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RAPID TESTS FOR MULTIDRUG RESISTANT TUBERCULOSIS IN LOW INCOME SETTINGS

Freddie Bwanga

This thesis is based on a joint Ph.D training program between
Karolinska Institute and Makerere University

Karolinska Institute
SE - 171 177 Stockholm, Sweden

Makerere University
P . O. Box 7062 Kampala, Uganda

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From Departments of Microbiology, Tumor and Cell Biology, Karolinska Institute Stockholm, Sweden; Bacteriology, Swedish Institute for Infectious Disease Control Solna, Sweden; and Medical Microbiology School of Biomedical Sciences Makerere University College of Health Sciences Kampala, Uganda

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SMITTSKYDDSSINSTITUTET
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"TB is the child of poverty - and also its parent and provider",
Archbishop Desmond Tutu, in this quote
encapsulated the link between tuberculosis (TB) and poverty!

ABSTRACT

Tuberculosis (TB) is at epidemic levels in the resource-limited settings (RLSs) due to HIV/AIDS, poverty and insufficient TB control programmes. These factors are also contributing to TB drug resistance. Patients with multidrug drug resistant tuberculosis (MDR-TB) do not respond to first line drugs. These patients require unique drug regimens, making it necessary to routinely screen for MDR-TB. Screening for MDR-TB with the Lowenstein-Jensen proportion method (LJPM), which is common in the RLSs is a very slow process – taking 2-3 months. More rapid tests suitable for RLSs are urgently needed. In this thesis, a comparison of the technical and operational performance of several rapid tests for MDR-TB was done, and the most optimal tests for RLSs are proposed.

In **paper I**, a meta-analysis of rapid tests for direct detection of MDR-TB was conducted. The direct nitrate reductase assay (NRA), microscopic observation drug susceptibility (MODS) and Genotype[®] MTBDR*plus* (GT-DR*plus*) were highly sensitive and specific, and far more rapid than the conventional indirect drug susceptibility testing (DST).

In **paper II**, the NRA, MODS, Mycobacterium Growth Indicator Tube (MGIT 960), GT-DR*plus*, Alamar blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and resazurin assays were compared head-to-head for indirect detection of MDR-TB at the National Tuberculosis Reference Laboratory (NTRL) Kampala. The NRA, MGIT 960, GT-DR*plus* and MODS were the most sensitive and specific tests, with significantly shorter time to results compared to the LJPM.

In **paper III**, the direct NRA and MODS assays were compared at the NTRL on consecutive sputum specimens from re-treatment TB patients. Interpretable results were obtained in over 90% of the samples with both assays. The median days to results were 10 with the NRA and 7 with MODS. The direct NRA was more sensitive and specific, and was cheaper.

In **paper IV**, the sensitivity, specificity, time to results (TTR) and reproducibility of the direct GT-DR*plus* against the MGIT 960 was assessed. Sensitivity and specificity were 100% and 96% for detection of rifampicin resistance; 81%, and 100% for isoniazid resistance; and 92%, and 96%, for MDR-TB, respectively. The TTR was 1-3 days, and concordance of results between the Molecular Laboratory at Makerere University and the FIND Diagnostics Laboratory was 98%.

In **paper V**, we applied spoligotyping to study the clustering rate and predominant genotypic strains of 99 MDR-TB strains isolated from patients in Kampala. Eighty three percent of the strains occurred in clusters, and the T2 lineage was the largest single cluster.

Conclusion. The direct NRA and the GT-DR*plus* appear to be the most appropriate tests for MDR-TB in RLSs. The NRA being the cheapest test can be applied where resources are extremely limited, while the ultra rapid but commercially available GT-DR*plus* can be used where resources permit.

LIST OF PUBLICATIONS

- I. **Bwanga Freddie**, Hoffner S, Haile M Joloba ML. Direct susceptibility testing for multi drug resistant tuberculosis: A meta-analysis. *BMC Infectious Diseases* May 2009, **9**:67.
- II. **Bwanga Freddie**, Moses L Joloba, Melles Haile, Sven Hoffner 2010. Evaluation of seven tests for rapid detection of multi drug resistant tuberculosis in Uganda. *Int J Tuberc Lung Dis* 14(7):890–895
- III. **Bwanga Freddie**, Melles Haile, Sven Hoffner, Emmanuel Ochom, Moses L. Joloba. Direct Nitrate Reductase Assay versus Microscopic Observation Drug Susceptibility for rapid detection of MDR-TB in Uganda. Manuscript.
- IV. Heidi Albert, **Freddie Bwanga**, Sheena Mukkada, Barnabas Nyesiga, Julius Patrick Ademun, George Lukyamuzi, Melles Haile, Sven Hoffner, Moses Joloba, Richard O'Brien. Rapid screening of MDR-TB using molecular Line Probe Assay is feasible in Uganda. *BMC Infectious Diseases* 2010, 10:41
- V. **Bwanga Freddie**, William George Muyombya, Sven Hoffner, Melles Haile, Benon Asiiimwe, David Kateete, Fred Katabazi, Jennifer Asiiimwe, Maria Wijkander, Moses L Joloba. High clustering of MDR-TB strains in Kampala, Uganda: Predominance of the T2 lineage. Manuscript.

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LIST OF ABBREVIATIONS

AcpM	acyl carrier protein
AIDS	Acquired Immunodeficiency Syndrome
bp	Base pair
BSL	Bio safety level
DNA	Deoxyribonucleic acid
DR	Drug resistant
DST	Drug Susceptibility Testing
FIND	Foundation for Innovative New Diagnostics
GI	Growth Index
HIV	Human immunodeficiency virus
IJTL	International Journal of Tuberculosis and Lung Disease
InhA	Enoyl-acyl carrier protein reductase
IUATLD	International Union Against Tuberculosis and Lung Disease
JCRC	Joint Clinical Research Centre
KasA	β -ketoacyl-ACP synthase
KatG	Catalase-peroxidase enzyme
KNO ₃	Potassium nitrate
LJPM	Lowenstein-Jensen proportion method
LiPA	Line probe assay
MDR	Multidrug resistant
MGIT 960	Mycobacterium Growth Indicator Tube
MIC	Minimum inhibitory concentration
MODS	Microscopic observation drug susceptibility
MTB	<i>Mycobacterium tuberculosis</i>
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADH	Reduced nicotinamide adenine dinucleotide
NRA	Nitrate reductase assay
NTRL	National Tuberculosis Reference Laboratory
OADC	Oleic Acid-Albumin-Dextrose-Catalase
PANTA	Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin
PAS	<i>p</i> -aminosalicylic acid
PCR	Polymerase chain reaction
PNB	Para-Nitrobenzoic Acid
RFLP	Restriction fragment length polymorphism
RLSs	Resource-Limited Settings
RNA	Ribonucleic Acid
sROC	Summary Receiver Operator Characteristic curve
TB	Tuberculosis
TTR	Time to results
VNTRs	Variable number of tandem repeats
WHO	World Health Organization
XDR-TB	Extensively drug resistant tuberculosis

1 INTRODUCTION

1.1 BACKGROUND

The discovery of anti-TB drugs in the 1940s followed by combination chemotherapy made TB a curable disease. In the developed countries, effective treatment and surveillance reduced tuberculosis dramatically with high hopes of total eradication (1-2). However, in the 1980s, it was realized that tuberculosis had not only ceased to decline in the developed countries, notably the USA, but was actually increasing, particularly in major cities (2). It was also soon realized that the disease was out of control and increasing at an alarming rate across most of the poorest regions of the world especially Africa due to HIV/AIDS (1, 3). Presently, the WHO estimates that one-third of the world's population is infected with *Mycobacterium tuberculosis* (MTB) - the bacterium that causes tuberculosis, and 9 million new cases of active TB and 2-3 million deaths occur annually – 95% in developing countries (4-5).

Global efforts to control the TB pandemic have been undermined by the emergence and spread of strains that are resistant to the commonly used first line anti-TB drugs isoniazid (H), rifampicin (R), ethambutol (E), and pyrazinamide (Z). Strains resistant to at least H and R, the two most efficacious TB drugs are termed multi drug resistant (MDR) (6). MDR-TB treatment is rather complicated as it requires second line drugs some of which are only injectables, are less efficacious, more toxic and more expensive than the first line agents (7). Treatment lasts for 18-24 months but only around 50% – 60% of MDR-TB patients will be cured compared with 95%–97% cure rate for patients with drug-susceptible strains treated with first line agents (8-9). The recent emergency of extensively drug resistant tuberculosis (XDR-TB) defined as MDR-TB strains with resistance to a fluoroquinolone and to at least one injectable second line drug (kanamycin, amikacin, or capreomycin) has further complicated the problem of MDR-TB (6). A study in South Africa found a mortality rate from XDR-TB of 90% among the HIV infected patients due to lack of treatment options (10).

1.2 EPIDEMIOLOGY OF MDR-TB

The Global Project on Anti-Tuberculosis Drug Resistance Surveillance has been gathering data since 1994. The latest data indicates that every region of the world has reported MDR-TB, as shown in figure 1 (11).

