The management of tuberculosis: epidemiology, resistance and monitoring

Rapid methods to improve treatment outcome

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2. Bang D, Lillebaek T, Thomsen VØ, Andersen ÅB. Multidrug-resistant tuberculosis: treatment outcome in Denmark, 1992-2007. Scand J Infect Dis. 2010;42:288-93.
3. Bang D, Andersen PH, Andersen ÅB, Thomsen VØ. Isoniazid-resistant tuberculosis in Denmark: mutations, transmission and treatment outcome. J Infect. 2010;60:452-7.
4. Bang D, Andersen ÅB, Thomsen VØ. Rapid genotypic detection of rifampin- and isoniazid-resistant *Mycobacterium tuberculosis* directly in clinical specimens. J Clin Microbiol. 2006;44:2605-8.
5. Vijdea R, Stegger M, Sosnovskaja A, Andersen ÅB, Thomsen VØ, Bang D. Multidrug-resistant tuberculosis: rapid detection of resistance to rifampin and high or low levels of isoniazid in clinical specimens and isolates. Eur J Clin Microbiol Infect Dis. 2008;27:1079-86.

PREFACE

The scientific work of this PhD thesis was conducted from 2006-2010 during my employment at the International Reference Laboratory of Mycobacteriology. A special thank you to my supervisors: *Åse Bengaard Andersen* and *Vibeke Østergaard Thomsen* for their guidance and supervision. I acknowledge *Troels Lillebaek*, *Roxana Vijdea*, *Anaida Sosnovskaja*, *Marc Stegger*, *Peter H. Andersen*, *Axel Kok-Jensen*, *Azra Kurbasic*, *Anders Mørup Jensen* and my colleagues for their input and research discussions. I thank my family and friends for their continued support throughout the study.

INTRODUCTION

Denmark (DK), a low-burden tuberculosis (TB) country, had an overall incidence of 6.7 per 100 000, with nearly 400 notified TB-cases in 2008 [1]. A steady increase in the number of notified TB-cases per year due to immigration was observed from the mid 1980´s until it levelled in the mid 1990´s before taking a downward trend [1,2]. For decades, the TB incidence amongst Danes has remained low and unchanged [1]. Although DK is a high-income country with the resources necessary to combat TB, the National TB control programme (NTP) has failed to curtail the TB-burden further and the disease remains a health problem of concern. Globally, the incidence of TB increased in 2008, to an estimated 9.4 million incident cases [3]. Tuberculosis continues to be a global challenge, despite the World Health Organization/Centers for Disease Control and Prevention (WHO/CDC) enforced interventions [4]. Insufficient treatment regimens, non-adherence, and poor drug availability led to treatment failure, recurrent disease and drug resistance (DR) [4]. Not only is TB a contagious infectious disease, life-long immunity is not gained by an episode of active TB disease and the disease may be contracted again at a later stage, either by relapse with the same strain or reinfection with a new strain [5].

RECURRENT TUBERCULOSIS

Recurrent TB has been defined as a serial episode of active TB disease occurring after treatment success [6-10]. Standardized IS*6110* restriction fragment length polymorphism (RFLP) genotyping of *Mycobacterium tuberculosis* isolates allows recurrent TB-cases to be categorized as relapse of disease from the original infecting strain or reinfection with a new strain of *M. tuberculosis* [11]. The nationwide TB surveillance and RFLP-genotyping coverage gives DK a stronghold in assessing treatment outcome and the risk factors for developing recurrent TB, with discrimination between relapse and reinfection.

MONITORING TUBERCULOSIS TREATMENT RESPONSE

Combined multidrug therapy is necessary to prevent selection and development of DR during treatment. Clinical assessment of treatment response can be difficult as symptoms improve earlier than bacterial death. Serial chest radiographic regression is even slower than culture conversion. A definitive diagnosis of TB is based on the presence of acid-fast bacilli (AFB) by smear microscopy and culture of *M. tuberculosis*. Acid-fast bacilli smear has the disadvantage of a low sensitivity of approximately 60% compared to culture and cannot distinguish viable from dead bacteria. As sputum smear AFB may persist for long periods after culture conversion, smear is of limited value in monitoring treatment [12-

14]. Culture has a high sensitivity in detecting *M. tuberculosis*, which increases with the number of analysed samples [15,16]. Previous studies have shown that in 80% of successfully treated patients with standard therapy, culture conversion occurred during the first 2-months of therapy [17,18]. Currently, culture is the golden standard of therapy follow-up. However, culture is time consuming (weeks-months). In order to prevent relapse and treatment failure faster methods are required to effectively monitor TB disease.

DRUG-RESISTANT TUBERCULOSIS

Drug resistance is a man-made amplification of a natural phenomenon. From a microbiological perspective, resistance is caused by a genetic mutation that makes a drug ineffective against the mutant bacteria. Patients with a large bacillary load have an increased risk of developing resistant bacteria because more spontaneous mutations occur in a large population of bacteria. An inadequate treatment regimen then allows for the selection of a drug-resistant strain to become the dominant strain in a patient infected with TB. Multidrug-resistant (MDR) TB is defined as disease due to *M. tuberculosis* resistant to at least rifampin (R) and isoniazid (H), the key elements of TB treatment. Multidrug-resistant TB is a serious public health problem, as it has been associated with higher rates of failure and mortality than drug-susceptible TB, especially in human immunodeficiency virus (HIV) co-infected patients [19,20]. Sputum culture conversion has been shown to be a predictor of outcome and is slower in patients with MDR-TB than those with drug-susceptible TB [21,22]. Drug-resistant TB is more complicated for both patients and health care systems, due to side-effects and costs of second-line drugs [4]. In low-burden TB countries, only few studies have addressed treatment outcomes of MDR-TB and even less is known about treatment outcome of the more prevalent H drug-resistant TB [23]. More reliable evaluations of combined treatment regimens can be carried out in this part of the world, as the costs of treatment are of less importance compared to some high-burden TB countries. With the global emergence and spread of drug-resistant TB, the project documents treatment outcome of MDR-TB and H-resistant TB cases in DK. In addition, treatment regimens chosen were described and the need for rapid methods to detect resistance and follow-up of treatment was established.

RAPID METHODS TO DETECT DRUG RESISTANCE

Detection of drug-resistant TB in clinical specimens is time-consuming (4-6 weeks), as it relies on the slow growth of *M. tuberculosis* before drug-susceptibility testing (DST) can be performed. Isoniazid and R are the most powerful bactericidal drugs, active against all populations of TB bacteria. Rapid methods to detect resistance are essential in order to ensure early adjustments of efficient treatment. Molecular methods have become commercially available for the detection of MDR-TB, but are dependent on culture of *M. tuberculosis*. The spread of MDR-TB can be prevented by rapid detection and correct treatment. For R resistance, the majority of the mutations responsible are located in the hotspot 81-base pair "core region" of the B-subunit RNA polymerase gene (*rpoB*) in >95% of isolates. Whereas, H resistance is more complex as mutations are located in several genes [24]. The majority of clinical H-resistant strains have mutations in the catalase-peroxidase gene (*katG*), 50-95% of which are located in codon 315, and 15-35% located in the NADH-dependent enoyl ACP reductase gene (*inhA*) [25-28]. Mechanisms of *M. tuberculosis* DR gene mutations are shown in table 1.

OBJECTIVES

The overall objective of this thesis was to analyse national Danish TB epidemiology data with specific focus on treatment outcome and establishing the need for new tools to improve TB treatment outcome. In addition, rapid methods for resistance detection and monitoring of treatment were optimized and evaluated.

SPECIFIC AIMS

1. To analyze recurrent TB disease over time and to determine the rate of relapse and reinfection. To compare the relapse and reinfection groups on the basis of selected characteristics.
2. To examine long-term treatment outcome of MDR-TB in DK, to assess if MDR-TB transmission occurs, and to determine whether analysis of the most frequent R and H conferring mutations would have identified MDR-TB cases found by conventional DST at an earlier stage.
3. To describe treatment outcome and transmission of H-resistant TB disease and to evaluate a mutation analysis of the most frequent H conferring mutations for high- and low-level H resistance.
4. To optimize and evaluate a rapid method for resistance detection directly in clinical specimens. Furthermore, genetic mutation results were compared with conventional DST.
5. To evaluate the performance of an extended assay for the rapid detection of mutations causing R and high- or low-level H resistance in *M. tuberculosis* isolates and directly in clinical specimens. The genetic mutation results were compared to conventional DST.

