Utilization of molecular resistance test results as tools to support public health efforts for improved control of rifampicin-resistant tuberculosis

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Chapter 1

Introduction
THEN AND NOW: TUBERCULOSIS AS A GLOBAL PUBLIC HEALTH BURDEN

Historical overview
Because there was no available treatment for tuberculosis in the 19th century, tuberculosis (TB) patients were prescribed with rest and fresh air. The absence of effective TB treatment, overcrowding, reduced exposure to sunlight, and overall poor standards of living (1-2) synergistically favored the spread of TB.

TB then became the single biggest killer of the urban poor in the US and Northern Europe, represented in Émile Poincaré’s 1848 TB distribution map (Figure 1) (3). Other regions such as Asia and Africa did not have TB incidence data yet during this period.

![Figure 1. Global distribution of TB cases by Émile Léon Poincaré (3).](image)

Notably, TB mortality declined dramatically (Figure 2) even before *Mycobacterium tuberculosis* was discovered (1, 2, 4, 5), and prior to administering anti-tuberculous chemotherapy (1) and Bacillus Calmette-Guérin vaccine (6). Thomas McKeown hypothesized that the reduced incidence of TB came about due to changes in lifestyle and standards of living at least in the US and Northern Europe (4). It was then found that countries with the lowest gross domestic product had the highest rates of TB (7). This was affirmed by the World Health Organization (WHO) estimates identifying the highest TB incidence rates in the most economically challenged countries, with South Africa as an exception due to its convergent HIV epidemic (8).
Today, TB continues to threaten global health even with potent anti-TB drugs delivered, and with the Bacillus Calmette-Guérin vaccine being the most widely administered vaccine worldwide, to protect infants and young children from severe TB. However, it has been unsuccessful in reducing the global TB burden. As such, newer and more effective vaccines are being developed (5).

Despite being preventable and treatable, TB remains the ninth leading cause of mortality and principal cause of death from a single infectious disease agent worldwide (8). TB has infected a quarter of the global population and caused 1.5 million deaths in 2018 (8). TB disproportionately places its heaviest burden on the poorest and most vulnerable populations globally. This inequity is most reflected in impoverished communities of Asia and Africa (Figure 3) (8, 9).

TB is transmitted via the aerosol route and could manifest as pulmonary, the more common type of active TB, or extrapulmonary. Symptoms of active pulmonary TB disease include persistent cough, fever, night sweats, and cachexia while extrapulmonary TB can be found in lymph nodes, pleura, pericardium, meninges, bones and joints, amongst others (5, 10). Since active TB symptoms resemble those of pneumonia or cancer, the physician’s clinical suspicion must be supplemented by diagnostics specific for confirming TB.
Among individuals infected by TB, 5 to 10% progress into active TB disease, the progression rate over lifetime in the absence of frank immunodeficiency, while the rest remain latently infected (5, 11). Although asymptomatic, latent TB patients generate a distinct immune response captured by a positive tuberculin skin test or TB-oriented Interferon-Gamma Release Assay. Individuals with HIV, diabetes, low BMI, amongst others, have increased risk of developing active TB disease upon infection (9).

**ANTI-TUBERCULOUS CHEMOTHERAPY**

Anti-tuberculous chemotherapy is highly effective. The current standard TB treatment regimen is two months of intensive phase - isoniazid, rifampicin, ethambutol, and pyrazinamide followed by four months of continuation phase - isoniazid and rifampicin (5).

Streptomycin and *para*-aminosalicylic acid were both discovered before isoniazid. A TB patient with a dim course of the disease was treated with streptomycin in 1944. She survived, regained weight, and resumed living an active life. Streptomycin and
para-aminosalicylic acid were effective but had unpleasant drug reactions, and when administered alone promoted development of drug resistance.

Isoniazid was discovered in 1951 and proved to be safe, effective, and cost-efficient, effective, but its sole administration was quickly recognized to induce resistance, which thus called the need for multiple drug therapy (12). ‘Triple therapy’ consisting of isoniazid, para-aminosalicylic acid, and streptomycin then became the standard treatment regimen for all manifestations of TB for almost two decades. Multiple drugs are necessary to tackle the mycolic acid-rich cell wall and unique metabolism of *M. tuberculosis* and its tendency to revert, under drug pressure, to a drug-tolerant “persister” and/or drug-resistant state (12).