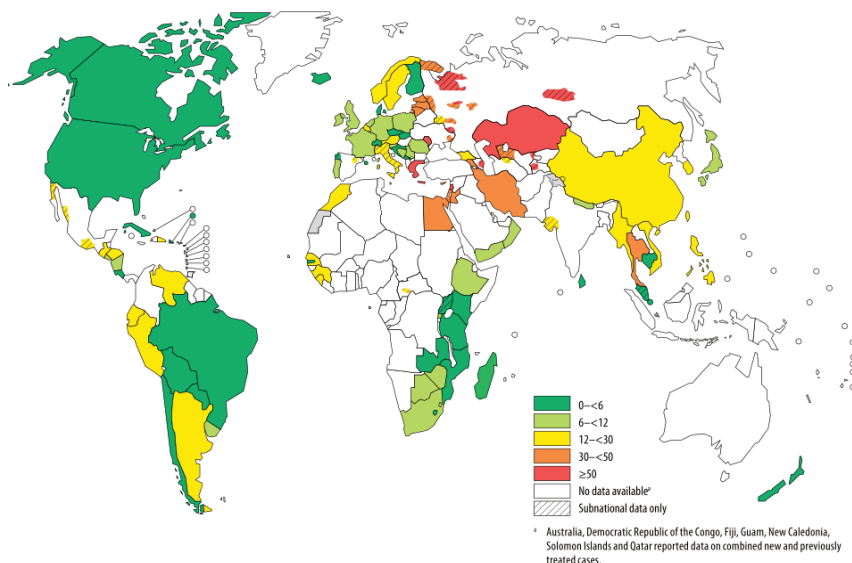


Figure 1. Proportion of MDR-TB among previously treated TB cases, 1994–2009

Source: World Health Organization (11)

1.2.1 MDR-TB Prevalence

The number of prevalent cases of MDR-TB in many parts of the world is estimated to be much higher than the number of incident case arising annually. Globally, the median prevalence of MDR-TB is reported to be 3% among the new and 15% among the re-treatment cases (12). Countries and territories in Eastern Europe such as Tajikistan, Uzbekistan and parts of China have the highest MDR-TB prevalence – up to 15% among the new and 60% among the previously treated cases (13). In sub Saharan Africa, inadequacy of laboratory services makes it difficult to estimate the actual burden of MDR-TB. However, surveillance data (2005-7) from Cote d’Ivoire, Ethiopia, Madagascar, Rwanda, and Senegal, reported a prevalence of 1-4% among the new and 4-17% among the previously treated TB cases (12). In Uganda, according to the first MDR-TB surveillance study done in 1996-7, a prevalence of 0.5% among the new and 4% among the previously treated cases was reported (14). Temple et al (2008)

found MDR-TB in 13% of the previously treated cases at Kampala (15). A more recent survey conducted in Kampala, found MDR-TB in 1.5% and 13% among the new and re-treatment cases, respectively (Joloba ML 2009, personal communication). These data indicate that if immediate control measures including improvements in laboratory infrastructure are not done, MDR-TB will be increasing dramatically in many African countries including Uganda.

1.2.2 MDR-TB Incidence

The estimated global number of incident MDR-TB cases among new and relapse TB cases in 2008 was 440 000 ((95% CI: 390 000–510 000) (16). Based on incident MDR cases, the WHO and the Stop TB Partnership identified 27 high MDR-TB burden countries responsible for 85% of the global estimated burden of MDR-TB. These countries refer to those Member States estimated by WHO in 2008 to have had at least 4000 MDR-TB cases arising annually and/or at least 10% of newly registered TB cases with MDR-TB (16). The countries are: Armenia, Azerbaijan, Bangladesh, Belarus, Bulgaria, China, Democratic Republic of the Congo, Estonia, Ethiopia, Georgia, India, Indonesia, Kazakhstan, Kyrgyzstan, Latvia, Lithuania, Myanmar, Nigeria, Pakistan, Philippines, Republic of Moldova, Russian Federation, South Africa, Tajikistan, Ukraine, Uzbekistan and Viet Nam. China and India account for almost 50% of the estimated global number of incident MDR-TB cases (16).

Due to the limitations in susceptibility testing in many countries, it is believed that the true magnitude of the MDR-TB problem in the World is larger than currently known (12). According to the Stop TB Partnership's Global Plan to Stop TB, 2006–2015, an estimated 1.3 million MDR-TB cases will need to be treated in the 27 high MDR-TB burden countries between 2010 and 2015 alone, at an estimated total cost of US\$ 16.2 billion.

1.2.3 Risk factors for MDR-TB

Exposure to anti-TB drugs. All worldwide drug surveys show that prior exposure to anti-TB drugs is the commonest risk factor for drug resistance (12, 16). Exposure to TB drugs helps to select for the pre-existing resistant mutant strains of *M. tuberculosis* in the patient, which then dominate the lesions.

HIV infection. Upcoming evidence suggests a possible association between HIV and MDR-TB. MDR-TB has been widely documented in nosocomial and other congregate settings among people living with HIV (10, 17-18). The 4th report on anti-tuberculosis drug resistance also reported a significant association between HIV-positive status and MDR-TB in Latvia and Donetsk Oblast of Ukraine (16). Furthermore, in Lithuania HIV-positive TB patients had an 8.4 (95% CI: 2.7– 28.2) times higher odds of harboring MDR-TB strains than TB patients for whom HIV status was unknown (16). Lastly, preliminary results of a survey conducted in Mozambique in 2007 have also found a significant association between HIV and MDR-TB(16).

Gender. While males predominate among TB cases in the World, an association between gender with MDR-TB has been controversial. Studies in South Africa, Australia, the Netherlands and the United States of America have reported slightly higher odds ratios among females than males, while other studies fail to find such associations (16). In general, it appears that the overall risk of harbouring MDR-TB strains is not influenced by gender.

To summarise this section, among all the factors studied so far, prior exposure to anti-tuberculosis drugs is the most important risk factor for MDR-TB.

1.3 ANTI-TUBERCULOSIS DRUGS

There was no known anti-TB chemotherapy until the 1940s. In that decade, streptomycin and *p*-aminosalicylic acid (PAS) were introduced as anti-TB drugs (19-21). The key steps that heralded the development of modern TB chemotherapy however were the demonstration in clinical trials, in 1947-48, that streptomycin was a viable drug for the disease (21). Several drugs have since been discovered and as of today, anti-TB drugs can be broadly categorized into 2 groups based on clinical uses, namely (i) first line and (ii) second line drugs. First-line drugs are used in treatment of new TB cases in whom the risk of resistant TB is low, and are usually given orally. Second line drugs are used for treatment of TB that is resistant to first line drugs, and can be further categorised into 4 subgroups, *i.e.* injectable second line drugs, fluoroquinolones, oral bacteriostatic anti-TB agents and anti-tuberculosis agents with unclear efficacy - not recommended by WHO for routine use in MDR-TB patients

(22-23). Table 1 shows examples under each of these drug groups, the doses, mechanism of action and the genes affected by resistant mutations.

Table 1. Anti-TB drugs, action and genes affected by resistant mutations

Drug	Mechanism of action	Genes affected by resistant mutations
First line drugs (Oral)		
Isoniazid (H)	Inhibits mycolic acid synthesis	<i>katG, HA, oxyR</i>
Rampin (R)	Binds to RNA polymerase inhibiting RNA synthesis	<i>rpoB</i>
Pyrazinamide (Z)	Activated to pyrazinoic acid, which is bactericidal	<i>pncA</i>
Ethambutol (E)	Inhibits cell wall synthesis.	<i>EA,B,C</i>
Second line drugs		
a. Injectable drugs		
Streptomycin (S)	Binds to ribosomal proteins and Inhibits protein synthesis	<i>rrs, rpsL</i>
Amikacin (Am)	Disrupts ribosomal function and Inhibits protein synthesis	<i>rrs, rpsL</i>
Kanamycin (Km)	Binds to 30S ribosomal subunit, Inhibiting protein synthesis	<i>rrs, rpsL</i>
Capreomycin (Cm)	Similar to aminoglycosides	<i>rrs, rpsL</i>
b. Fluoroquinolones		
Ciprofloxacin (Cfx)	Disrupts the DNA-DNA gyrase complex blocking DNA synthesis	<i>gyrA, gyrB</i>
Ofloxacin (Ofx)		
Levofloxacin (Lfx)		
Moxifloxacin (Mfx)		
Gatifloxacin (Gfx)		
c. Oral bacteriostatic anti-TB agents (second-line)		
Cycloserine (Cs)	Inhibits cell wall synthesis	-
Ethionamide (Eto)	Inhibits oxygen dependent mycolic acid synthesis	-
<i>P</i> -aminosalicylic acid (PAS)	Disrupts folic acid metabolism.	-
Rifabutin	Binds to RNA polymerase Inhibiting RNA synthesis	<i>rpoB</i>
Thioacetazone (Th)		
d. Anti-TB agents with unclear efficacy		
Clofazimine (Cfz)	-	-
Amoxicillin/Clavulanate (Amx/Clv)	-	-
Clarithromycin (Clr)	-	-
Linezolid (Lzd)	-	-

Isoniazid (H)

Discovery. In 1912, Hans Meyer and Josef Mally first synthesized isonicotinic acid hydrazide (H) from ethyl isonicotinate and hydrazine as part of their research work at German Charles University in Prague (24). The chemical structure of isoniazid is shown in figure 2.