METHODS

STUDY LOCATION AND LABORATORY METHODS

All studies were carried out at the International Reference Laboratory of Mycobacteriology, Statens Serum Institut. The laboratory performs all human culture-based mycobacteriological diagnostics in DK and has Quality Assurance accreditation. Specimens were subjected to auramine-rhodamine smear AFB microscopy and incubated on both Löwenstein-Jensen slants and Mycobacterial Growth Indicator Tube 960 system (MGIT) (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) media. Growth of *M. tuberculosis* was confirmed by InnoLipa species (InnoGenetics, Ghent, Belgium) and DST was performed using BACTEC 460 (BACTEC) system (Becton Dickinson Diagnostic Systems, Sparks, MD, USA).

Table 1

Mechanisms of *Mycobacterium tuberculosis* drug resistance gene mutations.

Drug	Gene(S)	Function of the gene	Mechanism of resistance
Rifampin	*rpoB*	*B* subunit of RNA polymerase	Inhibition of RNA synthesis
Isoniazid	*katG*	Catalase-peroxidase	Reduces the ability to activate the prodrug isoniazid
	inhA	Enoyl ACP reductase	Reduces the binding of NADH to inhA and attack by isoniazid
	ahpC	alkyl hydroperoxide reductase	Overexpression of the antioxidant enzyme AhpC, that removes peroxide necessary for isoniazid activation
	kasA	B-ketoacyl ACP synthase	Overexpression of KasA, involved in fatty acid and mycolic acid synthesis
	ndh	NADH dehydrogenase	Increases the NADH/NAD ratio, competes with the binding of isoniazid-NAD to inhA
Ethambutol	*embB*	Arabinosyltransferase	Decreases the binding to ethambutol
Pyrazinamide	*pncA*	Pyrazinamidase	Lack of conversion of pyrazinamide to pyrazinoic acid
Streptomycin	*rpsL*	S12 ribosomal protein	Decreased binding of streptomycin to S12 ribosomal protein
	rrs	16S rRNA	Decreased binding of aminoglycosides to 16S rRNA
Amikacin			
Kanamycin	*rrs*	16S rRNA	Decreased binding of aminoglycosides to 16S rRNA
Capreomycin	*tlyA*	2´-O-methyltransferase	
Fluoroquinolone	*gyrA, gyrB*	DNA gyrase	Inhibition of DNA gyrase
Ethionamide	*inhA*	Enoyl ACP reductase Flavin monooxygenase	Inhibition of mycolic acid synthesis

ACP = acyl carrier protein; NADH = nicotinamide adenine dinucleotide, reduced form

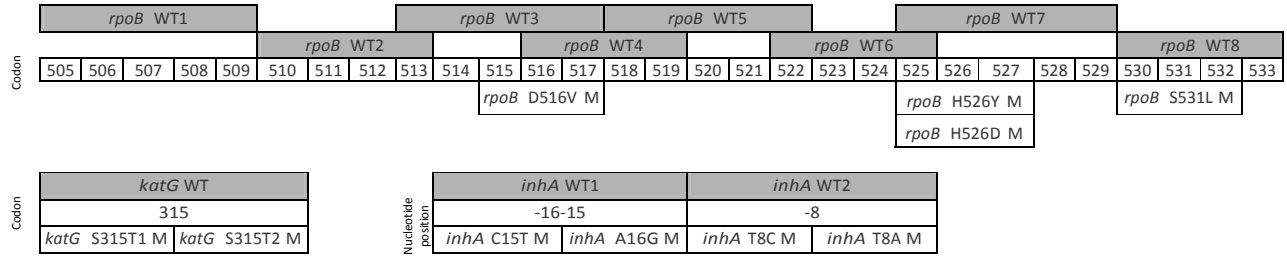

Figure 1
***rpoB, katG* and *inhA* mutation (M) and wild-type (WT) probe coverage of the GT*plus* assay.**

MOLECULAR GENOTYPING

Molecular genotyping was performed by the standardized IS*6110* RFLP. In 1992, the International Reference Laboratory of Mycobacteriology introduced nationwide RFLP-genotyping of all *M. tuberculosis* strains from all patients in DK. A database of all *M. tuberculosis* RFLP-genotype results was created and used in this study. Standardized RFLP-genotyping allows recurrent TB-cases to be categorized as being due to relapse or reinfection.

DATA COLLECTION

For all studies performed, identification codes, age, sex, site of disease, smear, culture, DST, RFLP results were collected from the Mycobacterial Registry. In the recurrent TB study and the MDR-TB treatment outcome study, data on treatment and characteristics were collected from patient files. For the H resistance treatment outcome study, treatment outcome data was collected from the voluntary national epidemiological surveillance data. Mortality and transfer out data was retrieved from the nationwide Danish Civil Registration System. All collected data was merged and standardized into databases.

STUDY DEFINITIONS

We defined serial cases, recurrent TB, relapse, reinfection, "default" recurrent cases as outlined in table 2. Treatment outcome categories including MDR treatment outcome were defined according to the WHO guidelines are shown in table 2 [21,29]. "Treatment completion" or "cure", were recognized as treatment success.

Gene mutation and wild-type (WT) coverage of the GenoType® MTBDR*plus* (GT*plus*) assay are shown in figure 1. Detailed descriptions on methodologies, study populations, design, data collection, follow-up, genetic analyses, statistical methods, assumptions and ethical considerations are stated in articles I-V (Appendix I-V).

RESULTS

Results of projects I-V are presented in each of the articles (Appendix I-V). The following relevant figures and tables are depicted in this thesis. GT*plus* strip pattern results are shown in figure 2. The performance of the GenoType® MTBDR (GT) assay compared to GT*plus* assay directly in clinical specimens is shown in table 4 (IV, V). Comparisons of the GT*plus* assay results with conventional DST for R and H in the 115 isolates tested are shown in tables 5 and 6 (V).

Table 2

Definitions of serial cases, recurrent tuberculosis, relapse, reinfection, "default" recurrent cases, WHO treatment outcome categories and sputum culture conversion (I, II, III).

Serial case	A culture-positive TB-case occurring ≥6 months after a previous episode
Recurrent TB*	A serial case with the original strain after treatment success or a case with a new strain irrespective of treatment outcome
Relapse	Recurrent TB episode caused by the original strain, based on the RFLP-pattern after treatment success
Reinfection	Recurrent TB episode caused by a new strain with a >2 band change in RFLP-pattern, irrespective of treatment outcome
"Default" recurrent	A culture-positive TB-case with the same strain with ≥6 months interval that defaulted (interrupted treatment for ≥2 months)
Cure	A patient who is sputum culture negative in the last month of treatment and on ≥1 previous occasion.
MDR Cure	A patient that completed treatment according to the NTP that either had ≥5 consecutive negative cultures or only 1 positive culture followed by 3 negative cultures from samples collected ≥ 30 days apart with no clinical deterioration in the final 12 months of treatment.
Treatment completion	A patient who has completed treatment but who does not meet the criteria to be classified as a cure or failure.
MDR treatment completed	A patient that completed treatment according to the NTP that did not meet the definition for cure due to the lack of bacteriological results.
Treatment failure	A sputum smear or culture-positive case at five months or later during treatment.
Died	A patient who dies for any reason during the course of treatment.
Defaulter	A patient whose treatment was interrupted for ≥2 consecutive months.
Transfer out	A patient that left the country to another reporting and recording unit with an unknown treatment outcome.
Sputum culture conversion	A culture-positive patient having ≥2 negative cultures taken ≥30 days apart after therapy initiation.

MDR = multidrug-resistant; TB = tuberculosis; NTP = national TB control programme.

*) Recurrent TB cases with RFLP-patterns differing ≤2 bands were considered genetic drift and thus relapse rather than reinfection.