Despite the availability of effective therapy, adverse drug effects and patient non-compliance (5) have been documented and drug-resistant strains continued to emerge. In 1961, ethambutol was shown to work on isoniazid-resistant strains and eventually replaced para-aminosalicylic acid. Rifampicin was discovered by Piero Sensi (13), and was first administered in 1966. It became a critical component of TB therapy in combination with isoniazid and ethambutol as these drugs in combination allowed reducing treatment duration to nine months. The addition of pyrazinamide further shortened the course of treatment to six months (1).

Drug-resistant TB strains have emerged within a decade of introducing the anti-TB drugs in medical practice (1, 14) and have since jeopardized TB control efforts and gains (9).

For management of rifampicin-resistant (RR) and multidrug-resistant (MDR)-TB (8), defined as having resistance to at least rifampicin and isoniazid (5), the WHO recommended until recently at least five drugs likely to be effective, including a fluoroquinolone, an injectable drug (kanamycin, capreomycin or amikacin), ethionamide or prothionamide, pyrazinamide, and either cycloserine or para-aminosalicylic acid (15). In 2016, WHO recommended a standardized shorter MDR-TB regimen (9 to 12 months). The efficacy of this combination was tested in patients from Bangladesh, Niger, and Cameroon (9). This regimen includes high-dose Isoniazid, kanamycin, and prothionamide for 4–6 months, with additional clofazimine, ethambutol, moxifloxacin, and pyrazinamide given throughout the course of treatment.
In 2018, the WHO endorsed the injection-free MDR-TB regimen which may be modified and tailored based on the patient’s response. TB drugs were reclassified into three groups (A, B and C) for the purpose of composing the longer regimen: Group A comprise levofloxacin/moxifloxacin, bedaquiline, and linezolid to be prioritized and used, if possible, in all regimens; Group B covers clofazimine and cycloserine/terizidone to be possibly added to all regimens; and Group C includes “other” agents (including injectables) to be used as a substitute to complete a regimen of at least four drugs when agents from groups A and B cannot be used (15, 16).

An MDR-TB patient who develops additional resistance to at least one of the fluoroquinolones and to at least one of the second-line injectables amikacin, capreomycin, or kanamycin, is said to have extensively drug resistant (XDR)-TB (5). For management of XDR-TB, the WHO recommends the recently developed drugs bedaquiline and/or delamanid, plus linezolid, including a later-generation fluoroquinolone, and addition of other drugs such as clofazimine, para-aminosalicylic acid, pyrazinamide, high-dose isoniazid, and other drugs depending on the likelihood of susceptibility is recommended (9).

Resistance to bedaquiline and delamanid included as add-on agents in the WHO guidelines on MDR-TB treatment (17) has already been detected (18).

**RIFAMPICIN-RESISTANT TUBERCULOSIS**

Approximately one fifth of TB patients globally are estimated to carry isolates resistant to at least one major anti-tuberculous drug (9). Drug resistance in *M. tuberculosis* may arise through mutations in genes that transcribe drug targets and those that activate enzymes, promoters (5, 19, 20), and potentially also in efflux pumps. *M. tuberculosis* bears no plasmids, and as a clonal and obligate intracellular bacillus, rarely undergoes horizontal gene transfer and recombination (5). The changes in the *Mtb* genome could be in the form of a single nucleotide polymorphism, an insertion, or a deletion (9).

Rifampicin continues to be regarded as the most potent core drug in standard TB treatment, with both bactericidal and sterilizing activity that largely protects
against treatment failure and relapse (21). Shortly after it was administered in clinical settings, it was shown to inhibit transcription of DNA into RNA. Rifampicin resistance in M. tuberculosis is linked to target modifications in the DNA-dependent RNA polymerase (13). Experiments on Escherichia coli and Bacillus subtilis helped to identify and confirm that the β subunit was the specific target within the RNA polymerase enzyme encoded by the rpoB gene (13, 14).

Rifampicin resistance persists as an urgent public health crisis. In 2018, there were an estimated 484,000 patients with RR-TB worldwide, of whom 78% were MDR-TB. Only 39% of estimated RR-TB patients world-wide were detected and notified as such in 2018, and only 1 in 3 had access to appropriate treatment (8, 22). These data reflect unaddressed gaps in detection and treatment of MDR and RR-TB, which require the same treatment regimen to achieve a cure.