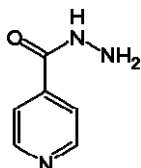


Figure 2. Chemical structure of isoniazid (H)

However, the antituberculosis properties of H were unveiled in the late 1940s when investigations at pharmaceutical companies Hoffman La Roche, Farbenfabriken Bayer, and Squibb Institute for Medical Research each independently discovered H as an antituberculosis agent (25). The efficacy of the drug was proved beyond doubt after a clinical trial in 1951-52 at Sea View Hospital in Staten Island, New York (26). In 1959, H and the earlier discovered streptomycin, later joined by R established a standard combined and effective drug regimen termed *triple* therapy at the time, for treatment against tuberculosis. A cure for tuberculosis was thus first considered reasonable.

Mechanism of action. Isoniazid is a prodrug, and must be activated by a bacterial catalase-peroxidase enzyme (KatG), but the actual form of H that is active *in vivo* remains elusive (27). However, it is believed that KatG couples the isonicotinic acyl with NADH to form isonicotinic acyl-NADH complex and other yet unknown inhibitors (28). These inhibitors bind tightly to their targets, an enoyl-acyl carrier protein reductase (InhA) (29-30) and a β -ketoacyl-acyl carrier protein synthase (31). This process inhibits the synthesis of mycolic acid, a long chain fatty acid-containing component of the mycobacterial cell wall (32-34).

Molecular Mechanism of resistance. Soon after introduction of H, it was realized that H-resistant MTB strains frequently lost catalase and peroxidase activity (35). In the 1990's the primary mycobacterial catalase-peroxidase gene (*katG*) was cloned and sequenced, and mutations in this gene were found in 42–58% of H-resistant clinical isolates, confirming the role of the KatG enzyme in H activity (27). A large number of different mutations have been characterized since then. However, the Ser315Thr mutation is found most often, occurring in approximately 40% of all isoniazid-resistant strains (36-38). The Ser315Thr mutation results in a catalase enzyme that cannot activate isoniazid, but retains approximately 50% of its catalase-peroxidase activity sufficient to enable the organism to detoxify host antibacterial radicals (39).

M. tuberculosis with low level H resistance have been found to also have mutations in the promoter regions, or less commonly in the genes *inhA*, *acpM*, and *kasA* that respectively encode for the mycolic acid-synthesis intracellular proteins: fatty-acid enoyl-acyl carrier protein reductase (InhA), acyl carrier protein (AcpM) and a β -

ketoacyl-ACP synthase (KasA) (8). It is proposed that over-expression of one or more of these target proteins may be the reason for isoniazid resistance in these strains. Mutations in the promoter region of a gene that encodes an alkyl hydroperoxidase reductase (*ahpC*) have been found in approximately 10% of isoniazid-resistant isolates, but mutations in *katG* were also found in these isolates [8,16,23]. The resulting over-expression of alkyl hydroperoxidase reductase may compensate for the loss of catalase-peroxidase activity in these mycobacteria [24].

Rifampicin (R)

Discovery. In 1957, Prof. Piero Sensi and colleagues at the Dow-Lepetit Research Laboratories in Milan, Italy discovered a new bacterium *Nocardia mediterranei* (formerly *Streptomyces mediterranei*) in a sample of soil from a pine wood on the French Riviera (40-42). This new species appeared immediately of great scientific interest since it was naturally producing a new class of molecules with antibiotic activity. These molecules were named "Rifamycins", in memory of the then popular French crime story *Rifi* - about a jewel heist and rival gangs (43). Several Rifamycins were characterized but subsequent studies leading to highly active derivatives were performed on Rifamycin B that was itself practically inactive. After two years of attempts to obtain more stable semi-synthetic products, in 1959 a new molecule with high efficacy and good tolerability was produced and was named "rifampicin" (40), whose chemical structure is shown in figure 3.

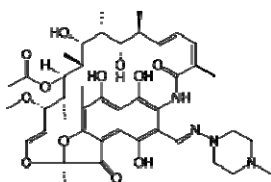


Figure 3. Chemical structure of rifampicin (R)

Thus, R is a semisynthetic bactericidal antibiotic drug of the rifamycin group. Rifampicin was introduced for clinical use in 1967 as a major addition to the cocktail-drug treatment of tuberculosis and meningitis, along with isoniazid, ethambutol, pyrazinamide and streptomycin (44).

Mechanism of action. Rifampicin acts by binding to the beta subunit of the DNA-dependent RNA polymerase. During transcription, DNA enters through the jaw side of

the RNA polymerase and both the DNA and the new RNA strands get out at the exit channel, as shown in figure 4 (45). Rifampicin binds to the exit end of the RNA polymerase in bacterial cells and directly blocks the channel of the elongating RNA when the transcript becomes 2 to 3 nucleotides long (46). This inhibits transcription of DNA to RNA and subsequent translation to proteins (45, 47).

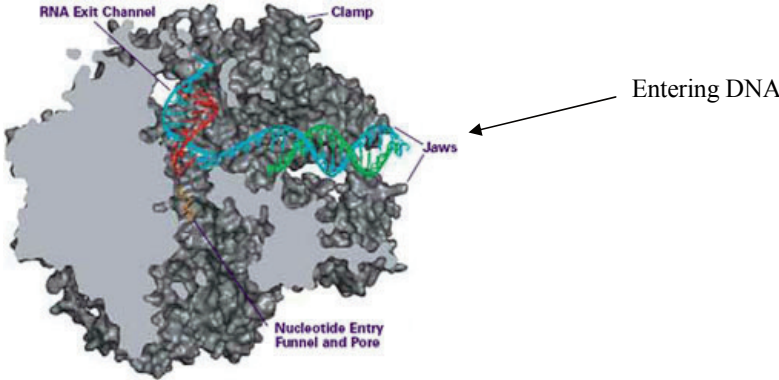


Figure 4. Crystal Structure of the RNA polymerase enzyme

The human RNA polymerase variant is not affected by rifampicin even at 10 times the inhibitory concentration in mycobacteria (47). The rifampicin-RNA polymerase complex in mycobacteria is extremely stable yet experiments have shown that this is not due to any form of covalent linkage (48). It is hypothesized that hydrogen bonds and $\pi-\pi$ bond interactions between naphthoquinone and the aromatic amino acids (phenylalanine, tryptophan, and tyrosine) are the major stabilizers (49).

Mechanism of resistance. The DNA-dependent RNA polymerase (the target for rifampicin binding) is encoded by the *rpoβ* gene as shown in figure 5 (37, 48).

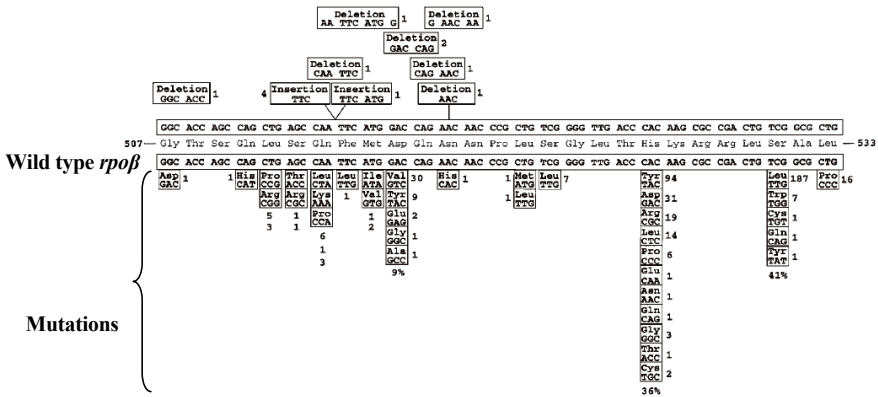


Figure 5. Mutations in codons 507 - 533 of the *rpoβ* gene (37)

Spontaneous mutations (deletions/substitutions/insertions), occurring in the 81-bp hot-spot region of the *rpoβ* gene result into replacement of the aromatic with non-aromatic amino acids in the target RNA polymerase enzyme (figure 5). This results into poor bonding between rifampicin and the RNA polymerase (37, 48), and activity of the enzyme (transcription) is preserved, thus explaining resistance to rifampicin in bacteria.

1.4 DEVELOPMENT OF DRUG RESISTANT TUBERCULOSIS

In the 1940's, when mono therapy with PAS or S was used as treatment for TB, high rates of treatment failures were observed, but was controlled by combining two or more drugs (50). Molecular genetic studies dating to the 1970s showed that resistance to anti-TB drugs resulted from naturally occurring mutations in the genome of *M. tuberculosis* (51). Subsequent studies demonstrated that within a population of *M. tuberculosis* there are mutants that arise due to spontaneous point or deletion mutations (52). Mutations in genes encoding drug targets or drug activating enzymes are responsible for resistance, and such mutations have been found for all first-line drugs and some second line drugs (37).

For a given drug, resistant mutants occur approximately once in every 10^7 to 10^{10} cells (53-54). Therapy with one drug therefore results into rapid selection for the drug-resistant mutants, which will dominate the lesions in the patient. The occurrence of one mutant strain with resistance to two drugs simultaneously, requires a theoretical population of 10^{16} mycobacterial cells. Thus, combining two or more anti-TB drugs reduces effectively, the risk of selecting for resistant mutants in a predominantly susceptible population of *M. tuberculosis*. Indeed by combining PAS and S in the 1940s, the treatment failure rate was reduced to 9% (55). Since then, combination chemotherapy remained the cornerstone for TB treatment. Multi drug resistant and extensively drug resistant TB strains arise by sequential accumulation of resistant mutants to individual drugs until the strain is resistant to drugs that define these forms of resistance.