DISCUSSION

TREATMENT OUTCOME

Antibiotic treatment of TB has remained unchanged for decades. In DK, standard short-course therapy for drug-susceptible TB cases consisted of HR for 6 months with ethambutol (E) and pyrazinamide (Z) included for the first 3 months until 2008, when the initial phase was shortened to 2RHEZ/4RH in accordance with international WHO/ CDC recommendations [4,17,30]. Whereas, regimens for MDR-TB or H-resistant TB were individually modified [30]. In 2001, the WHO/International Union Against Tuberculosis and Lung Disease (IUATLD) standardized TB treatment outcome monitoring was implemented in DK [31]. This thesis addressed treatment outcome in DK with focus on recurrent TB, MDR-TB and H-resistant TB. Nationwide, the study showed that recurrent TB accounted for 1.8% (*n* = 73) of a total 4154 cases during 13.5-years in DK (I). Of these recurrent cases, 1.3% represented relapse, indicating a high treatment efficacy. An observational study performed in a low-burden country, where the risk of TB re-exposure is sparse, the rate of relapse may be a good indicator of treatment efficacy. These findings confirm other studies performed in low-burden TB countries, where recurrent TB rates ranged from 0.4-7% [6,7,32,33]. After the first TB episode the risk of recurrent TB due to relapse declined to reach baseline four-years later. This may warrant a longer individualised follow-up to be included in the NTP for selected cases (I). The rate of reinfection with a new strain was found to be very rare (0.5%), and the cumulative risk of reinfection increased with time. The proportion of recurrent TB due to reinfection was comparable to those found in other low-burden countries (I) [34-36].

Poor treatment adherence, cavitary disease, DR, being underweight, a positive 2-months treatment culture, HIV co-infection, smoking, alcohol and drug abuse have previously been associated with an increased risk of recurrent TB [7,18,37-39]. Comparison of demographic characteristics between the patient subgroups with relapse and reinfection revealed that only the presence of cavernous disease was significantly associated with a four-fold increase of relapse compared to reinfection (I). A patient with cavitary disease may hold up to 1×10^9 bacteria in the lungs [40]. The presence of cavitation has been shown to be an important risk factor of relapse, probably due to the higher bacterial load and lower accessibility of cavities to antibiotics [40,41]. Cavitation increases the risk of developing DR [41]. Having cavitary disease and a positive sputum culture after 2-months of treatment was associated with an even higher relapse rate [17]. The American Thoracic Society (ATS)/CDC guidelines recommend close monitoring for patients with cavitary disease or those who have a positive 2-months treatment culture, and an extension to a total of 9-months therapy if both are found [17]. Current recommendations on treatment of cavitary TB disease in DK follow these guidelines.

In the initial TB episode, any single resistance was observed in 19% of the patients with relapse, and the development of DR in relapse patients during the serial episode was rare (7%) (I). The WHO recommends the monitoring of treatment progress in smear-positive or culture-positive patients through sputum smears and cultures: at 2-months, at 5-months, and at the end of treatment [4]. In contrast, the ATS/CDC recommends for pulmonary TB patients that sputum specimens for AFB smear and culture to be obtained at monthly intervals until two consecutive specimens are negative on culture [17]. Ensuring sputum culture conversion, especially in patients with cavitary TB is stressed. The monitoring of treatment progress may be improved by the development of more rapid surrogate markers than culture that may earlier identify patients that are prone to develop relapse.

In drug-free environments, R resistance occurs at an estimated rate of 1 in 10^8 bacteria, while H resistance arises in approximately 1 in 10^6 bacteria [42]. The genetic mechanisms of H resistance in *M. tuberculosis* are complex, and several genes participate in H resistance. Strains of MDR *M. tuberculosis* appear to result from the stepwise acquisition of mutations in the genes encoding drug targets or drug-converting enzymes [41]. Three relapse patients initially polyresistant, were susceptible during the serial episode over a time span of 4.9-6.5 years (I). New evi dence suggests that a strain can revert from resistant to susceptible over time [43]. The WT susceptible bacteria may have had a better "fitness" and overgrown the mutated drug-resistant

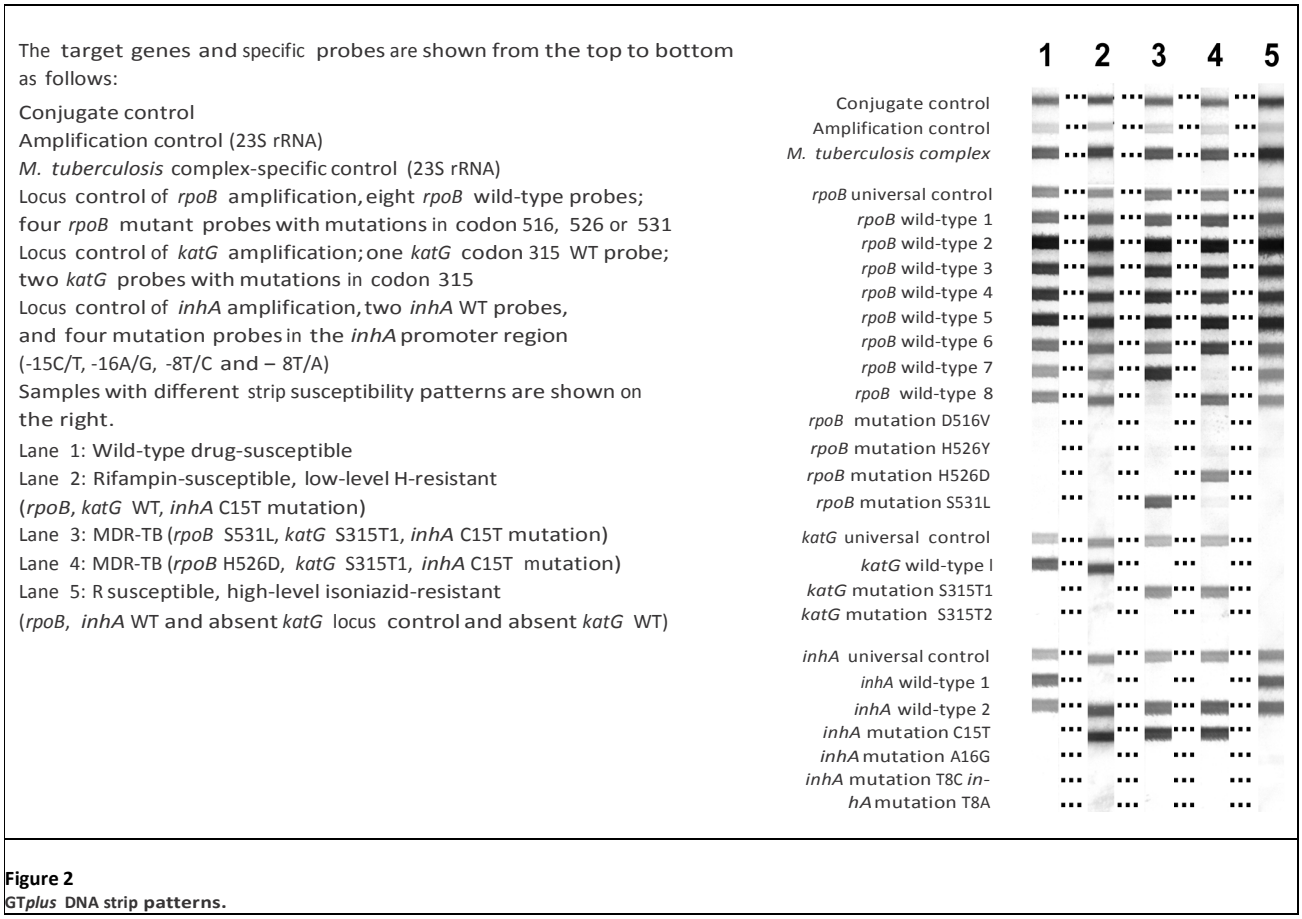

Figure 2
GT*plus* DNA strip patterns.

clones. Alternatively, the patients may have been reinfected with another strain with the same RFLP-pattern or DST results were erroneous.

MULTIDRUG-RESISTANT AND ISONIAZID-RESISTANT TUBERCULOSIS

Among all incident TB cases globally, 3.6% were estimated to have MDR-TB in 2008 [44]. Global estimates indicate that there are about half a million MDR-TB cases, where 5-7%, are the serious variant of extensively drug-resistant (XDR) TB [44,45]. Extensively drug-resistant-TB is defined as MDR-TB and resistance to a fluoroquinolone (Q) and at least one of the second-line injectable drugs (amikacin (Am), capreomycin or kanamycin). Nationwide, the incidence of combined H-resistant TB was found to be 6.7% (2002-2007), and the incidence of MDR-TB was confirmed in only 0.5% (1992-2007) of all culture-verified TB cases (II, III). These levels are similar to levels previously reported in DK [46]. XDR-TB has not as yet been found in DK. The proportion of H resistance found is close to the WHO target of any resistance among new cases in ≤5%, as being the level of a good NTP [47]. The majority of MDR-TB and H-resistant TB cases were imported, whereas acquisition and active transmission was rare. However, due to migration XDR-cases may be expected in the future.