**Rifampicin resistance-conferring mutations**
The majority of rifampicin-resistance conferring mutations are situated at codon positions 426-452 (23) within the 81-bp Rifampicin Resistance Determining Region (RRDR) of the rpoB gene [4–6]. Databases documenting mutations associated with rifampicin-resistance include the TB Drug Resistance Mutation database (20), MUIIB-TB-DB (24), and ReSeqTB (25). Coll and co-workers performed genome-wide association studies of MDR- and extensively-drug-resistant TB strains and determined specific rpoB mutations significantly associated with rifampicin resistance (26). Mutations Ser450Leu, Asp435Val, His445Asp, and His445Tyr were among the most frequently detected in the global M. tuberculosis complex strain population and have the greatest association with RR-TB (26, 27). Miotto and colleagues correlated a panel of RR-conferring mutations detected in whole genomes of strains with the MIC levels obtained from phenotypic drug susceptibility tests (pDST). From the association found, they were able to classify the level of resistance caused by the different mutations (28).

“Disputed” mutations in the rpoB gene bring about resistance that is comparable with the more frequently detected RR mutations. However, these mutations are often not captured by the commonly deployed phenotypic DSTs such as Mycobacterium Growth Indicator Tube (MGIT), thereby posing a huge threat in RR-TB control. In Bangladesh, Pakistan, and Zimbabwe, disputed mutations such as Asp435Tyr and Leu452Pro comprise up to 25% of retreatment cases (G. Torrea, unpublished data). Such missed disputed mutations in strains of
retreatment patients were associated with poor treatment outcomes akin to those of undisputed RR-conferring mutations such as Ser450Leu causing high-level rifampicin resistance (29-32).

Accurate and timely detection of rifampicin-resistance conferring mutations is critically important for the individual TB patient, to receive effective treatment, and for the community, to interrupt ongoing transmission. After detecting RR-TB, it is key to check for resistance to fluoroquinolone, the most potent second-line core drug (21).

**Laboratory diagnosis of Mycobacterium tuberculosis and rifampicin-resistant TB**

The modern TB laboratory detects and identifies mycobacterial species, and determines the susceptibility to the various anti-tuberculous drugs to optimize the treatment plan for each patient. For pulmonary TB, the diagnosis is typically made on a sputum sample from the patient, whereas for extrapulmonary TB, an aspirate or biopsy from the infected area may be tested (5). The WHO recommends the use of both phenotypic and genotypic rapid DSTs to detect RR-TB in adults and children (5, 9). The new all-oral MDR-TB regimen recommended by the WHO prioritizes phenotypic DST results for new drugs such as bedaquiline, linezolid, clofazimine, and delamanid where genotypic DST (gDST) is still under development due to partially identified mutations conferring resistance to these drugs (16, 33). Thus, pDST and gDST will be done in tandem for a while to come. Furthermore, only 4% of laboratories globally can actually perform pDST for bedaquiline. New drugs must be accompanied by reliable diagnostic tests which can be implemented in laboratories worldwide.

**Phenotypic drug susceptibility tests**

Traditional culture-based pDSTs require high biosafety requirements and have slow turnaround time. Conventionally, fresh cultures of patient samples are inoculated on solid media such as Löwenstein Jensen, and Middlebrook 7H10 or 7H11 agar or on liquid media such as 7H9 and Mycobacteria Growth Indicator Tube (MGIT) (34, 35). The proportion method, using LJ, 7H11, or other agar, determines the presence of at least 1% resistant subpopulation in clinical samples (36, 37).
MGIT is widely employed, both in low to middle income high RR-TB burden countries and high-income countries, as it could detect susceptibility or resistance faster than the other culture-based methods. The pre-set standard conditions of MGIT include the 1 µg/ml critical rifampicin concentration that inhibits growth of 99% phenotypically wild-type strains, and pre-set incubation time, both of which were previously validated (38-40).

The WHO has also recommended the non-commercial DST methods such as Microscopic Observation Drug Susceptibility Testing, Nitrate Reductase Assay, and Colorimetric Redox Indicator such as Resazurin Microtiter Assay for use in TB reference laboratories (41, 42).

The Nitrate Reductase Assay is based on the ability of M. tuberculosis to reduce nitrate to nitrite, and detects rifampicin resistance in sample or culture by the presence of nitrite in a solid medium with rifampicin, observed through a color change (43). The Microscopic Observation Drug Susceptibility Assay on the other hand, is a tissue culture plate-based assay with enriched Middlebrook 7H9 liquid medium tested directly on patients’ samples. Reduced susceptibility to rifampicin is microscopically detected by the cord-like growth of M. tuberculosis in the rifampicin-containing well (44).

The Resazurin Microtiter Assay Plate determines rifampicin resistance when resazurin is reduced in the presence of the drug evidenced by a blue to pink color change (45), whereas the Sensititre MYCOTB plate ascertains the MIC of rifampicin when tested on the sample (46).