1.5 TB DRUG SUSCEPTIBILITY TESTING

1.5.1 General considerations

Determination of resistance to a given drug is performed as an *in-vitro* assay in the laboratory, a process called drug susceptibility testing (DST). Where resources are limited, the WHO recommends a hierarchy of DST that should include at least R and H

- the two most efficacious drugs that define MDR-TB (22). For more than 40 years, DST in the developing countries has relied on conventional *indirect* susceptibility methods on Lowenstein-Jensen (LJ) solid medium (56). Indirect testing involves primary isolation of pure colonies of *Mycobacterium tuberculosis*, which are then used as inoculum for DST. In contrast, *direct* DST involves inoculation of processed smear positive samples rather than pure MTB colonies. Results of direct testing are much more rapid and help to triage MDR from non-MDR-TB patients promptly.

1.5.2 Conventional Susceptibility Tests

Three conventional techniques - the proportion method, the absolute concentration and resistance ratio have been standardized, and are widely used in the developing countries (57).

Proportion method. With this method, an equal quantity of a standardized inoculum of *M. tuberculosis* is seeded on a drug-free and drug-containing medium. The drug free medium is seeded with an inoculum that is 100 times diluted compared with that seeded on the drug-containing medium. Distinct, countable colony-forming units (CFU) should be present on the drug-free medium. On the drug-containing medium, only pre-existing resistant mutants are expected to grow. Although the proportion of pre-existing mutants based on a mutation rate of 1 in 10^{7-10} would be much lower, for ease of interpretation, it is theoretically assumed to be 1%, and this has been determined to predict therapeutic outcome (58). Assuming that 1% of the inoculum on the drug medium are resistant mutants, only these mutants will grow, and by dividing the number of CFU on drug medium by those on drug free medium it is possible to deduce that the isolate is susceptible ($\leq 1\%$) or resistant ($>1\%$). Thus to interpret as susceptible, the number of CFU on the drug medium must not exceed those on drug free medium. This is the principle underlying the proportional method of DST in MTB (56, 58).

The proportion method can be performed on LJ or Middlebrook agar medium (59). The LJ medium is recommended by the WHO and the IUATLD for developing countries as it is cheap, easy to read, has low contamination rates and DST results are highly reproducible (60). With the PM, estimation of the inoculum size from the colony-forming units (CFU) counts is easy. However, a single CFU could arise from a clump of bacilli rather than from an individual cell, resulting in an inaccurate calculation of

the proportion of resistant mutants and thus false results. The LJPM is the DST method commonly used in Uganda and in many other developing countries.

Critical concentrations of drugs: The critical concentration (CC) is defined as the concentration that Inhibits *in-vitro* growth of most MTB cells within the population of wild type strains without appreciably affecting the growth of pre-existing resistant mutants (58). Table 2 shows the CC for selected commonly tested anti-TB drugs for the different tests. If resistant mutants exceed 1%, the CC may not inhibit growth, and this predicts therapeutic failure.

Table 2. Critical concentration of commonly tested anti-TB drugs

Drug	Critical concentration, µg/ml				
	Indirect LJPM	Middlebrook agar,		BACTEC 460	MGIT 960
		7H10	7H11		
First line drugs					
Isoniazid	0.2	0.2	0.2	0.1	0.1
Rampin	40.0	1.0	1.0	2.0	1.0
Ethambutol	2.0	5.0	7.5	2.5	5.0
Pyrazinamide	-	-	-	100.0	100.0
Injectable Anti-TB drugs					
Streptomycin	4.0	2.0	2.0	2.0	1.0
Kanamycin	30.0	5.0	6.0	4.0	-
Amikacin	-	-	-	1.0	1.0
Capreomycin	40.0	10.0	10.0	1.25	2.5
Fluoroquinolones					
Ciprofloxacin	2.0	2.0	2.0	2.0	1.0
Ofloxacin	2.0	2.0	2.0	2.0	2.0
Levofloxacin	-	2.0	-	-	2.0
Moxifloxacin	-	-	-	0.5	0.25
Gatifloxacin	-	1.0	-	-	-
Oral Bacteriostatic second-line Anti-TB agents					
Ethionamide	40.0	5.0	10.0	2.5	5.0
Prothionamide	40.0	-	-	1.25	2.5
Cycloserine	40.0	-	-	-	-
PAS	1.0	2.0	8.0	2.0	-

LJPM = Lowenstein-Jensen proportion method; MIC = Minimum Inhibitory concentration; MGIT = Mycobacterium Growth Indicator Tube; LJ = Lowenstein-Jensen; PAS = p-amino salicylic acid.

Source: World Health Organization (61)

The absolute concentration method. An inoculum of *M. tuberculosis* is added to LJ or 7H10/7H11 agar containing several sequential dilutions of each drug. Resistance is indicated by the lowest concentration of the drug that Inhibits growth, *i.e.* fewer than 20 colonies by the end of 4 weeks (62).

The resistance ratio method. The resistance ratio (RR) is the ratio of the minimum Inhibitory concentration (MIC) for the patients' strain to the MIC of the drug-

susceptible reference strain, H37Rv, both tested in the same experiment (63). After 4 weeks of incubation, growth on any slope is defined as the presence of 20 or more colonies, and MIC is defined as the lowest drug concentration where the number of colonies is less than 20. A resistance ratio of 2 or less indicates sensitive strain, and a resistance ratio of 8 or more indicates resistant strains (58). The RR method is the most expensive of the three conventional methods (63).

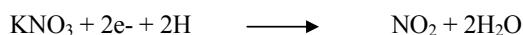
Conventional tests have been time tested to offer very reproducible DST results and have been considered as the gold standard tests for TB susceptibility testing. However, when performed on solid medium - typical of RLSSs, the DST process is very slow (2-3 months), necessitating the need for more rapid assays.

1.5.3 New Rapid Susceptibility Tests

At the beginning of this research programme in September 2006, the new rapid susceptibility tests in the literature included **Solid media culture-based techniques** such as Nitrate Reductase Assay (NRA) (64), E test (AB Bio Disk Solna, Sweden) (65-66), and Phage-based susceptibility tests (Biotec Laboratories Ltd., Ipswich, UK, (67-68); **In-house liquid media culture-based tests** such as the microscopic observation drug susceptibility (MODS) assay (69), Alamar blue (70), the MTT test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (71), and resazurin assays (72); **commercial liquid media culture-based tests** such as the BACTEC 460 radiometric system (Becton Dickinson, Sparks, Maryland), Mycobacterium Growth Indicator Tube - MGIT (Becton Dickinson, Sparks, Maryland), and MB BacT/Alert system (bioMe'rieux, Marcy l'Etoile, France) (73-74); and **Molecular tests** (Line Probe Assays) such as the INNO-LiPA Rif. TB Assay (Innogenetics, Ghent, Belgium) (75) and the Genotype[®] MTBDR and its newer version - the Genotype[®] MTBDR_{plus} (Hain Life sciences, Nehren, Germany) (76).

Solid Media Culture-Based Tests

Nitrate reductase assay (NRA). *Mycobacteria tuberculosis* has nitro- reductase enzymes that catabolically reduce nitrate (NO₃) to nitrite (NO₂), in the reaction pathway:



In 1879, Griess, a German chemist working at the University of Marburg, described the diazotization reaction, which now forms the basis for the Griess test for the detection of nitrite (77). By incorporating 1mg/mL potassium nitrate (KNO₃) in the medium, the reduction of nitrate to nitrite can be detected using the Griess reagent. When Griess reagent is added on the 7th-21st day of incubation, the nitrite in the medium causes a pink-purple color. In the presence of R or H at the critical concentrations, the appearance of a pink-purple colour represents resistance to the drug (64). Susceptible strains do not grow, as they are inhibited by the antibiotic thus producing a non-coloured reaction. As the NRA uses the detection of nitrate reduction as an indicator of growth, DST results can be obtained faster than by waiting for visual detection of colonies.

Progress had been made in the use of the NRA for indirect DST in *M. tuberculosis* showing sensitivity and specificity of 92-100% (78). The NRA test is technically easy to set and read, and gives a clear cut answer on susceptibility. When NRA is used for indirect DST, the bio safety and cost is almost similar to the LJPM since it needs a minor modification to perform the test. However, data on the performance of the NRA in RLSs in Africa was limited (78). It was therefore essential to further evaluate the NRA test procedures before it gets considered for routine MDR-TB diagnosis in RLS.

E test susceptibility testing. This method uses plastic strips that contain exponential gradients of antibiotics for susceptibility testing of mycobacteria (AB BIODISK, Solna, Sweden). The antibiotic diffuses into the medium and thereby inhibiting growth of susceptible strains. The minimum inhibitory concentration (MIC) is read and the isolate interpreted as resistant or susceptible. Initial studies of the E test showed high accuracy estimates of close to 100% when compared with the conventional agar proportion and BACTEC radiometric tests (65-66). However, it is now known that the diffused antibiotics degrade fast amidst the slowly growing mycobacteria resulting in a blurred cut off point for MIC reading. The other disadvantage of the E test is the need for a heavy inoculum *i.e.* #3 MarcFarland (MF) equivalent, which may not be achievable with direct DST on sputum sediments, but which also poses a major risk of aerosol generation and inhalation by the staff in the safety level 2 laboratories of developing countries. The E test antibiotic strips are also very expensive (up to USD 30 per strip), which may not be affordable in RLSs. With these issues, the E test was not found to be suitable for further evaluation in this research programme.