Short- and long-term treatment outcome

Previous studies have documented that MDR-TB treatment outcomes after individualized or standardized short-course therapies were poor [20,48]. Improved short- and long-term MDR-TB treatment outcomes in low-burden countries have been reported [49-51]. Successful short-term MDR-TB outcomes of 85-87% have been found in the USA, Canada and Germany in series including up to approximately 200 patients [49-51]. MDR-TB treatment outcome studies in settings with low TB burdens and high-incomes are shown in table 3. These studies were heterogeneous and not comparable as methodologies differed. Applying WHO MDR treatment outcome definitions, we likewise report a high short-term treatment success of 89% in a series with 27 patients, with no TB-related deaths among MDR treated patients (II). Our findings are similar to a previous small study performed in DK [52].

Table 3

Multidrug-resistant tuberculosis treatment outcome studies from high income low-burden settings.

Country	Period	Number of patients	HIV (%)	Short-term success (%)	Long-term success (%)	TB mortality rate (%)	Follow-up (years)	Definitions of treatment success (consecutive months)	Reference
USA	1973-1983	171	0	65	56	22	1.7	≥3 CN (3)	***Goble et al* [20]**
USA	1991-1993	38	89	63	55	45	1-2.8	≥2 CN (≥2 wks apart)	***Turett et al* [57]**
USA	1991-1994	25	0	-	68	0	1.9#	≥2 CN (≥2 wks apart)	***Telzak et al* [55]**
USA	1984-1998	205	0	85	75	12	15 (up to)	≥3 CN (3)	***Chan et al* [50]**
Netherlands	1985-1998	44	0*	-	75	2	4.4	≥2 CN (≥2 wks apart)	***Geerligs et al* [54]**
Canada	1986-1999	40	0	85	60	12.5	4.8	3 CN (3)	***Avendano et al* [51]**
UK	1995-1999	13	0*	-	62	7.7	3.6	≥2 cons CN	***Hutchison et al* [53]**
Germany	2004-2006	177	4.9 (*n*=142)	87	-	7.9	-	WHO (MDR)	***Eker et al* [49]**
Denmark	1992-2007	27	7	89	100	0	8.9	WHO (MDR)	***Bang et al* (II)**

*** = or presumed negative; # = median; CN = culture negative; wks = weeks; WHO (MDR) treatment outcome definitions [21].**

Long-term success was achieved in all 26 MDR patients 2 years after initial diagnosis, and 22 had success after censoring during a mean passive follow-up of 9.3 years (II). Long-term MDR-TB treatment success has ranged from 60-75% in the Netherlands, UK, Canada and the USA [50,51,53-55]. Again methodologies differed, see table 3. In a recent meta-analysis, definitions were homogenised and short-term outcome has been named "end of treatment" and long-term "follow-up outcomes" [56]. Only few studies on treatment outcomes in HIV-infected MDR-TB patients have been reported in high-income countries. In the USA, a 59% short-term treatment success was reported in HIV-infected patients with a long-term success of 50% and a 50% mortality rate [57]. However, patients were few and most were severely immunosuppressed. Recently, a large meta-analysis showed that the presence of HIV was not associated with a worse MDR-TB outcome, however the level of immune status was not stated [56]. In our study, only 7% of the MDR-TB cases were HIV-infected and received antiretroviral treatment (II). We found a high treatment success, despite the slow sputum culture conversion occurring >2 months after treatment initiation. Slower sputum culture conversion has been observed for MDR-TB compared to drug-susceptible TB [58]. This was possibly due to lower effectiveness of the second-line drug therapy and the initial use of drugs to which the isolate was resistant. In comparison to MDR-TB, XDR-TB has been associated with lower treatment responses, long-term outcomes and survival rates, particularly in HIV co-infected patients [59-61].

Isoniazid resistance has previously been found to be a predictor of poor outcome [48,62]. Similarly for H, a high successful short-term treatment outcome of 80% was found for 110 patients with available data (III). Our short-term outcome finding for H resistance was similar to the 80-87% treatment success that has been documented for all notified cases in DK [1,63]. Successful long-term treatment outcome was achieved in 95% of 97 patients during a mean passive follow-up period of 4.4 years (III). True relapse was only observed in 1% of H-resistant cases (III). Similar to a study conducted in the USA, a high treatment success comparable to that of susceptible TB was found [64].

Recent surveys indicate that the global rate for treatment success of new smear-positive cases was 86% and thus the goal target of 85% was reached, whereas for MDR-TB overall treatment success was lower at 60% and therefore continued reporting of treatment outcomes is of great importance, especially for MDR-TB [3,44]. Larger global meta-analysis studies have confirmed these findings with 62% successful MDR-TB outcomes [56,65].

Treatment of multidrug-resistant and isoniazid-resistant tuberculosis

Denmark has an individualised treatment approach when designing MDR-TB and H-resistant TB treatment regimens, where therapy adjustments are made according to DR patterns and information on prior treatment. Fluoroquinolone´s have been documented to improve treatment outcome and together with the injectable drug Am have been favoured for use against MDR-TB [19,50]. The exact number of drugs necessary to treat MDR-TB is unknown. When MDR is suspected, a minimum of five or six drugs are suggested to be used initially including H, R, Z, E, a Q, and injectable agents such as streptomycin, capreomycin, or Am [66]. However, when full DST is known, a minimum of four drugs is appropriate including an aminoglycoside or capreomycin, a Q, ethionamide, cycloserine and p-aminosalicylic acid [66]. Recently, WHO guidelines suggest the use of at least four drugs with either certain or almost certain effectiveness [45]. Four MDR-TB patients in our study were treated with H, despite H resistance, as it was believed that a subpopulation of the bacteria remained susceptible. In one study, a high-dose H regimen (16-18 mg/kg) has been shown to improve sputum culture conversion and chest radiography in MDR-TB patients [67]. However, high-dose H contribution to the efficacy of a MDR regimen is still unclear [67,68].

Treatment of H-resistant TB in DK was either modified standard daily therapy with 3RE(H)Z/3-6RE(Z) or REZ supplemented with a Q. The WHO and ATS/CDC guidelines recommend 6 months treatment with REZ throughout, and the WHO suggests the regimen may be extended to 9 months, and that for patients with extensive disease an additional Q may strengthen the regimen [17,21]. Recent evidence supports the use of the newer Q´s, such as moxifloxacin opposed to the older Q´s and may be equally efficacious to H when substituted in the initial 2-month phase of standard therapy of susceptible TB with REZ [69,70]. The role of supplementary Q´s for the treatment of H-resistant TB still remains to be determined and the most effective regimen is still not known. The critical concentration of a drug is defined as the lowest concentration that inhibits the growth of 95% WT susceptible strains and allows growth of all resistant strains from patients not responding to therapy [71]. For H, the Clinical and Laboratory Standards Institute (CLSI) suggests testing at an additional higher concentration than the critical concentration that may provide information about the level of resistance [71]. Some experts recommend using H in the presence of low-level (≤0.1 mg/l) H resistance, however there are no randomised trials to provide the basis for this recommendation [71]. We did not find that treatment success was correlated to a high (≥0.4 mg/l) or low-level H resistance concentration, and it appears that the advantage of

Table 4

Performance of the GenoType and the GenoType*plus* assays in clinical respiratory and nonrespiratory specimens (IV, V).

			No. of specimens with interpretable DNA-strip reading/no. of specimens tested (%)			
			GT assay		GTplus assay	
Smear[a]	*M. tuberculosis* culture[b]	SDA[c]	Respiratory	Nonrespiratory	Respiratory	Nonrespiratory
>2+	+	ND	32/32 (100)	13/14 (93)	35/35 (100)	2/2 (100)
1+	+	ND	4/6 (67)	2/4 (50)	7/7 (100)	4/4 (100)
0	+	ND	2/14 (14)	3/8 (38)	3/9 (33)	3/4 (75)
0	+	+	0/5	1/3 (33)	-	-
0	+	÷	0/1	0/3	-	-
0	÷	+	2/6 (33)	0/2	2/2 (100)	6/6 (100)
0	÷	÷	0/6	0/2	0/8	0/4
All positive (%)			36/38 (95)	15/18 (83)	42/42 (100)	6/6 (100)
All negative (%)			4/32 (13)	4/18 (22)	5/19 (26)	9/14 (64)
Total (%)			40/70 (57)	19/36 (53)	47/61 (77)	15/20 (75)

a) Smear grade for acid-fast bacilli; b) + = positive; ÷ = negative; c) SDA = strand displacement amplification; ND = not done.