**Genotypic drug susceptibility tests**

To address the challenges of long turnaround time and high-level biosafety requirements of phenotypic DSTs, genotypic probe-based tests were developed to rapidly detect rifampicin-resistant TB. The WHO currently recommends the cartridge-based Xpert MTB/RIF (Xpert Classic), Xpert MTB/RIF Ultra (Xpert Ultra), and the line probe assays GenoType MTBDRplus v2.0 (LPA-Hain) and GenoscholarNTM+MDRTB II (LPA-Nipro) (9, 47). These molecular genotypic tests detect rifampicin resistance-conferring mutations in the RRDR or hotspot of the rpoB gene (23, 47-49).
Xpert MTB/RIF is an automated cartridge-based assay that detects the presence of *M. tuberculosis* and resistance to rifampicin in less than 2 hours. The core of the Xpert assay is a single sample cassette in which sample extraction, processing, and analysis occurs. The test employs heminested real-time polymerase chain reaction assay through molecular beacon technology. The molecular beacons comprise a probe sequence flanked by two complementary “arm oligonucleotide sequences”. When the assay is run, the two ‘arm sequences’ hybridize forming a stem-and-loop structure (Figure 4B) with a fluorophore at the end of one arm and a quencher on the other. Binding of the probe to its complementary target DNA induces separation of the fluorophore and quencher from the two arms, emitting fluorescence.

The Xpert assay involves binding of five short overlapping fluorescent probes to wild-type regions of the RRDR (48, 49, 51) (Figure 4A). *M. tuberculosis* is detected by Xpert when at least 2 of the 5 probes (A, B, C, D, and E) produce a positive signal with cycle threshold of ≤38; whereas rifampicin resistance is reported if the difference between the first and last cycle threshold is >3.5 cycles (Figure 5). Within its coverage, each of the 5 probes corresponds to few or several mutations (51) which inhibit probe binding, thereby reducing or eliminating the signal from the respective probe. Thus, when a mutation is detected by the test, at least one of the probes will not bind. Certain mutations completely interfere with probe binding, considered as ‘drop-out’ or absent; whereas mutations that allow limited probe hybridization are referred to as ‘delayed’ (49). Probes detected and reported by Xpert thus represent circulating rifampicin resistance-conferring
mutations which may be common or infrequent in a particular geographic setting (country). For example, the most frequently detected mutation globally in association with rifampicin-resistant TB, mutation Ser450Leu (26, 27), is captured by ‘absent’ probe E, whereas mutation Leu452Pro, also covered by probe E, is associated with delayed binding rather than complete absence (26).

**Figure 5.** The mechanism of detecting rifampicin-susceptible and -resistant strains by the Xpert MTB/RIF assay. Here, the rifampicin-resistant strains are captured by the cycle threshold difference of at least 3.5. Xpert probes A to E cover short segments of the hotspot and classify resistance-conferring mutations based on their location within the *Mtb rpoB* gene (51).

Xpert Ultra, the second generation Xpert assay, was released and recommended by the WHO in 2017 (3). The core of this re-engineered assay are the four sloppy molecular beacon probes, rpoB1 to rpoB4, with longer sequences and labelled with different fluorophores. High temperature-induced separation of the probe-amplicon hybrids generates a characteristic melt peak temperature (Tm) that will classify the sample as rifampicin susceptible or resistant and help identify which mutation is present. Xpert Ultra probes that can discriminate between the different rifampicin resistance--conferring mutations have been validated in a previous study (52).
The first-line “line probe assays” - LPA-Hain and LPA-Nipro- rely on multiplex amplification of the target gene and reverse hybridization of target to both wild-type and mutant probes immobilized on a membrane strip (5, 9, 47, 53). The LPAs include mutant probes that capture the commonly observed RR-conferring mutations - Asp435Val, His445Asp, His445Tyr, and Ser450Leu (53, 54).

Whereas Xpert is an automated assay that rapidly provides results, the LPAs entail manual steps that are at risk for amplicon contamination, and thus require specific technical skill and laboratory infrastructure to generate results in 1 to 2 days. The LPAs are therefore suitable for tertiary test centers and TB reference laboratories, while Xpert is targeted for use in district or sub-district health facilities (9).

Xpert Classic and Xpert Ultra were recommended for scale up to diagnose TB and RR-TB worldwide. Integrating these RDTs in national TB diagnostic algorithms has dramatically improved detection of RR/MDR-TB (8, 47). The implementation of Xpert was a colossal breakthrough in TB diagnosis as it revolutionized detection of RR-TB globally, allowing for prompt identification of patients who need to undergo treatment.