Phage-based susceptibility tests. Phage assays rely on the ability of live and thus resistant *M. tuberculosis* pre-incubated with the test drug to support the growth of an infecting mycobacteriophage - a virus that infects mycobacteria (68, 79). Both the commercially available *FastPlaque* TB assay and the in-house versions have been mainly studied for the detection of rifampicin resistance of either *M. tuberculosis* isolates or directly on clinical specimens with good results and rapid time to results of 2-3 days (67-68). An evaluation of this assay in Uganda also showed high sensitivity and specificity (80). However, the phage assay can be technically complex, labour-intensive, and can have high failure rates with inability to have interpretable results (Joloba M personal communication). This test was excluded from further evaluation.

In-House Liquid Media Culture-Based Tests

Microscopic-observation drug-susceptibility (MODS) assay. The MODS assay is a broth-based technique for the detection of tuberculosis and multidrug-resistant tuberculosis, indirectly or directly from sputum. The test relies on three principles (69): first, *Mycobacterium tuberculosis* grows faster in liquid medium than in solid medium; second, characteristic cord formation occurs and these cords can be visualized microscopically in liquid medium at an early stage; and third, incorporation of drugs permits rapid and direct drug-susceptibility testing concomitantly with the detection of bacterial growth. Resistant strains are detected due to the ability of *M. tuberculosis* to grow with characteristic cord-like structures detected with an inverted microscope. Visualization of cord-like structures in liquid medium containing the tested drug indicates resistance. Recently, the protocol for the MODS assay has been updated to include a well with Para-Nitrobenzoic Acid (PNB) to help identify MTB from atypical mycobacteria. PNB inhibits growth of MTB complex but not atypical mycobacteria (81).

Studies on MODS have shown sensitivities and specificities ranging from 86-100% for rifampicin and isoniazid resistance (69, 82). The MODS assay requires minimal training, is easy to set and results are rapid (7-14 days). However, microscopic observation of the cords may be subjective. Being a liquid culture-based test performed on tissue culture plates, concerns have been raised over the bio safety of staff working with this test. However, the main bio safety concern is at the point of sputum processing and inoculation after which the plate is supposedly sealed and never re-

opened even at microscopic examination. On basis of rapidity (7-14 days), technical ease, and low cost, the MODS assay was thought to be a promising DST method for poor countries, and was therefore listed for further analysis of the accuracy and operational issues in Uganda.

Alamar blue, Resazurin and MTT assays. These tests are referred to as colorimetric assays since they involve oxidation-reduction reactions with a colour change (83). They all use liquid medium on 96-well micro titre plates although tube assays have been reported (84). Supplemented 7H9 broth containing the test drug is inoculated with mycobacteria and incubated for 7 days at 37°C. After addition of Alamar blue or resazurin reagents to wells and if there is bacterial growth, the blue oxidized reagent is reduced to a pink dye visible with the naked eye or with a colorimeter. A change of colour from blue to pink in a drug-containing well indicates presence of growing resistant *M. tuberculosis* (85). For the MTT assay, detection of resistance is based on the ability of mitochondrial dehydrogenase enzymes from viable mycobacterial cells to cleave the tetrazolium rings of the pale yellow MTT, resulting in formation of violet-purple or dark blue formazan, visible with a naked eye or with a colorimeter (72). After the 7 days incubation the yellow MTT is added to the wells, and the plate incubated for 24 hours to allow the MTT to precipitate in the cytoplasm. A lysing buffer is then added to the wells to lyse the bacterial cell and release the MTT into the medium. Development of a strong violet-purple colour in the drug-containing well indicates presence of resistant mycobacterial strains (72).

Each of these colorimetric methods has been assessed in previous studies with reported sensitivity and specificity of 94-100%, and the results obtained within 10 days (83). These tests use micro titre plates with around 10 samples per plate, thus high throughput, which would be good for TB high-burden settings. However, after 7 days of incubation, the Alamar blue and resazurin plates are opened once while the MTT plate must be opened twice to add the detection reagents. This is not only cumbersome but also carries a serious bio safety risk to the laboratory personnel. On basis of high throughput these tests were listed for further analysis to assess their performance in Uganda even though they but may not be optimal for safety level-2 TB laboratories.

Commercial Liquid Media Culture-Based Tests

BACTEC 460. The BACTEC 460 (Becton Dickinson, Sparks, Maryland) relies on radiometric detection of $^{14}\text{CO}_2$ as an indicator of bacterial growth. The Bactec vials contain Middlebrook 7H12 medium and fatty acid substrates labeled with ^{14}C . Growing mycobacteria release $^{14}\text{CO}_2$ as a metabolic end product. The gas is removed, analyzed and the amount of radioactive ^{14}C is expressed as a numerical value called the Growth Index (GI). When the GI value in the control vial reaches 30 interpretation of drug tube begins on the next day as follows. Susceptible: Change in GI in the control vial $>$ GI in drug vial; Resistant: Change in GI in control vial $<$ GI in drug vial and Border line: GI in control vial = GI in drug vial. This test is highly sensitive and specific but it uses radioactive carbon whose half life is 5,000 years, which makes it difficult and expensive to dispose. Due to these issues, the BACTEC 460 is being phased out and has been replaced by non-radiometric systems such as the Mycobacterial Growth Indicator Tube and the MB/BactAlert system. The BACTEC 460 test was therefore not evaluated further in this study.

Mycobacterial Growth Indicator Tube. The Mycobacterial Growth Indicator Tube (MGIT; Becton Dickinson, Sparks, Maryland, USA) is based on fluorescence detection of mycobacterial growth in a tube containing a modified Middlebrook 7H9 medium together with fluorescence quenching-based oxygen sensor (a ruthenium pentahydrate substance embedded in silicone rubber) at the bottom of the tube (86-87). As the bacteria grow and consume oxygen, the indicator fluoresces under ultraviolet light, and growth in a tube with the test drug indicates resistance (86). The MGIT system, introduced around 15 years ago, in its manual and now automated versions, is part of the new-generation of rapid tests for detection of drug resistant TB. Studies of both the manual and automated MGIT 960 system have shown very high correlation with conventional DST methods for rapid detection of resistance to the first and second-line anti-TB drugs (88-90).

The automated MGIT 960 system has the advantage of high throughput (>900 samples can be tested on one instrument at ago), it is rapid (4-13days), and very easy to interpret results. All these aspects would be suitable for the TB high-burden settings. However, the test has been studied only as an indirect assay mainly in the developed countries.

Thus, there is limited data on the technical performance of the test in RLS, and on how the operational issues such as cost, contamination rates, and power failures would impact on the DST results in the low income settings. In this study, the automated MGIT 960 system was evaluated as both a direct and an indirect DST assay at Kampala.

The BacT/ALERT® 3D System. The BacT/ALERT® 3D System (bioMe´rieux, Marcy Etoile France) is a liquid based automated assay performed in a tube with a liquid emulsion sensor. Growing bacteria produce CO₂, which reacts with the sensor, resulting in a colour change from gray to a lighter colour, detected colorimetrically as growth. Growth in a drug tube indicates resistance. The BacT/ALERT® 3D System is slightly slow compared with the MGIT 960. This test was thus excluded from further analysis.

Molecular Assays

Molecular methods for MDR-TB detect the common mutations conferring resistance to R and H, rather than the resistance phenotype. The commercially available line probe assays involve DNA extraction, polymerase chain reaction (PCR), and solid phase reverse hybridization of amplified DNA to probes covering the core region of the target gene, immobilized on a nitrocellulose strip. These tests can be applied on MTB isolates or on sputum smear positive sputum (91-92).

The **INNO-LiPA Rif TB Assay** (Innogenetics, Ghent, Belgium) detects the common mutations in only the *rpoB* gene for rifampicin resistance (92). Evaluation studies of the INNO-LiPA Rif. TB Assay showed high sensitivity and specificity (75). Rifampicin resistance predicts MDR-TB in over 90% of cases, and may be sufficient for MDR diagnosis. However, isoniazid testing as well may be helpful in the design of second line drug regimens for MDR-TB patients. The GenoType® MTBDR assay (Hain Lifesciences, Nehren, Germany) simultaneously detects the common mutations in the *rpoB* and *katG* gene (93). The GenoType® MTBDR*plus*, a newer version of the genotype MTBDR detects more of the common mutations in the *rpoB* and *katG* genes, and also mutations in the *inhA* promoter region, making it the most sensitive line probe assay for detection of resistance (94). Evaluation studies of these assays have reported sensitivity and specificity of 98-100% for rifampicin, and of 70-100% for isoniazid, with results in 1-3 days (95).

Most of these studies were performed in developed countries and there was limited data on the performance of the tests in developing countries (95). A major limitation of these assays in developing countries could be the expertise in molecular biology required to perform them correctly, the unidirectional work flow laboratory infrastructure and the cost of molecular assays. However, a study on the INNO-LiPA Rif. TB assay in Rwanda demonstrated that the required skills could be learnt in a matter of weeks (96). Additionally, many of the developing countries now have facilities for basic molecular biology as used in monitoring viral load in HIV treatment. Thus, based on high sensitivity and specificity, rapidity, and potential ease of use, we considered the GenoType[®] MTBDR assay and GenoType[®] MTBDR*plus* for further assessment in Uganda.