DST at an additional higher concentration of H is less clear (III). Possibly, modified standard treatment of H-resistant TB is sufficient to yield treatment success. The mean treatment duration for successfully treated patients was prolonged (8.6 months), which may reflect NTP guidelines and different expert treatment practises [30]. Recently, a standardised policy on second-line DST has been approved and therefore improved treatment outcomes may be expected in the future [72].

The implementation of routine directly observed therapy (DOT) in DK may lead to a further reduction in relapse and the frequency of DR, especially in "default" recurrent cases [73]. Directly observed therapy may however be difficult in "default" patients due to other factors such as homelessness and alcohol abuse. For MDR-TB patients, treatment for longer than 18 months and DOT throughout has been shown to improve outcome [65]. The WHO encourages a minimum of 18 months MDR-TB treatment after culture conversion and DOT throughout treatment [21]. However, the use of DOT in DK may be debatable as studies were heterogeneous both from high- and low-income settings [65].

Treatment outcome study limitations

In the observational studies performed in this thesis, treatment data was collected retrospectively from patient files and surveillance data, which are subject to differences in recording and inaccuracy of data. The RFLP-genotype method cannot always distinguish a true relapse from reinfection, especially if specific strains occur more frequently. Patients with identical RFLP-genotypes during initial and recurrent episodes may have been re-exposed and reinfected by a strain with the same RFLP-genotype rather than having a relapse. The assumption of treatment success for the whole cohort in our analysis may slightly have underestimated the true recurrent TB rate. Patients with invalid civil status were excluded from the recurrent analysis, as we were unable to follow these patients, and this may have led to a slight underestimation of the rate of recurrency. Estimations of reinfection rates may be affected by differences in *M. tuberculosis* strain virulence factors, or behavioural differences such as travels abroad, or residing in sub-communities leading to higher exposure and thereby increased risk of reinfection. Possibly, some of our apparent reinfection strains were due to initial mixed infections, where relapse of a "minor" strain occurred in the recurrent episode. However, we would expect a low-burden setting to yield a reduced mixed infection rate. As DK is a low TB incidence country, only few patients were included in the MDR-TB and H-resistant treatment outcome studies, which may limit conclusions drawn from these studies. Given the nationwide centralized laboratory system and long observation periods, we believe that our findings are highly representative for recurrent TB and treatment outcome of MDR-TB and H-resistant TB in a low-burden setting.

MOLECULAR DETECTION OF DRUG RESISTANCE

Molecular DST relies on identifying mutations in the *M. tuberculosis* genome conferring resistance. Several molecular methods including real-time polymerase chain reaction (PCR) have been developed for DR detection. Sequencing and hybridization-based methods directly detect precise mutations, whereas PCR-based electrophoresis methods indirectly detect the presence of mutations without characterising the exact nucleotide change involved.

MULTIPLEX PCR HYBRIDIZATION METHODS

Simultaneous detection of mutations in the rpoB and katG genes

Our study was the first to optimize the first version of the GT multiplex PCR membrane hybridization assay to identify R and H resistance conferring mutations in both the *rpoB* and codon 315 of the *katG* genes directly in pretreated clinical specimens as opposed to conventional DST after culture (IV). Several studies have shown that the GT assay had a high performance in isolates, detecting 92-100% and 67-89% of the *M. tuberculosis* strains resistant to R and H, respectively [74-76]. Evaluation of the optimized GT assay directly on smear-positive pretreated clinical specimens, revealed interpretable results in 95% of 38 respiratory and 83% of 18 non-respiratory specimens (IV). A 95% concordance between GT results and conventional DST was found, and all specimens with MDR *M. tuberculosis* were identified (IV). These findings were later confirmed by other studies. A German study, tested the GT assay directly in 42 smear-positive sputum specimens and found 100% concordance with conventional DST [77]. In a study conducted in the USA, the GT was applied directly on 143 smear-positive specimens, and a sensitivity of 94% for *katG* and 91% for *rpoB* was achieved [78]. In the same study, the assay was shown to correctly identify H resistance in 84% of specimens with high-level H resistance and 96% of specimens with R resistance [78]. Our optimized protocol for use directly in clinical specimens was used in the manufacturer's protocol and the "Foundation for Innovative Diagnostics" incorporated our GT results into the WHO policy on the use of molecular tests (IV) [79,80].

Table 5

GenoType*plus* assay versus conventional rifampin DST inall isolates (V).

Isolates	GT*plus* result	Conventional DST		Total
	Mutation/absent WT	R^r	R^s	
All isolates	***rpoB* gene**	**21**	1	22
	R^s	-	93	93
Total		**21**	94	115

R = rifampin; r = resistance; s = susceptible; WT = wild-type;
DST= drug-susceptibility testing; R drugtest concentration = 2 mg/l.

Table 6

GenoType*plus* assay versus conventional high- and low-level isoniazid DST in all isolates (V).

Islates	Gt*plus* result	Conventional DST			Total
	Mutation/absent WT	H^r high-level	H^r low-level	H^s	
All isolates	*katG* gene	**31**	-	-	31
	inhA gene	-	24		24
	H^s	-	4	56	60
Total		**31**	28	56	115

H = isoniazid; r = resistance; s = susceptible; WT = wild-type; DST= drug-susceptibility testing; drug test concentrations; high-level= 0.4 mg/l; low-level = 0.1 mg/l.

The original line probe assay (LPA) INNO-LiPA Rif.TB® (Innogenetics, Ghent, Belgium) only covered the most frequent R resistance mutations in the *rpoB* gene. A meta-analysis showed an overall sensitivity and specificity of 95% and 100% when applied on isolates, respectively [81]. Two studies compared the GT with INNO-LiPA Rif.TB and found a high concordance for R [82,83]. The main advantage of the GT assay compared to INNO-LiPA Rif.TB is that the assay also detects H resistance.

Simultaneous detection of mutations in the rpoB, katG and inhA genes

The second-generation extended GT*plus* assay that covers *rpoB* mutations responsible for R resistance, *katG* codon 315 mutations and additionally *inhA* mutations responsible for H resistance revealed interpretable results in all 115 isolates tested (V). Overall, GT*plus* results were 96% concordant with conventional DST in isolates (V). The GT*plus* identified all 21 MDR-TB isolates and overall detected 98% of the 56 susceptible isolates. The *rpoB* S531L mutation was found to be the most prevalent in 86% of Lithuanian isolates, and has been shown to be the most frequent worldwide (V) [24]. The GT*plus* assay identified the *katG* S315T1 mutation in all high-level H-resistant isolates from Lithuania and was fully concordant with conventional DST. Of these, 12% additionally had the *inhA* C15T mutation. Of the 28 low-level H-resistant isolates a correlation of 86% was found between GT*plus* and conventional DST indicating that the new GT*plus* detects low-level H resistance effectively, although additional mutations in other genes may also cause low-level resistance (V).

A systematic meta-analysis of the GT assays with 14 R and 15 H comparisons to conventional DST including our first GT study, was conducted (IV) [84]. Compared to conventional DST, the GT assays revealed excellent accuracy with a pooled sensitivity of 98.1% (95% confidence interval (CI) 95.9-99.1) and a pooled specificity of 98.7% (95% CI 97.3-99.4) for detecting R resistance in isolates or directly from clinical specimens. The accuracy for H was variable with a lower pooled sensitivity of 84.3% (95% CI 76.6-89.8) and specificity of 99.5% (95% CI 97.5-99.9). Isoniazid sensitivity increased to nearly 90% when mutations in the *inhA* gene were included [84]. More recent evaluations of the GT*plus* assay in strains confirmed these results with a 98-100% detection rate for R resistance and 79-86% for H resistance in studies conducted in France and Spain [85-87]. A large Chinese study that included 242 MDR strains similarly found a sensitivity of 96% for R, 82% for H, and 79% for MDR detection and a 100% specificity [88].