In contrast with the LPAs, targeted sequencing of the \textit{rpoB} gene detects all genetic variants in the gene, with Sanger technology as the most employed genetic sequencing method for surveillance by high TB burden countries worldwide (55).

**Challenges in rifampicin-resistant TB detection**

Rifampicin resistance jeopardizes TB control efforts because of high rates of poor outcome on first-line treatment and the resulting ongoing spread of resistant strains. In 2017, estimated 71% of patients with rifampicin-resistant TB worldwide were not diagnosed (56). This wide detection gap is mainly attributed to the lack of access to culture-based pDST and Xpert testing for new patients in settings where the initial diagnostic test is still smear microscopy. Furthermore, discordant phenotypic and genotypic diagnostic test results linked with disputed mutations, rifampicin heteroresistance, and rifampicin resistance-conferring mutations outside the 81 bp hotspot of the \textit{rpoB} gene aggravate the RR-TB detection gap.

**“Disputed” mutations**

Discordant results of phenotypic MGIT and genotypic RDTs are chiefly attributed to “disputed” \textit{rpoB} mutations. Disputed mutations only reduce the affinity of
rifampicin to bind with rpoB but do not totally prevent it from attaching to the protein. This was evidenced by the effect of His445 mutations causing a different conformation of rifampicin-rpoB protein binding (57). The said mutation still allowed RNA synthesis to proceed, observed as partial inhibition of mycobacterial growth on rifampicin-containing media. Further, RR-TB strains harboring disputed mutations may grow slower than mutant strains with undisputed mutations in culture DST due to a fitness cost (58, 59), thus growth-based DST techniques may not be able to detect their reduced susceptibility to rifampicin (60, 61).

The 1 µg/ml critical concentration of rifampicin used in MGIT is higher than the epidemiological cutoff values (ECOFF) which represent the highest wild-type MIC. This results in a breakpoint artefact, in which the minimum inhibitory concentrations of the wild-type and mutant strains overlap, and strains harboring disputed mutations are classified as rifampicin susceptible (62). The pre-standard conditions of MGIT therefore do not optimally detect strains with disputed mutations.

**Rifampicin heteroresistance**

A small portion of the rifampicin-resistant TB detection gap may be attributed to the co-existence of rifampicin-resistant and susceptible strains in patient samples, termed as rifampicin heteroresistance. Due to detection limits of currently implemented tests, rifampicin heteroresistance is reported as rifampicin susceptible. Minority rifampicin-resistant variants may emerge from an evolving population of *M. tuberculosis* bacilli or from a mixed infection of resistant and susceptible bacilli, and may lead to full blown resistance due to drug selection (63-65). Thus, failure to detect rifampicin heteroresistance can result in unsuccessful treatment and untraceable spread of rifampicin-resistant TB strains (66).

The limit of detection (LOD) of rifampicin heteroresistance is defined as the minimum percentage of mutant bacilli in a total mycobacterial population necessary for rifampicin resistance to be detected (66). The LOD of the WHO-recommended RDTs is insufficiently documented in association with the specific *rpoB* mutation. In the case of Xpert Classic, previous studies report LOD values ranging from 65 to 100% for mutations Ser450Leu and Leu452Pro (49, 67), for Xpert Ultra, only the LODs for mutations L430P, H445N, and S450L were reported (52), while LODs of LPA-Nipro have not been determined.
Complete documentation of the rifampicin heteroresistance LODs for the most commonly detected rifampicin resistance-conferring mutations and for the state-of-the-art RDTs is crucial for timely and more accurate detection of rifampicin resistance and initiation of appropriate treatment.

**Rifampicin resistance-conferring mutations outside the hotspot: non-RRDR mutations**

The currently implemented RDTs fail to detect mutations located outside the RRDR (71). Rifampicin resistance-conferring mutations not picked up by the RDTs include mutations Val170Phe and Ile491Phe. Mutation Ile491Phe is significantly associated with RR-TB (26) and critically found in 30.4% of TB strains in Eswatini, 14.3% of isoniazid monoresistant strains in South Africa, and in lower percentages in Hong Kong and Australia (72). It is linked with poor treatment outcomes and resistance to pyrazinamide and ethambutol (70).

**RIFAMPICIN-RESISTANT TB TRANSMISSION**

Critical gaps in RR-TB detection and subsequent treatment delays can inevitably result in failed standard treatment. While the prevalence of MDR-TB is higher in patients who were previously treated for TB (retreatment cases) than in new cases (73), most retreatment patients with MDR-TB already had MDR-TB on starting their first TB treatment episode yet missed the opportunity for resistance testing. MDR/RR-TB strains could then potentially spread and circulate within populations resulting in RR-TB outbreaks (9).