To conclude this section, apart from the intrinsic properties, the performance of diagnostic tests also depend on the prior probability of disease in the study population, and the design of the study (97). The design of some of the studies cited above were typical of in stage 1 or 2 of test development, and were mostly conducted in the resource-rich settings (98). Some of the studies were done on very diverse or intentionally biased study populations, casting uncertainty on the wider applicability of the results, particularly in the RLSs (78, 83, 95). Furthermore, recent MDR-TB diagnostic research has focused on *direct* susceptibility testing. However, data on the listed tests when used as direct assays was very limited. The WHO in July 2010 recommended the use of colorimetric tests, MODS and the NRA for TB susceptibility testing in RLSs, but the available data to support the recommendation of for example the NRA in RLSs was admittedly limited (99). In this thesis we provide more recent data and experience with these assays in a typical RLS.

2 ABOUT THIS THESIS

This thesis is based on a 4-year research program that begun in September 2006. The overall goal of the research program was to find and recommend highly accurate, affordable and easy to use test(s) for diagnosis of MDR-TB in RLSs. We compared several rapid tests for detection of drug resistant tuberculosis on sensitivity, specificity, time to results, contamination rates, cost, bio safety and reproducibility in the perspective of resource-limited settings.

2.1 CONCEPTUAL FRAMEWORK

In the RLSs, there is limited capacity to perform TB DST even at national reference laboratories. DST with the LJPM, which sometimes is available in these settings, is considered to be cheap and the results accurate but come too late to be useful in patient care. As a result, TB treatment is based on standard WHO drug regimens. For the new TB cases oral drugs are given, and the two-months intensive phase involves use of R, H, Z and E, followed by a 6-months continuation phase with H and E (*i.e.* 2RHZE/6HE). For the re-treatment patients, the intensive phase goes on for 3 months. For the first two-months injectable S plus oral R, H, Z, and E are used; followed by one month of H, E, R, Z; and then five months of H, E, R (*i.e.* 2SRHZE/1RHZE/5RHE).

In case of MDR-TB particularly among the re-treatment cases, the standard regimen may not be effective since only streptomycin is added to a failing regimen. Patients may thus deteriorate and remain infectious to many new contacts, which may lead to MDR-TB outbreaks (17-18, 100). In case of susceptible TB, which constitute 80-85% of the re-treatment cases in sub-Saharan Africa, the simpler oral regimen used in the new TB cases could as well be effective. Thus, using injectable streptomycin may represent unnecessary treatment and wastage of resources; moreover the drug is more toxic than the simpler oral regimens. Therefore, rapid tests to screen for MDR-TB would help Physicians to triage patients early for the appropriate treatment. This would improve the patients' condition, curtail the spread of MDR-TB and optimize drug usage (see figure 6).

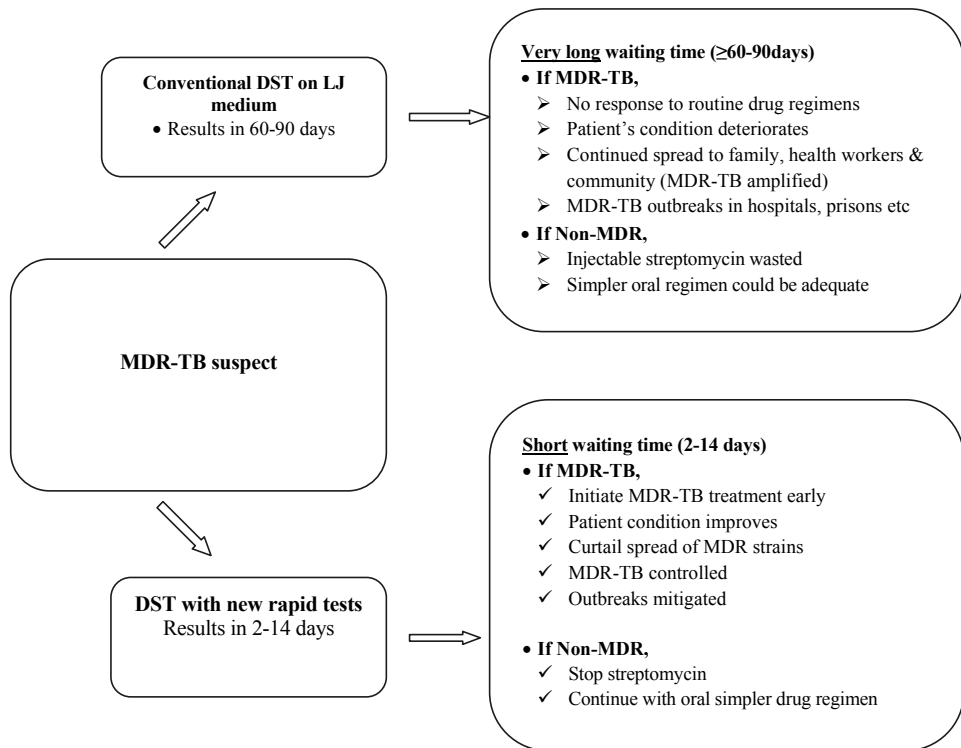


Figure 6. Conceptual framework

2.2 SPECIFIC OBJECTIVES

1. To conduct a meta-analysis comparing the sensitivity, specificity and time to results of rapid MDR-TB tests.
2. To compare the sensitivity, specificity and time to results of rapid indirect tests for MDR-TB against the LJPM.
3. To compare the proportion of interpretable susceptibility results, results obtained at initial testing, contamination rates, sensitivity and specificity, time to results, cost of testing, and reproducibility of direct assays for MDR-TB at Kampala, Uganda.
4. To investigate the clustering rate and the predominant genotypic lineages of MDR-TB strains spreading in Kampala, Uganda.

3 OVERVIEW OF THESIS PAPERS

This thesis is based on five papers. In paper I, a meta-analysis of direct rapid susceptibility tests was conducted. In paper II, the rapid tests were compared as indirect susceptibility assays in Uganda. In paper III, the tests were compared as direct assays in Uganda. In paper IV, one molecular test was studied for technical performance and reproducibility in two laboratories in Uganda, while in paper V, we studied the clustering rate and predominant strains of MDR-TB spreading in Kampala.

3.1 STATISTICS AND DATA ANALYSIS

In paper I (meta-analysis), The Meta-Disc[®] software (101) was used to analyse the reports and tests for sensitivity, specificity, and area under the summary receiver operating characteristic (sROC) curves. Heterogeneity in accuracy estimates was tested with the Spearman correlation coefficient and Chi-square.

In papers II and III, the data was entered in Microsoft Excel 2007 (Microsoft corporation, Redmond, WA, USA), and then imported into SPSS 11.0 for Windows (Statistical Package for the Social Sciences, Chicago, IL, USA) where two-by-two tables on true-resistant (TR), false resistant (FR), false-susceptible (FS), true susceptible (TS) and *kappa* agreements were generated. A *kappa* agreement of greater than 0.75 was considered excellent agreement beyond chance between the new rapid test and the reference test. Sensitivity, *i.e.*, the proportion of drug-resistant TB strains correctly identified by the new rapid test (true-positive), specificity, *i.e.*, the proportion of susceptible isolates correctly identified (true-negative) and the 95% confidence intervals were computed with the Meta-Disc[®] software. Time to results and cost estimates were computed in Microsoft Excel 2007 (Microsoft, Redmond, WA, USA).

In paper IV, statistical tests were performed using Intercooled STATA 8.0 software (Statacorp LP, College Station, TX, USA) and Microsoft Excel 7.0 as referenced above. Results were considered significant at *p value* of < 0.05.

In paper V, spoligotypes were entered into an online spoligotyping database of the Pasteur Institute of Guadeloupe (http://www.pasteur-guadeloupe.fr/tb/bd_myco.html), which assigned them octal numbers and lineages.

3.2 PAPER I

META-ANALYSIS COMPARING THE SENSITIVITY, SPECIFICITY AND TIME TO RESULTS OF RAPID MDR-TB TESTS

The main aim of this phase of the research program was to find out which rapid tests were available, and which ones had prospects for applicability in RLSs. We reviewed literature in journals, textbooks and electronic resources, and based on high accuracies as reported by authors, a priority list of tests for further study was prepared. The priority tests included the nitrate reductase assay (NRA), E test, microscopic observation drug susceptibility (MODS), alamar blue, MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), manual and automated mycobacterium growth indicator tube (MGIT: Becton Dickinson, Sparks, Maryland), Genotype MTBDR[®] and Genotype[®] MTBDR^{plus} (Hain life sciences, Nehren, Germany)

We then conducted meta-analysis of the rapid assay reports on direct susceptibility testing for MDR-TB. In the meta-analysis we compared the sensitivity, specificity and time to results (TTR) of four direct drug susceptibility testing assays with the conventional indirect testing for detection of resistance to R and H in *M. tuberculosis*. The four direct tests included two in-house phenotypic assays – Nitrate Reductase Assay (NRA) and Microscopic Observation Drug Susceptibility (MODS), and two commercially available tests – Genotype[®] MTBDR and Genotype[®] MTBDR^{plus} (Hain Life Sciences, Nehren, Germany). The MetaDisc software (101) was used to compute the sensitivity, specificity, and area under the summary Receiver Operator Characteristic Curves (sROC).