When applying the GT*plus* assay directly on 48 smear-positive clinical specimens using the optimized protocol, interpretable results were achieved in all specimens tested (V). However, interpretable results were only obtained in 42% of smear-negative specimens (V). Likewise, 100% interpretable results in smear-positive specimens and only 46% in smear-negative specimens was found in a study performed in Spain on 65 specimens [86]. In a German study, that included 72 smear-positive specimens, interpretable results were obtained in 99% similarly to our study. In this study, R was correctly detected in 97% of 31 MDR specimens and 90% of the 41 H-resistant specimens [89]. A large study on 536 smear-positive specimens in South Africa found valid interpretable results in 97% of specimens [90]. Sensitivities, specificities, positive predictive value (PPV)´s and negative predictive value (NPV)´s were ≥98% for R and H resistance and MDR detection, except the sensitivity for H resistance was lower at 94% compared to conventional DST [90]. The study also included 100 smear-negative specimens and found interpretable results in 16 of the 20 (80%) specimens that were culture-positive but none of the culture-negative specimens [90]. The high detection rate in the smear-negative samples was higher than our findings. This may reflect differences in smear microscopy skills. Recently, a study performed in the Cape region of South Africa found a somewhat lower sensitivity for MDR and H-monoresistant strains at 92% and 56%, respectively [91]. The presence of local resistance mutation patterns not covered by the assay may explain these findings [91]. The GT*plus* assay has been evaluated in 168 smear-positive sputum specimens in Russia, where ≥96% sensitivities and specificities of 91%, 83% and 90% for the resistance detection of R, H and MDR-TB were found, respectively [92]. After the recent WHO approval of the LPA to screen smear-positive samples in low-income settings, the GT*plus* assay was evaluated in Uganda after minimal training [93]. An extraordinary performance with interpretable results in 95.8% of smear-positive specimens from 118 previously treated patients was achieved [93]. For R, the sensitivity, specificity, PPV and NPV´s were 100%, 96%, 83% and 100%; for H the same parameters were 81%, 100%, 100% and 93% and for the detection of MDR-TB; 92%, 96%, 80% and 99% [93]. Results in this study were found to be highly reproducible [93]. Overall sensitivity for R was high and for H slightly lower and comparable to those found in our study (V).

Studies comparing the GT with the GT*plus* assay have shown improved sensitivity for H [84,85,89]. Overall, we found that the inclusion of the *inhA* probes for H resistance in the new GT*plus* version improved the ability of the assay to detect H resistance (V).

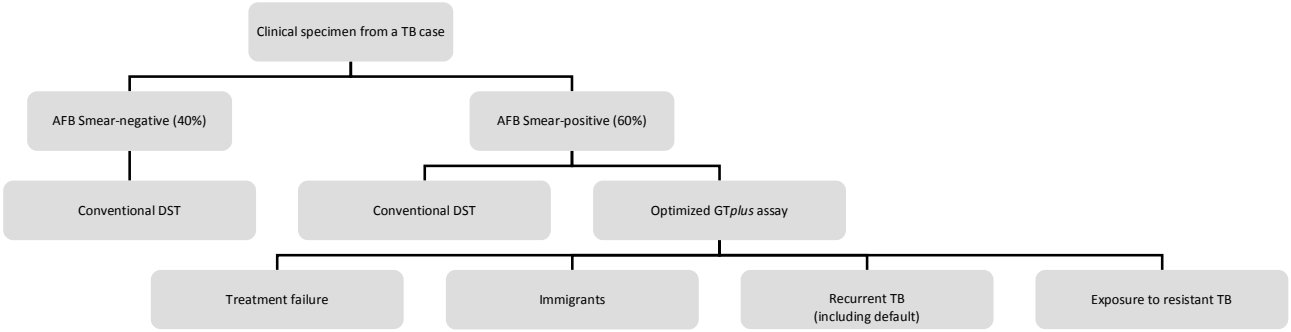

Figure 3
Proposed algorithm for the use of the optimized Genotype*plus* assay for the rapid detection of rifampin (*rpoB*), isoniazid (*katG/inhA*) gene mutations in patients with tuberculosis in Denmark. AFB: acid-fast bacilli smear microscopy.

Resistance conferring mutations in the rpoB, katG and inhA genes and treatment outcome

Applying the GT*plus* to subcultured isolates from patients with MDR-TB and H-resistant TB from the treatment outcome studies revealed that for all MDR-TB isolates tested, the GT*plus* assay correctly detected R resistance in the *rpoB* gene (II, III). As most R-resistant isolates are also resistant to H, R resistance can be used as a reliable surrogate marker for MDR [24]. Isoniazid resistance in the *katG* and *inhA* genes was correctly determined in 82% of the MDR- TB isolates tested (II). This emphasizes the effectiveness of rapid detection of MDR-TB conferring mutations directly in smear-positive specimens (V). Recently, the WHO has incorporated rapid molecular DR testing for use in smear-positive patients in the guidelines for programmatic management of drug-resistant TB and treatment guidelines [21,45]. Several advantages of rapid molecular testing include screening of patients at risk for MDR-TB, earlier identification of patients on inadequate regimens and the prompt ability to isolate MDR-TB patients. To our knowledge no previous studies have correlated MDR-TB treatment outcome with mutation analysis findings.

Overall, for H resistance the GT*plus* would have identified 94% of the 111 H-resistant strains (III). The *katG* S315T1 mutation correctly identified high-level H resistance in 84% of the strains, whereas 14% of the high-level strains had the *inhA* C15T mutation, which normally confers low-level H resistance (III). However, the *inhA* C15T mutation was correctly identified in 84% of the low- level H-resistant strains (III). Thus, *inhA* C15T mutations may also result in high-level H resistance or the strains had other mutations located elsewhere in the H resistance coding genes. The presence of a mutation may therefore not always be able to distinguish phenotypic high- or low-level resistance. This has been confirmed in one study [86]. For MDR-TB or H-resistant cases, we did not find particular mutations associated with poor treatment outcomes, although the number of patients studied was few (II, III). Utilization of the rapid mutation analysis may have reduced the spread of disease in the smear-positive pulmonary patients by direct analysis in the sputum specimens. We found that the mutation analysis may be a supplementary tool to distinguish whether transmission clustering occurred with the same strain. Our study emphasizes the need for increased use of rapid mutation analysis in smear-positive cases from TB endemic countries, from patients with a prior history of TB or relapse, and contacts of TB-cases with known R and/or H resistance. A proposed algorithm for use of the GT*plus* assay in clinical specimens is shown in figure 3.

COMPARISON TO OTHER MOLECULAR DRUG RESISTANCE PCR-BASED METHODS

DNA microarray (Biochip)

Although technically a hybridization assay, microarrays, also known as biochips, have been developed to detect DR in *M. tuberculosis*. They are based on the hybridization of DNA from clinical samples to immobilised probes, such as miniature glass, and comparison of fluorescence intensities between mutation and WT binding. The biochips enable massive parallel molecular analyses to be carried out in a miniaturized format with a very high throughput. Commercial biochips are available for R and H DST [94-97]. The Biochips™ detected >95% R resistance and almost 80% H resistance in 220 *M. tuberculosis* isolates within 12 hours [94]. In the same study, an almost similar performance was found when the Biochips™ was applied directly on 131 clinical specimens [94]. In studies, the Combichip™ Mycobacteria DR chip has found sensitivities ranging from 93-100% for R (*rpoB*) and 71-84% for H resistance (*katG*, *inhA*) detection, and specificities of 95-98% for R and 98-100% for H compared to phenotypic DST [96,97]. Although promising, high costs, interpretation difficulties, and risk of contamination during handling are limitations of this assay.

Sequencing

DNA sequencing gives the most accurate information and allows differentiation of mixed WT and mutant strains, and information on silent mutations. Pyrosequencing technology involving real-time sequencing has accurately found mutations in the *rpoB* gene [98]. Pyrosequencing has recently been combined with a rapid *M. tuberculosis* nucleic acid amplification (NAA) test for detection in clinical specimens and one study has demonstrated a sensitivity of 95% in respiratory and 89% in non-respiratory specimens and specificities of >99.9% for the detection of R resistance [99]. However, sequencing is time consuming and expensive as several reactions for several drugs for each specimen would be required.

REAL-TIME PCR METHODS

Molecular beacon

The molecular beacons are hybridization probes that emit fluorescence when hybridized to their target. The molecular beacons can

discriminate targets differing by a single nucleotide. The semi-nested real-time PCR method was developed to target R resistance in the *rpoB* gene and H resistance in the *katG*, *inhA* and *ahpC* genes [100]. In strains, the overall sensitivity and specificity of the assay compared to conventional DST were 98% and 100% for R resistance, and 85% and 100% for H resistance, respectively [100]. In a similar study, sensitivity and specificity was 98% and 100% for R resistance, and 83% and 100% for H resistance (*katG* and *inhA* genes), respectively [101]. The assay was found applicable in a few smear-positive samples. The molecular beacon has recently been commercialized for the simultaneous detection of *M. tuberculosis* NAA and R resistance including an internal control in an automated closed single tube system [102,103]. The Cepheid GeneXpert® MTB/RIF assay has shown 98-100% sensitivities for smear-positive samples and 72-85% for smear-negative culture-positive samples [103]. Specificity was 100% for both smear-positive and smear-negative samples [103]. The molecular beacon has promising aspects, although is disadvantaged by expenses and the lack of ability to simultaneously detect H resistance.