Whole genome sequencing and statistical modeling analyses revealed that the current patterns of RR-TB in high RR-TB settings, especially in the former Soviet Union, can only be explained by transmission (74). This supports previous findings that transmission rather than acquired resistance is the primary driver of the global RR/MDR-TB burden particularly in highly endemic settings (73, 75, 76).
Compensatory mutations

RR-TB strains exhibit a fitness cost - a reduced competitive ability against RS strains in the absence of rifampicin. This fitness cost is dictated by the genetic background of the strain and the RR mutation it harbors (77). For example, clinical strains harboring mutation Ser450Leu have little to no fitness cost.

While resistance comes at a fitness disadvantage, the average fitness of resistant strains tends to increase over time and approach that of susceptible strains due to out-selection of mutants with low fitness cost. Compensatory mutations that counteract fitness loss may also be present such as those in rpoA and rpoC genes. Compensatory mutations in rpoA and rpoC genes have allowed successful transmission of MDR-TB strains in Uzbekistan, Central Asia and South Africa (74, 78). Multiple regression analyses revealed that compensatory mutations are positively associated with successful transmission of drug-resistant TB strains in various settings and outbreak clades.

Early detection of rifampicin-resistant TB clusters

Early detection of TB clusters, and the identification, isolation and treatment of patients with incident active cases may help curb transmission, that will benefit not only the individual patient but also their wider community. Surveillance programs were then implemented, for instance by high-income countries where they have complemented contact investigations with classical molecular genotyping methods such as IS6110 restriction fragment length polymorphism (RFLP) (79), CRISPR-based spoligotyping (80), and 24-loci mycobacterial interspersed repetitive units variable number tandem repeat (MIRU-VNTR) (81) to identify transmission clusters of M. tuberculosis strains (82). These methods however are rather slow to identify transmission hotspots in an actionable manner.

Early detection of RR-TB clusters would entail rapid and systematic collection and analysis of rifampicin resistance data at the population level. The increasing use of Xpert as a frontline diagnostic test for all presumptive TB patients in low and high TB burden settings (5, 9, 47) provides an opportunity to exploit routinely produced rifampicin resistance data for timely detection of RR-TB clusters. Analyzing Xpert test results in a systematic manner may benefit surveillance efforts and inform public health decisions by flagging the national TB control programs to target resources where they are urgently needed. Clinicians may then deliver prompt
and appropriate interventions and community health workers may conduct active case finding, both of which are known to reduce the transmissibility of TB. The systematic screening and testing for RR-TB among individuals who do not voluntarily consult in health facilities, also known as active case finding, aims to promptly identify additional patients who need to undergo treatment to reduce the duration of infectiousness. In settings with high prevalence of undetected TB, the WHO recommends active case finding for high risk groups such as household contacts of TB patients, children and elderly, HIV patients, prisoners, and populations with limited access to health services (83). Thus, molecular and epidemiological analysis of Xpert data may provide opportunities that will may serve both the individual patient in receiving prompt effective treatment and public health objectives for reducing the burden of RR-TB.

Previous studies have shown that routine data are useful for identifying clusters that are likely to become TB/RR-TB outbreaks. Althomsons and colleagues gathered and analyzed patient data, genotyping results, and geospatial information routinely reported to the Centers for Disease Control and Prevention (CDC) to predict TB clusters that were most likely to progress into TB outbreaks in the United States (85). Their retrospective data analysis showed that social risk factors such as homelessness, excess alcohol use, illicit drug use, or incarceration increased occurrence of a TB outbreak, and that this information combined with genotyping results can be utilized to identify clusters that were likely to become outbreaks. Using routine surveillance data, Kammerer et al. found that the three statistical methods, namely county-based log-likelihood ratio, cumulative sums, and spatial scan statistic, had comparable abilities to retrospectively and timely detect known TB outbreaks in the United States (86). Althomsons and co-workers fit negative binomial hurdle models using epidemiologic data from the CDC US National Tuberculosis Surveillance and combined the generated information with whole genome sequences from the National Tuberculosis Genotyping Service (87). They detected 15 possible outbreaks among endemic tuberculosis clusters. They expect their method to be incorporated into CDC’s existing surveillance system for large outbreaks of TB in the United States.

