*: policy analysis [62].

Within the new conceptual framework of diagnostic tools evaluations, the approach used in the studies included in this thesis is to some extent limited. However, the evidence they provided belongs to the first layer of the IAF structure and in the particular case of the study III results, our results also shed light on aspects included in layer 3.

The evaluation of the direct NRA in Honduras (study III) clearly exposed the challenges and limitations that a weak infrastructure poses for the successful implementation of new diagnostic tool. In the specific case of this setting, priority must be given to strengthening the laboratory network. Especially since the Honduran NTP wants to implement the indirect NRA by 2011 at the NRL, as is stated in the National TB Strategic Plan 2009-2015 [47].

There is also a need to identify the health-system important outcomes in Honduras. The overall improvement of the quality assurance process, procurement of supplies and human resources availability also need to be considered.

### 5.3 MUTATIONS RELATED TO DRUG-RESISTANT TUBERCULOSIS

The study IV is the first characterisation report of mutations related to resistance in MDR-TB isolates from Honduras, Romania and Iraq. Overall, the distribution of mutations related to resistance to RIF, INH and OFX were in agreement with earlier studies.[15, 25, 28, 40, 52, 115] However, less common mutations in our study, for instance the *katG* mutations Arg385Pro and Ser446Arg have not been previously reported in the TBDRaMDB database[96]. The frequency and distribution of mutations between different settings varied, which also may imply differences in the behaviour of the MDR-TB development and spread. Whereas isolates from Honduras, Iran, Iraq and Uganda had more heterogeneous mutations, strains from Belarus and Romania had less diversity. This might indicate a significant ongoing transmission of MDR-TB in the latter mentioned sites.

Similar inferences can be done with OFX-resistance. Well characterised OFX-resistance-related mutations were detected in isolates from Belarus and Romania. In contrast, these mutations were absent or rare in strains from Honduras, Uganda and Iran. This finding may indicate that fluorquinolone-resistance is less frequent in Central America and Africa than in Eastern Europe.

The Ugandan genotype *gyrA* Thr80Ala + Asp94Gly has been previously associated with hyper susceptibility to FQ [12]. Further studies are needed to characterise the mechanisms underlying this phenomenon.

Knowledge of geographic-specific mutations allows the development and implementation of in-house, PCR-based methods targeting mutations relevant in a specific setting.

For Honduras in particular, these findings indicate that the problem of MDR-TB, is more related to acquired resistance rather than clonal spread of MDR-Tb strains.

### 5.4 GENETIC DIVERSITY OF *M. tuberculosis* IN HONDURAS

The LAM family is the backbone of the Honduran *M. tuberculosis* population (study V). This finding correlates with reports from other Latin American countries, where also the Haarlem and T families are among the most prevalent. In fact, the LAM genotype is widely distributed in the world as well as the three most prevalent SIT found in this study (SIT 53, 42 and 33).

The three predominant families found in study V are included in the Euro-American lineage, one of six phylogeographic clades based on large sequence polymorphisms (LSP)[32]. This phylogeographic description links the mycobacterial populations to ancient human migration and geographic locations. In the case of the Euro-American lineages in particular, which are also present in southern Europe and the Mediterranean area, they might be associated to the population movements during the European colonial era. However, this association to the human host can also be influenced by other causes, such as cultural, social or epidemiological factors [86].

These genotypes might have adapted to the Latin-American population, either because they did not have competitors or because they are host-specific due to selective advantages acquired during their evolutionary process. Recently, a new LPS lineage derived from a LAM9 ancestor has been reported in Brazil[59]. The RD<sup>Rio</sup> *M. tuberculosis* strains may be associated with high virulence, transmissibility and clinical presentation of active disease [60]. This host-specific adaptation may be also the reason why only a low frequency of *M. tuberculosis*- Beijing type in Latin America has been previously reported [3,

11, 21, 73, 75, 90]. Therefore, the presence of only one, fully susceptible, Beijing strain in our study is not surprising.

Although we observed a high proportion of clustering, the presence of several subfamilies within the LAM lineage is an indicator of the high level of biodiversity in this population. The genetic variability of the study population was further confirmed with the complementary RFLP typing of the major spoligotype cluster. This observation is in agreement with the previous genotyping study conducted Honduras [83]. Therefore, this finding suggests that TB transmission in this setting is not related to clonal spread of the disease. Nevertheless, prospective, population-based studies are needed to assess more in detail this possibility. Furthermore, association with HIV status, clinical manifestation of the disease as well as epidemiological links also needs to be considered. In the mean time, our findings provide a baseline for future analyses.