TaqMan probes

Similarly to the molecular beacon technique, the TaqMan probes releases fluorescence when annealing the amplified target that is measured by real-time. The absence of fluorescence indicates a mismatch and the presence of a mutation as probe binding is impaired. The TaqMan probe assay has been applied directly on pretreated clinical specimens for the detection of mutations for R resistance in the *rpoB* gene and H resistance in the *katG* and *inhA* genes. Sensitivities ranging from 95-99% in smear-positive samples and from 30-35% in smear-negative samples were found [104]. Likewise, the TaqMan minor groove binder probe (*rpoB*, *katG* and *embB* arabinosyl transferase gene) has been found to have a high sensitivity when tested in 45 isolates and directly in 27 clinical specimens, of which 9 were AFB smear-positive. However, additional nested PCR testing was necessary in 11 specimens [105]. For the *katG* gene codon 315, overall sensitivity was found to be 75% in 30 of the 40 samples tested [106].

Fluorescence resonance energy transfer probes

Fluorescence resonance energy transfer (FRET) is a real-time PCR technique that utilizes a sensor probe that is designed to bind the mutation and that allows the adjacent anchor probe to emit fluorescence during the annealing step of PCR. The technique relies on the measurement of the melting temperature (Tm) of the (deoxy) ribonucleic acids at which the sensor probe detaches from the complementary DNA strand that differs in the presence of a mutation compared to WT. The FRET probe was developed to detect specific mutations in the *rpoB*, *katG* and the *inhA* genes [107,108]. A simplified assay to simultaneously detect multiple R and H resistance mutations in a single tube was developed [109]. In 100 H-resistant and 50 susceptible strains the FRET probes for *katG*, *inhA* and *ahpC* genes had a sensitivity of 76% and specificity of 100% compared to conventional DST [110]. Similarly to our findings the FRET probes have been shown to detect R and H mutations directly in clinical specimens and have found high sensitivities of 98% for *rpoB*, 97% for *katG* and 98% for *inhA*, and specificities of 100% in comparison to conventional DST, when applied directly in 205 smear-positive specimens [111,112].

Single-stranded conformation polymorphism

Single-stranded conformation polymorphism (SSCP) is based on the ability of DNA to undergo a conformational change when a single nucleotide is altered resulting in a different electrophoretic migration in comparison to WT fragments. The SSCP has been developed for indirect detection of R and H mutations in the *rpoB*, *katG*, *inhA* and *ahpC* genes [28,113]. Compared to phenotypic DST, sensitivities have varied from 82->96% and 80-87% for R and H when tested in clinical isolates, respectively [28,114]. The specificity was 92-100% and 100% for R and H, respectively [28,114]. The advantage of the SSCP is that it is rapid, easy to perform and inexpensive. Limitations encountered with the assay are difficult interpretation, false detection of nontuberculous mycobacteria (NTM) and silent mutations. A major drawback with the SSCP was failure to discriminate the most frequent R mutation from susceptible WT strains in one report [115].

Mismatches in heteroduplexes temperature gradient high performance liquid chromatography analysis

In this method, target DNA from PCR amplification is mixed with DNA from a reference drug-susceptible strain and denaturation produces double stranded hybrid DNA. When a mutation is present, a mismatch occurs between the two strands and mobility will differ compared to the susceptible reference fragments during electrophoresis. Heteroduplexes have for R resistance detection shown promising results, however only a few studies have been performed, and the same limitations may be encountered as for the other electrophoresis assays [116]. High performance liquid chromotography has for R shown varying sensitivity and specificity and is expensive to perform.

Advantages of the GTplus assay compared to other drug resistance PCR-based methods

The GT*plus* assay is rapid, easy to perform with minimal training requirements and can be performed both automated and manually. In general, visual interpretation is easy with clear band intensities on the DNA strips. The assay includes control bands for *M. tuberculosis* and locus control bands for *rpoB*, *katG* and *inhA*. The biochips, although promising are still expensive to perform. The GT*plus* assay has recently become affordable in countries with limited resources. Throughput has recently been improved by the availability of automated strip reading. Similar to our findings, other rapid PCR-based assays utilizing TaqMan, Molecular Beacons and FRET probes have demonstrated high sensitivities in AFB smear-positive specimens [102,104,105,111,112].

The GT*plus* assay has the advantage as it relies on visual interpretation of bands and not real-time PCR curve analysis or melting temperatures as used in the molecular beacon, TaqMan probes and FRET probes. Furthermore, nested PCR which increases the risk of cross-contamination is not necessary. Sequencing is still too expensive and would be difficult to implement routinely as several reactions are required for each drug tested. Although pyrosequencing and the molecular beacons have recently been developed to detect R resistance in a single tube simultaneously with *M. tuberculosis* NAA detection and thereby increasing the detection in smear-negative samples considerably, they are only designed to detect R resistance, where the GT*plus* assay detects both R and H resistance (V) [99,103].

PHENOTYPIC DRUG-SUSCEPTIBILITY TESTING

BACTEC and MGIT

Conventional phenotypic DST methods detect growth or metabolism of *M. tuberculosis* in vials containing anti-TB drugs compared to controls without drugs. The rapid proportion methods in liquid medium are preferred to the slower solid Löwenstein Jensen or agar media. The semi-automated radiometric BACTEC system that has shown a high performance and been used exten-

sively for DST of first-line drugs has the disadvantage of utilizing radioactivity and syringes in the process [117]. The non-radioactive commercial broth-based liquid medium method, the MGIT system has replaced BACTEC, and is recommended by the WHO for first- and second-line DST [72]. The DST turnover time of BACTEC and MGIT is approximately 4-6 weeks. Several studies have evaluated the MGIT for DST and found a good performance [72]. However, it has been shown that low-level R resistance linked to specific *rpoB* mutations, may be missed by standard broth-based methods such as MGIT and BACTEC [118]. The critical concentration of some drugs such as E is close to the minimal inhibitory concentration for WT susceptible strains, therefore DST may yield poorly reproducible results and further standardisation is required [119]. Furthermore, limitations of the MGIT are contamination of NTM or other microorganisms [120].

MODS assay

An alternative to the rapid liquid-based DST methods is the "in house" microscopic observation broth drug-susceptibility assay (MODS) that relies on microscopic observation of *M. tuberculosis* growth in plates with or without anti-TB drugs. For R and H the MODS assay has been evaluated with good results within approximately 22 days, however for E, and streptomycin concordance was low [121]. Although cheap, the main disadvantage of the MODS assay is misidentification of *M. tuberculosis* with NTM during microscopic growth evaluation and technical requirements to prevent cross-contamination and biosafety issues.

Slide DST and Microcolony methods

Another alternative is the slide DST method that depends on microscopy of microcolonies after incubation in a liquid medium for 10 days. The slide DST method has been found to be highly predictive of MDR-TB and has the advantage compared to the MODS assay in requiring less handling in closed instead of open plates [122]. The slide DST method is however limited to application in smear-positive sputum samples and has only been tested for R, kanamycin and a Q. The microcolony method or thin layer agar method performed on solid media has been evaluated for R and H in a small study [123]. Again the limitations of microscopy, differentiation from NTM and lack of studies on accuracy are disadvantages of its use.

Colorimetric redox indicator and Nitrate reductase methods

The colorimetric redox indicator methods rely on oxygen consumption by *M. tuberculosis* growth with or without anti-TB drugs that produce a change in colour of the oxygen reduction indicator that may be visually interpreted in a microtiter plate. High accuracy has been shown with the same turnover time as MGIT [124]. However, the method is hampered by biosafety concerns. The nitrate reductase assay is based on the ability of *M. tuberculosis* to reduce nitrate to nitrite creating a colour change that may be performed in a solid medium. Good concordance has been observed for the first-line drugs and Q [125,126]. However, the assay is limited as it cannot detect *M. tuberculosis* strains that lack nitrate reductase.