The application of RDT data for RR-TB surveillance has only been explored by McIntosh and colleagues (88) where they gathered retrospective LPA-Hain and DST routine data to locate RR-TB cases and generate heatmaps across the Western Cape province.
Potential use of Xpert Classic data for early detection of rifampicin-resistant TB clusters

Xpert Classic is the most widely deployed RDT globally, implemented as the front-line diagnostic tool for all patients with suspected active TB disease by 32 out of 48 WHO high burden countries (5, 8). More countries are rapidly moving towards using Xpert as the initial diagnostic test for all presumptive TB patients (5, 8, 89, 90), for which large volumes of Xpert test results remain stored in local computers in health facilities and laboratories, with the risk of loss or corruption. Understanding the relationship of Xpert Classic probe reactions and specific rifampicin-resistance conferring mutations is key for the national TB control program to utilize Xpert test results for RR-TB surveillance.

Xpert probe reactions are potentially useful for early detection of unusual rifampicin-resistant tuberculosis clusters in specific settings, defined as the occurrence of geographically defined number of RR-TB cases above expected case counts. If associated with rare Xpert probe reactions, these may define potential transmission hotspots. The probability of detecting a potential transmission hotspot will be relatively higher for rarely observed probe reactions representing low-frequency mutations in a specific setting, and lower for frequently observed probe reactions representing common RR mutations (26).

Digital linking of Xpert machines: Leveraging connectivity platforms for rifampicin-resistant TB surveillance

In the LMICs, digitally-linked routine RDT data could allow the NTP to fully utilize the data they produce regularly and transform the information contained in the data to actionable solutions towards improved RR-TB control.

In line with the consensus of global TB experts on the potential of diagnostic connectivity for tuberculosis elimination (91), we hypothesize that RDT results could be employed as a molecular epidemiological tool in field conditions to trace RR-TB transmission hotspots in high burden TB settings. These readily available data could also aid in resolving the discordance between pDST and RDT results, primarily for disputed mutations (31).

Few years after roll out of Xpert in 2010, connectivity platforms were developed to digitally link Xpert machines together with generated test results, examples of which include GxAlert (SystemOne, Springfield, MA, USA), DataToCare (Savics,
Brussels, Belgium), and C360 (Cepheid, Sunnyvale, CA, USA). These connectivity platforms consolidate test results from all Xpert machines in the setting into a central server with built-in analytics system. The information gathered is then securely shared with different stakeholders—clinicians, national TB control programs, and patients—through text messaging or email (91).

**Whole genome sequencing to validate and complement use of RDT data for rifampicin-resistant TB surveillance in the local setting**

The abovementioned limitations of the RDTs can be solved by direct whole genome sequencing (WGS) of patients’ samples to determine its full drug resistance profile (92, 93). Prior to WGS being established as a high-resolution tool for TB surveillance, RFLP and MIRU-VNTR have been used to differentiate patients with relapse from those with exogenous reinfection (94). The increased resolution of whole genome sequencing made it more effective in distinguishing recurrent TB as relapse or reinfection particularly in settings with high infection pressure and closely related circulating strains (95-97).

**Use of WGS for RR-TB surveillance programs and epidemiological investigations**

WGS has been widely studied for improved detection of drug-resistant TB in diverse laboratory settings worldwide (26-28, 100). The CRyPTIC consortium, a collaboration among TB research institutions globally, was established for improved TB control, detection and treatment of MDR-TB patients through drug resistance and susceptibility prediction that may now inform clinicians which first-line drugs to prescribe (101). The ReSeqTB platform is a curated database of genomic variants associated with pDST and clinical drug resistance data which serve as reference standards for researchers, clinicians, and public health officials (25).

Resistance mutations and other markers of transmission can be accurately identified through WGS (93). The ability of WGS to distinguish between pathogenic strains made it highly suitable for TB epidemiological investigations utilizing single nucleotide polymorphism differences, phylogenetics, mutation rates, and transmission network based on contact tracing and timing of infectious periods to either confirm or exclude transmission (102-108).

WGS of strains isolated from all drug resistant-TB patients is already being widely implemented in various low TB incidence settings, aimed towards completely replacing pDST in the clinic (101, 109). WGS implemented in high TB burden countries was shown to accurately estimate the prevalence of DR-TB (55). Whereas the conventional periodic TB drug resistance surveys have been gathering data representative of the *M. tuberculosis* population in poor resource settings, several high-income countries apply continuous surveillance (8, 55) to help improve the choice of standard TB treatment before the full drug sensitivity profile is known (8). There is however, the TB data gap that needs to be overcome to establish and evaluate country-level digital-linking of Xpert machines.