Eighteen direct DST reports were analysed: NRA – 4, MODS- 6, Genotype MTBDR[®] – 3 and Genotype[®] MTBDR^{plus} – 5. The pooled sensitivity and specificity for detection of resistance to rifampicin were 99% and 100% with NRA, 96% and 96% with MODS, 99% and 98% with Genotype[®] MTBDR, and 99% and 99% with the new Genotype[®] MTBDR^{plus}, respectively. For isoniazid it was 94% and 100% for NRA, 92% and 96% for MODS, 71% and 100% for Genotype[®] MTBDR, and 96% and 100% with the Genotype[®] MTBDR^{plus}, respectively. The area under the summary receiver operating characteristic (sROC) curves was in

ranges of 0.98 to 1.00 for each of the four tests. Molecular tests were completed in 1 – 2 days and the phenotypic assays were also much more rapid than conventional testing. Results of the meta-analysis on direct testing were published in paper 1 (102)

Based on the high test accuracies and performance characteristics revealed during the literature review and meta-analysis, seven test methods (NRA, MODS, Genotype[®] MTBDR*plus*, MGIT 960, Alamar blue, Resazurin and MTT) were selected for further evaluation as indirect susceptibility tests at Kampala, Uganda.

3.3 PAPER II

SENSITIVITY, SPECIFICITY AND TIME TO RESULTS OF SEVEN RAPID TESTS FOR MDR-TB STUDIED AS INDIRECT ASSAYS AT KAMPALA

In research on diagnostic tests, before any test is recommended for routine use in a new setting, it is critical to study the performance of the test in that specific setting (98).

The current phase of the research program was undertaken at the NTRL Kampala, Uganda - a typical resource-limited setting which is the target of the tests studied.

Thirty-one well-characterized strains of *M. tuberculosis* were tested for susceptibility to R and H with seven rapid assays (NRA, MODS, MGIT 960, Genotype[®] MTBDR*plus*, Alamar blue, MTT and resazurin), which were compared head-to-head for sensitivity, specificity and time to results, against the LJPM as reference test.

The NRA correctly identified all the resistant strains with 100% sensitivity and specificity. The MGIT 960 detected all MDR strains but missed one R-mono resistant strain. The Genotype[®] MTBDR*plus* detected all R-resistant strains and the sensitivity for detection of H resistance was 88%. Sensitivity and specificity ranged from 86% to 100% with MODS, and from 57% to 100% with Alamar blue, MTT and resazurin assays for both drugs. Test results were obtained within 2–14 days.

Additionally, major cost areas, bio safety needs and technical ease were qualitatively assessed with regard to the existing TB laboratory infrastructure at the NTRL in Kampala. The NRA did not require additional instrumentation, while the MODS required an inverted microscope. The Genotype[®] MTBDR*plus* required laboratory re-designing and several instruments for molecular testing but their estimated total cost was not as expensive as for the MGIT 960 instrument (table 3).

Table 3. Qualitative assessment of major cost areas, bio safety needs and technical ease of new tests compared with the LJPM

Indirect DST	Cost (H & R testing)		Bio safety hood needed		Technical ease assessment
	Major investment / Infrastructure cost areas	Running Costs (Reagents & supplies)	DST inoculation	DST reading	
LJPM	<ul style="list-style-type: none"> • BSL- 2 laboratory (Room with air lock door and a Bio safety cabinet) • Centrifuge • Incubator • Autoclave • Fridge and Freezer 	<ul style="list-style-type: none"> • LJ reagents 	Yes	No	Cumbersome to set but easy to read. Slow
NRA	<ul style="list-style-type: none"> • As for LJPM 	<ul style="list-style-type: none"> • As for LJ plus • KNO₃ • Griess reagent 	Yes	Yes	Easy to set and simple to read
MGIT 960	<ul style="list-style-type: none"> • BSL-3 laboratory (Room with negative air flow and Bio safety cabinet) • MGIT instrument • Other instruments as for LJPM 	<ul style="list-style-type: none"> • MGIT Medium • Supplements • Other supplies 	Yes	No	Cumbersome to set but easy to read
Genotype® MTBDR <i>plus</i>	<ul style="list-style-type: none"> • Laboratory re-design • BSL-2 laboratory • UV work station • Microcentrifuge • Sonicator • Thermocycler • Twincubator® • Other instruments as for LJPM 	<ul style="list-style-type: none"> • Kits expensive but may be cost-effective 	Yes	No	Training in PCR & Hybridization Many steps
MODS	<ul style="list-style-type: none"> • BSL-2 laboratory • Inverted microscope • Other instruments as for LJPM 	<ul style="list-style-type: none"> • Tissue culture plates • 7H9 broth • OADC and PANTA 	Yes	No	Easy to set but reading may be subjective
Alamar blue MTT Resazurin	<ul style="list-style-type: none"> • BSL- 3 laboratory • Other instruments as for LJPM 	<ul style="list-style-type: none"> • Alamar blue reagent • Micro titre plates • Tape seal 	Yes	Yes	Cumbersome to set and read

In the study setting, the NRA was the most accurate assay for indirect detection of MDR-TB, followed by the MGIT 960, Genotype® MTBDR*plus* and MODS. Time to results was significantly shorter compared to conventional testing. These results were published in paper II (103).

Indirect susceptibility testing requires prior isolation of *M. tuberculosis* in pure colonies, which may take 21-60 days on solid medium. Additionally, even after isolation of the MTB in pure colonies, RLSs face unique challenges such as staff inadequacy, suboptimal work habits, stock outs and lost/misplaced culture tubes, which all tend to delay inoculation, reading and reporting of the susceptibility result. Therefore, the overall time from the date of sample receipt in the laboratory to obtaining a valid DST result is markedly prolonged – in most cases to at least 90 days at the NTRL Kampala, Uganda. Some of these issues could be minimized by performing direct DST. Therefore, based on high test accuracy revealed in the above study and also in the meta-analysis, two in-house assays (NRA and MODS) and two commercially available tests (Genotype[®] MTBDR*plus* and MGIT 960) were further evaluated as direct DST assays at Kampala, Uganda.

3.4 PAPER III

PROPORTION OF INTERPRETABLE SUSCEPTIBILITY RESULTS, RESULTS OBTAINED AT INITIAL TESTING, CONTAMINATION RATES, SENSITIVITY, SPECIFICITY, TIME TO RESULTS AND COST PER SAMPLE OF DIRECT ASSAYS FOR MDR-TB AT KAMPALA, UGANDA

Direct susceptibility testing in which decontaminated respiratory samples are inoculated in drug-free and drug-containing medium or amplified for detection of MDR-TB straight away eliminates the time needed for prior isolation of MTB and minimizes the related obstacles mentioned above. Furthermore, preliminary evidence from the meta-analysis indicated that direct testing for MDR-TB with the NRA, MODS and Genotype[®] MTBDR*plus* was highly sensitive and specific, and far more rapid than the conventional indirect DST (102). However, some of direct DST reports meta-analysed had insufficient study designs, that may limit generalization of the findings (102).

In this part of the research program, the NRA and MODS were compared on proportion of interpretable susceptibility results, results obtained at initial testing, contamination rates, sensitivity, specificity, time to results, and cost of testing per sample as direct MDR-TB assays at the NTRL Kampala. The performance of the direct MGIT 960 was also assessed. Retreatment TB patients were consecutively recruited at the TB clinic of Mulago National Referral Hospital. Ziehl-Neelsen smear positive sputum was collected

from 245 patients and was processed at the NTRL. Sediments were tested for susceptibility to R and H with the direct NRA, MODS and MGIT 960 at the NTRL, while a portion of it was tested with the direct Genotype[®] MTBDR_{plus} at the department of Medical Microbiology Makerere University College of Health Sciences and at the FIND diagnostics laboratory at Kampala, Uganda. Methodological details for the direct NRA and MODS assays are shown in paper III (Manuscript), and for the molecular assays in paper IV(104).

Results

Interpretable results. Interpretable results were obtained in 225 (92%), 229(93%), 211 (86%) and 226 (92%) of samples with the direct NRA, MODS, MGIT 960 and indirect LJPM, respectively.

Interpretable results obtained at initial testing were 90%, 85% and 80% with the direct MODS, NRA and MGIT 960, respectively. Repeat testing was mainly due to contamination for NRA, and lack of growth in the control well/tube for MODS and MGIT 960. For the samples with no interpretable results - even after repeat testing, insufficient growth rather than contamination was the main reason.

Contamination rates. Following initial inoculation, tubes/wells that contaminated were 22(9%), 12(5%) and 17(7%) of the NRA, MODS and MGIT 960 assays, respectively. However, contamination caused total failure to obtain interpretable final results in only a few tubes.

Sensitivity and specificity. Samples with interpretable results with both the study test and the LJPM were 218, 217 and 203 with the NRA, MODS and MGIT 960, respectively. Based on these samples, sensitivity, specificity and *kappa* agreement for MDR-TB diagnosis (R and H resistance) was 95%, 98% and 93% with the NRA, and 81%, 95% and 75% with the MODS, and 68%, 99% and 75% with the MGIT 960. These results are shown in table 4, and more detailed for NRA and MODS in paper III (Manuscript).