## **5.5 METHODOLOGICAL CONSIDERATIONS**

### **5.5.1 Studies of diagnostic accuracy**

Taking into account the items included in the QUADAS tool, the following reflections regarding the methodological limitations of the studies I-III can be mentioned:

- The spectrum of patients/mycobacterial isolates was appropriate for the evaluation conducted in study I. The MODS assessment as a DST technique was part of a larger, prospective, cross-sectional study [10] where patients were enrolled based on clinical suspicion of TB and further evaluated based on risk factors for having MDR-TB. In study II possible effect of spectrum bias is minor since the archived strains were selected with the intention to replicate a broad range of drug-resistance profiles. However, the prospective selection of consecutive smear-positive cases done in study III, might have introduce bias, since the majority of cases would have been expected to be drug-susceptible.
- Blinding of the results was carried out in all the studies, except study II. This may also have been a source of bias.
- Misclassification bias was observed in the MODS evaluation due to the interpretation criterion used, in combination with the break point concentration for INH. Both MODS and NRA may be prone to subjective interpretation of the growth indicator (cord-form or colour intensity) since both assays are more qualitatively interpreted.
- Un-interpretable results were reported in all the studies.
- In study III we observed that loss in follow-up of some of the samples was due to limitations of the reference method used. The insufficient or lack of growth in the primary isolation on LJ or in the proportion method did not allow the final comparison with direct NRA.
- In general, the validity of the tests evaluated was high, as reflected on their performance parameters (sensitivity and specificity)

### **5.5.2 Genetic studies**

The use of relatively small and convenient sample size may have introduced bias in studies IV and V. In study IV, most of the MDR-TB isolates included were isolated from consecutive cases. However, the number of isolates provided from each setting is likely representative of a localized region or city, rather than representative of the country. In the same study we could not reach complete phenotypic verification of resistance to OFX,

since some of the collaborating centres do not perform routinely DST for second-line drugs.

In the genotyping characterization (study IV) the study population represented  $\approx 1\%$  of the total amount of TB cases reported during the same time period when the samples were initially collected. This may have caused an underestimation of the clustering proportion [35, 36].



## 6 CONCLUSIONS

- The microscopic-observation drug-susceptibility assay (MODS) showed a good sensitivity and high negative predictive value when used for direct detection of resistance to isoniazid and rifampicin in respiratory samples. This characteristics may make MODS suitable as a screening test in order to prioritize *M. tuberculosis* isolates for subsequent confirmatory indirect susceptibility testing using conventional methods.
- The nitrate reductase assay (NRA) demonstrated a high sensitivity and specificity for detection of isoniazid and/or rifampicin resistance, both as direct or indirect test. NRA is a reliable option for detection of MDR-TB, also suited for low- and middle-income countries.
- Our study showed that NRA is also a promising diagnostic tool for detection of resistance also to second-line drugs. Further studies are needed to improve NRA sensitivity, especially for kanamycin.
- The most frequent resistance-conferring mutations among MDR-TB clinical isolates from Honduras were *rpoB* Ser531Leu and *katG* Ser315Thr. No mutations related to OFX-resistance were detected in Honduran *M.tuberculosis* strains. We also observed a very clear difference in the prevalence of these mutations between different study settings. The understanding of geographical differences on the prevalence of mutations related to resistance is important for the development and implementation of new diagnostic tools for drug-resistant tuberculosis.
- The Latin American Mediterranean (LAM) family was identified as the most common spoligotype among clinical *M.tuberculosis* isolates in Honduras. This is a reflection of the LAM-family's adaptation to the human host-population over time. The high biodiversity within the Honduran *M.tuberculosis* population was demonstrated using RFLP in the identification of several DNA-fingerprints among strains belonging to the major spoligotype-cluster (SIT33, Lam3). Association between clustering and demographic characteristics was not significant.

## 7 RECOMMENDATIONS

- Prior implementation of any new diagnostic tool in a resource-limited setting it is necessary to assess the impact of such implementation in terms of infrastructure, human resources, sample referral system and financial investment required. In the particular case of Honduras, it must be a priority to strengthen the laboratory-health system and to improve the quality assurance process in TB laboratory diagnosis.
- The indirect NRA is the more appropriate technique for implementation in the current conditions observed within the Honduran National Laboratory Network. The possibility to scale-up and decentralised drug susceptibility testing in Honduras should be carefully considered after validation and evaluation of the initial phase of such implementation.
- Implementation of line-probe assays could also be considered now that there is data available about resistance-related mutations in Honduran MDR-TB cases. The partnership between the National Reference Laboratory and the National University (UNAH) should be sustained and expanded in order to explore new collaborations for development and implementation of genotypic detection of drug-resistant TB in Honduras.
- Operational research in relevant areas for TB control in Honduras is urgently needed. These types of investigations will provide valuable information that will help to improve the health care service provided to TB patients in the country.
- More evidence is required to assess the operational impact of non-commercial, low-cost, rapid DST methods in field conditions. Preferably, these evaluations should be done as randomised trials in combination with cost-effectiveness estimations.

## 8 FUTURE RESEARCH

- The potential use of NRA for detection of resistance to second-line drugs should be further investigated. Also it could be interesting to develop the technique for specific detection of *M. tuberculosis* (by adding p-nitro benzoic acid), as well as its use with Ogawa culture medium. The latter will allow the direct inoculation of decontaminated samples without prior centrifugation. This technical modification, if proven to be as effective as the original platform, would allow the implementation of the assay in primary attention level.
- Further molecular epidemiology studies for prospective evaluation of transmission in Honduras are needed. Population-based studies, combined with contact tracing can provide a more detailed picture of the transmission and its association with HIV status and clinical manifestation of the disease.

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