**Potential use of new tools in support of public health surveillance for improved rifampicin-resistant TB control**

To support public health efforts with new tools for controlling the spread of RR-TB, it is key to have an exhaustive diagnostics implementation approach (112) and take into consideration the local contextual factors and practices that affect implementation. These factors go beyond health systems indicators; thus, it is necessary to increasingly engage in-country frontline stakeholders. This may then generate critical programmatic data for addressing potential obstacles in the implementation, prospective assessment, scale-up, and sustainability of new RR-TB surveillance tools. By doing so, we could be a step closer to reducing the burden of RR-TB transmission.
OBJECTIVES, SCOPE, AND STRUCTURE OF THIS THESIS

This thesis aims to introduce and investigate on a novel approach that extracts and analyzes critical information from routinely produced Xpert and line probe assay data which can then be utilized as a public health tool to boost setting-specific RR-TB surveillance.

The introductory Chapter 1 provides the context and rationale of this thesis.

The first part of this thesis addresses how the limitations of the WHO-recommended RDTs, and resulting phenotypic and genotypic discordance undermine efforts to interrupt rifampicin-resistant tuberculosis (RR-TB) transmission. To this end, we aim to investigate how the RDTs detect the different RRDR mutations including the “disputed” rpoB mutations, minority rifampicin resistant variants denoting rifampicin heteroresistance, and how the pDSTs detect RR mutations outside the RRDR, to help resolve discordant phenotypic and genotypic test results.

Chapters 2 and 3 determine and visualize the RDT probe signatures for the majority of documented RR-conferring rpoB mutations. Chapter 2 describes the laboratory validation of Xpert Classic, LPA-Hain, and LPA-Nipro results, and plots the RR mutations detected by the RDT probes. It also proposes a novel approach of fully utilizing connected TB diagnostics and readily available test results for real-time monitoring of RR-TB transmission towards improved RR-TB surveillance. Chapter 3 highlights the ability of the re-engineered Xpert Ultra to unambiguously identify specific RR-conferring mutations through unique combinations of Ultra probes and melting temperature shifts.

Chapter 4 investigates disputed RR-conferring mutations undetected by MGIT standard conditions due to the wide overlap between wild-type and mutant MICs. It also reports the variable ability of commercial and non-commercial phenotypic and genotypic RDTs to detect occult rifampicin resistance by disputed RR mutations.

Chapter 5 approaches the inadequate documentation of the threshold of the RDTs for detecting minority rifampicin resistant variants that comprise rifampicin heteroresistant samples. In here, we aim to determine the limits of detection of Xpert Classic, Ultra, LPA-Hain, and LPA-Nipro for detecting rifampicin
heteroresistance linked with the four RR mutations S450L, D435V, H445D, and H445Y, in relation with the different probes used in each RDT.

The second part of this thesis describes extracting critical information from large volumes of routine diagnostic resistance data, and translating this information into tools that would support public efforts for improved RR-TB control. This part also presents viewpoints of target end-users of the tool and algorithm, primarily the national TB control program managers and staff and other key stakeholders such as key TB scientists and advisers from the WHO, connectivity software developers, RDT manufacturers, and the medical doctor caring for a TB patient.

Chapter 6 deals with the increasing use of whole genome sequences in research and public health initiatives that can lead to a disconnect from the RDT-based data being generated routinely in the clinic, and the majority of surveys, as well as the challenge in comparing large volumes of RDT data within and between countries due to use of differing technologies, widening the TB data gap. This chapter tries to address this gap through the computational tool Myc TB Genome to Test (MTBGT), which transforms whole genome sequencing-derived data into validated results of the RDTs. It allows comparison of data from different collection periods and underlying platform i.e. previous historical strains with current isolates, and bridges the TB data gap between and among periodic drug resistance surveys, continuous surveillance programs, routine laboratory tests, and research settings.

Chapter 7 addresses the potential utilization of large volumes of Xpert data for RR-TB surveillance, exploring its utility for early detection of potential RR-TB transmission hotspots in specific settings. It describes a proof-of-concept modelling of longitudinal routine Xpert test results, in which we projected the number of expected RR-TB cases to quantitatively identify Xpert sites with unusual case counts, and examined the recorded Xpert probe reactions in all sites to identify areas with at least two rare Xpert probes representing low-frequency mutations, a proxy for an epidemiological link between two patients. This chapter also features the perspectives, needs and preferences of the of various stakeholders on improving RR-TB management and control in the context of this PhD project. Chapter 8 describes a false Xpert Ultra RR result obtained from testing a lymph node aspirate of a TB-HIV patient in Antwerp, Belgium.

Chapter 9 aims to synthesize and discuss the findings of this thesis, its policy implications, and future perspectives.
REFERENCES


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