Table 4. Sensitivity, specificity and kappa agreement of the direct NRA, MODS and MGIT 960

Drug		Direct NRA	Direct MODS	Direct MGIT 960
Rif	Sensitivity, % (95% CI)	98 (88-100)	83 (68-92)	68 (52-81)
	Specificity, % (95% CI)	98 (91-98)	94 (88-97)	99 (96-100)
	Kappa agreement	0.93	0.74	0.76
INH	Sensitivity, % (95% CI)	92 (83-97)	88 (77-95)	87 (76-94)
	Specificity, % (95% CI)	96 (94-99)	93 (87-96)	97 (93-99)
	Kappa agreement	0.87	0.79	0.86
Rif and INH	Sensitivity, % (95% CI)	95 (84-99)	81 (70-91)	68 (51-81)
	Specificity, % (95% CI)	98 (95-100)	95 (90-98)	99 (96-100)
	Kappa agreement	0.93	0.75	0.75

Time to Results (TTR). The median TTR was 10, 7, 8 and 64 days with the direct NRA, MODS, MGIT 960 and indirect LJPM, respectively.

Cost. The cost of laboratory supplies per sample was \$3.58, \$5.56 and \$4.12 with the direct NRA, MODS and indirect LJPM, respectively, thus the NRA was the cheaper in Uganda's settings.

To conclude this section, the direct NRA was the most sensitive, specific, and cheapest test for MDR-TB in Uganda's settings.

3.5 PAPER IV

RAPID SCREENING OF MDR-TB USING MOLECULAR LINE PROBE ASSAY IS FEASIBLE IN UGANDA

In this paper, we assessed the performance of a commercial line probe assay (LPA) the Genotype[®] MTBDR*plus* for direct detection of R and H resistance. Smear-positive sputum specimens from 118 previously treated TB patients were tested. LPA testing was performed at the Department of Medical Microbiology Makerere University. To assess the reproducibility of results, testing was also performed at the FIND diagnostics laboratory located within the TB reference laboratory at Kampala, Uganda. Results were compared with the indirect MGIT 960 liquid culture and DST.

Overall, 96% of smear-positive specimens gave interpretable results within 1-2 days using LPA. Sensitivity and specificity were 100% and 96% for detection of R resistance; 81% and 100% for H resistance; and 92% and 96% for MDR compared with MGIT 960 results. Concordance of susceptibility results between the two laboratories was 98%, implying high reproducibility. We concluded that rapid screening for MDR-TB with LPAs is possible in Uganda. Details of the methods and results are shown in paper IV (104)

3.6 PAPER V

CLUSTERING RATE AND PREDOMINANT GENOTYPIC LINEAGES OF MDR-TB STRAINS IN KAMPALA, UGANDA

Recent field epidemiological studies suggest a high rate of TB transmission among the peri-urban populations of African cities (105-107). Molecular fingerprinting studies with restriction fragment length polymorphism (RFLP), variable number of tandem DNA repeats (VNTRs) and spoligotyping (108-111) also reveal high clustering rates and recent TB transmission in Africa (112-114). These studies have brought to light a firm insight into the transmission dynamics of *M. tuberculosis* in Africa, but they did not focus on MDR-TB.

Knowledge of the transmission and predominant genotypes of MDR-TB strains in a given geographical region is important for TB control, particularly in understanding the transmissibility (115), virulence, immunogenicity and vaccine design (116). Whereas the Beijing genotype is highly prevalent and in most cases associated with multi drug resistance in Asia, Eastern Europe and New York (117-122), there is no parallel data on the MDR-TB strains spreading in Kampala.

In this study we applied the spoligotyping technique to determine the clustering rate and predominant genotypic strains of *M. tuberculosis* causing MDR-TB in Kampala, Uganda. A total of 99 MDR-TB isolates were studied. These isolates were from retreatment TB patients attending different health facilities in Kampala. Twenty-four strains were isolated consecutively from patients aged 19-56 years (median age 32 years) at Mulago National Referral Hospital during 2008. These strains had been identified as members of the MTB complex using the Capilia TB Neo (TAUNS

Corporation, Japan), confirmed MDR with the LJ-PM and kept at -40°C at the NTRL. Seventy-five strains were from TB patients recruited in several research projects at one or more of the health facilities in Kampala between 1997 – 2006. The latter strains had been identified as members of the MTB complex with PCR for *IS6110*, tested for susceptibility to R and H using the BACTEC 460 or the MGIT 960 and archived at the JCRC laboratory.

A commercially available spoligotyping kit (Isogen Bioscience BV, Maarsse, The Netherlands) was used to genotype 99 MDR-TB strains. Spoligotypes were entered into the international spoligotyping database of the Pasteur Institute of Guadeloupe (http://www.pasteur-guadeloupe.fr/tb/bd_myco.html) and assigned octal numbers and lineages. Clustering was defined by presence of two or more strains with identical spoligotypes.

Eighty-two isolates (83%) were part of 10 clusters. The T2 lineage was the largest cluster with 26 strains, followed by CAS –Delhi and LAM II ZWE with 13 and 10 strains, respectively. Seventeen strains did not cluster, twelve of which were total orphans while five already were in the database. One Bovis1_BCG lineage was also found. We concluded that 83% of the MDR-TB strains in Kampala occur in clusters, suggesting a high level of recent MDR-TB transmission. The T2 lineage was the largest single MDR-TB cluster, and it was also found earlier to be the most frequent genotype responsible for TB in Kampala (123). Thus there is a local epidemic of the T2 genotype causing TB and MDR-TB in Kampala, Uganda.

4 CONCLUSIVE REMARKS

The aim of this research program was to compare the technical and operational performance of new rapid MDR-TB tests with prospects for applicability in RLSs. Her below a summary of the findings is given.

From the meta-analysis of direct tests, the NRA, MODS and Genotype[®] MTBDR*plus* were highly sensitive and specific.

With indirect DST at Kampala, the NRA, MODS, Genotype[®] MTBDR*plus* and MGIT 960 tests were highly sensitive, specific and results were obtained much earlier than for the LJPM. The NRA did not require any additional instrument with regards to the existing infrastructure at the national TB reference laboratory Kampala, Uganda, while the MODS assay required an inverted Microscope as the only main additional instrument. The Genotype[®] MTBDR*plus* required the laboratory to be redesigned for the 3 rooms needed for molecular testing and additional instruments that were not so expensive. However, the MGIT 960 required a very expensive instrument and reagents. On laboratory infrastructure, the NRA, MODS, Genotype[®] MTBDR*plus* can be performed in a BSL-2 laboratory (room with air lock door and bio safety cabinet) – the type of laboratory in Uganda and in many RLSs, but MGIT culture and DST requires a BSL-3 laboratory with negative air flow due to high risk of aerosol generation.

With direct susceptibility testing, the NRA emerged as the most accurate, cheapest and easiest test in the study setting. Technologists in RLSs are familiar with TB culture and DST on LJ medium, thus introduction of the direct NRA test appears to be easier compared to the other techniques since it is also performed on LJ medium. Both the direct NRA and MODS require BSL-2 laboratory for sample processing, inoculation and later addition of Griess reagent - in the case of NRA. Since most TB laboratories in sub Saharan African countries are of BSL-2 at best, in these settings, the highly accurate direct NRA is likely to be the optimal test for rapid screening for MDR-TB. Whereas the MODS assay had slightly less sensitivity for MDR-TB detection, it can be a very good test for settings with many TB samples coming to the laboratory per day since one tissue culture plate is adequate for four samples. This

means the incubator space that would be needed by the many NRA tubes would be saved. Thus, a modestly sized incubator with less energy consumption could be adequate for the MODS assay. Furthermore, MODS uses much less drugs in the assays than NRA, and this may reduce the cost of testing. The WHO in July 2010 recommended the use of NRA, MODS and colorimetric assays as non-commercial susceptibility tests for MDR-TB in RLSs. Data from this research program represents more recent experience with these assays in a typical RLS, and it may be helpful in guiding RLSs on which tests to select for routine MDR-TB screening.

On the other hand, the commercially available Genotype[®] MTBDR*plus*, which was the most rapid assay, and which is one of the line probe assays recommended by WHO can be used in the RLSs where resources permit. Being a highly sensitive and specific assay for R resistance, it remains an important test for rapid screening of smear positive patients for MDR-TB (within 1-2 days of TB diagnosis). Patients can then be managed appropriately as further testing is ongoing. This would help to minimize patient mismanagement and the uncontrolled spread of MDR-TB. Whereas training, supervision and adherence to protocols in molecular biology remains a priority, more recent studies in Africa indicate that molecular testing is becoming feasible in RLSs (96, 104, 124) .

Lastly, Uganda has been granted permission by the Green Light Committee (GLC) of the World Health Organization (WHO) to treat patients with MDR-TB using second line anti-TB drugs, and the isolation ward has been earmarked at the Mulago National referral Hospital. Therefore, results from this study are timely, and will be directly applicable for rapid screening of TB patients for MDR-TB not only among patients from Uganda, but from neighboring countries in the lake Victoria region (Uganda, Kenya, Tanzania, Rwanda, Burundi, Democratic Republic of Congo (DRC), Ethiopia, Somalia and Sudan).

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