Mycobacteriophage DST methods

Mycobacteriophage DST methods depend on specific mycobacteriophages that infect and replicate in mycobacteria. The commercial FastPlaque (Biotec Labs ltd, Ipswich, UK) was developed to detect R resistance in *M. tuberculosis* by detecting lytic phages in a plate of rapidly growing NTM that may be visually interpreted within 2 days. The assay has been evaluated in isolates showing a high sensitivity, but variable and low specificity [127]. In a few studies, mycobacteriophage based assays for R resistance detection have also shown potential for direct application in sputum samples [128-130]. The method is limited by high contamination rates, false resistance and indeterminate results and lacking accuracy when applied directly in sputum specimens.

Advantages of the molecular methods compared to phenotypic methods

Molecular methods can reduce the time required for detection of DR to 1-2 days, taking advantage of the speed of NAA, while conventional culture DST methods provide results in 4-6 weeks. Compared to phenotypic methods molecular methods require biosafety conditions only during specimen processing and DNA extraction, which renders them non-infectious. However, following this step, handling is eased as NAA and hybridization laboratory facilities are necessary. These techniques therefore offer the possibility of DST even in low-income settings. Molecular methods, such as the GT*plus* assay may provide specific mutation information in comparison to phenotypic DST. Isolates that contain nonviable *M. tuberculosis* bacteria, are contaminated with other microorganisms or mixed with other NTM may yield results with the molecular methods, whereas conventional DST does not. Furthermore, detection of precise *rpoB* mutations by the GT*plus* allows for the choice of a rifamycin drug based on the correlation between mutations and phenotypic DST [131]. Mutations in codon 526 and 531 have shown cross-resistance between R and rifabutin, whereas mutations in codon 516 are correlated with rifabutin susceptibility [131].

Limitations of molecular drug resistance detection

Phenotypic DST relies on the detection of >1% DR present in the bacterial population. As the GT*plus* assay is unable to determine the proportion of resistant bacteria, and is limited to the detection of R and H resistance mutations in smear-positive specimens, the assay cannot in its present format replace phenotypic DST, which remains the golden standard. However, quantitative analyses may become available in the future. The molecular DST tests, as they are performed today, are valuable supplements to physicians, contributing to the assessment of the overall clinical picture and monitoring of the patient.

The mutations that cause resistance are not well characterised for some drugs, which is why phenotypic resistance is not always accompanied by a molecular mutation, and some mutations even in the structural target genes do not necessarily lead to phenotypic resistance. The *rpoB*, *katG* and *inhA* genes do not account for all R and H resistance and relevant mutations may be located elsewhere. In two H high-level resistant specimens the *katG* WT was absent, however, no *katG* mutations were detected (V). This was most probably due to the *katG* mutation being located elsewhere in the gene. One of our R phenotypic susceptible strains had an absent *rpoB* WT band with no *rpoB* mutations by GT*plus*, revealed an *rpoB* L533M mutation by sequencing (V). This rarely reported mutation did not result in a phenotypic DST change, and it remains to be determined whether the *rpoB* L533M mutation is associated with R resistance [132].

Likewise, for H-resistance the GT*plus* assay revealed false susceptibility in four low-level H-resistant strains, where sequencing revealed a previously uncharacterised point mutation in position -49 (G→A) in the *oxy-ahpC* region (V). Three of the isolates either had a weak or no amplification of the C-terminal region of the *katG* 463 gene (V). The GT*plus* assay cannot always distinguish high-level or low-level H resistance. In our study, there were four

clinical specimens that had high-level H resistance with the *inhA* mutation C15T, which normally confers low-level H resistance, confirmed by sequencing (V). Likewise, 14% of the high-level strains in the H-treatment outcome study had the *inhA* C15T mutation (III). The frequency and distribution of resistance conferring mutations varies geographically. In order to broaden the knowledge of global DR mutations and phenotypic results, a free access database, where sequences may be deposited, has been established [133].

The main risk of the molecular assays is cross-contamination which may be prevented by separating work areas, improved technique and the inclusion of internal controls. Limitations may be the presence of PCR inhibitors and the low sensitivity in smear-negative samples. For the GT*plus* assay this may lead to weak band intensities of controls, WT and mutation probes resulting in interpretation difficulties. Molecular DST can yield false susceptible results with incomplete coverage of phenotypic resistance with known mutations. Sources of molecular DST error are "heteroresistance" i.e. mixtures of WT and emerging mutant resistant strains, the presence of multiple mutations and the presence of silent mutations that have nucleotide changes that do not result in amino-acid changes and hence phenotypic resistance. One isolate carried the *katG* S315T1 mutation, however WT was not absent, possibly indicating a mixture of H-resistant and susceptible bacteria (V).

CONCLUSIONS AND PERSPECTIVES

In the studies of the present thesis, treatment outcome with focus on recurrent TB, MDR-TB and H-resistant TB was addressed in DK. We found a low rate of recurrent TB of 1.8%, during 13.5 years of passive follow-up. To our knowledge, this is the largest study performed with the longest observation period, in a low-burden setting. Relapse accounted for 1.3% of the recurrent TB cases. The risk of relapse in a low-burden country is an indicator of the efficacy of the NTP in that region. The initial phase of standard treatment of susceptible TB has in 2008 been shortened to 2-months instead of 3-months to conform with international WHO/CDC guidelines, and a future observational study on the treatment efficacy may be useful to compare with relapse rates found in the present study [4,17,30]. The definition of recurrent TB remains controversial with regard to time interval between episodes and treatment achievement. Our recurrent study seeks international consensus on the definitions of recurrent TB, relapse and reinfection. An increased hazard rate of relapse was observed up to four years after the initial episode. This may indicate the need for individualized prolonged monitoring after treatment. Resistance was observed in 21% of the first TB episodes of those that developed recurrent TB. Although debatable, the implementation of routine DOT for selected cases, may be a step further towards preventing the development of recurrent TB, drug-resistant TB and control of TB in DK. Comparing selected characteristics between the two subgroups relapse and reinfection revealed that only the presence of cavernous disease was associated with relapse. We found that patients with initial cavernous disease may have higher risk of relapse and need prolonged treatment and monitoring. Randomised clinical trials on therapy and duration for patients with cavitary disease are warranted in the future. Recurrent TB was due to reinfection in 0.5% of cases and thus rare, however the cumlative risk of reinfection increased slightly with time.

Overall, we confirmed that the combined incidence of H resistance was 6.7% during 2002-2007 and the MDR-TB incidence was low in DK at 0.5% during 1992-2007. Active transmission of H-resistant TB or MDR-TB was rare. For MDR-TB patients, successful short-term and long-term treatment outcome was high in 89% and 100%, of the cases. Likewise, a high successful short-term and long-term treatment outcome for H-resistant TB-cases was achieved in 80% and 95%, respectively. MDR-TB and H-resistant TB has a good prognosis in DK. High- and low-level H resistance did not affect treatment outcome of modified standard treatment.

This study was the first to optimize the multiplex PCR reverse hybridization commercial GT LPA assay that simultaneously detects the most important *rpoB* and *katG* gene codon 315 mutations conferring R and high-level H resistance in *M. tuberculosis* for application directly in pretreated clinical specimens as opposed to being performed on culture. The optimized GT was directly applicable in smear-positive specimens. The assay was easy to perform and allowed rapid (<48 h) detection of MDR-TB and was predicted to have a major impact on the future management of DR TB.

The second-generation GT*plus* assay that more broadly covers R (*rpoB*) resistance and high-level H (*katG*) resistance mutation detection was extended to include detection of low-level H resistance in the *inhA* gene. GT*plus* assay evaluation in 115 *M. tuberculosis* isolates and directly in 81 pretreated specimens revealed a high concordance with conventional DST. The GT*plus* assay is rapid (<48 hours), easy to perform, for the simultaneous detection of R and high- or low-level H resistance in *M. tuberculosis* isolates and clinical specimens obtained from patients suspected of having TB. We proposed that in well established laboratories with *M. tuberculosis* DST, the GT*plus* assay may supplement the golden standard conventional DST in obtaining a rapid preliminary DST result, as not all clinically relevant H mutations are covered by the assay. However, in countries that have yet to implement DST, the GT*plus* may be a valuable tool for the detection of MDR-TB until conventional DST facilities become available. The assay may be applied to smear-positive samples from patients suspected of treatment failure, recurrent TB, drug-resistant TB exposure or originating from countries with high levels of DR. We predicted that the new extended GT*plus* version may be a useful tool to combat and prevent new cases of MDR-TB and XDR-TB.

The performance of the GT*plus* assay on sub-cultured MDR and H-resistant *M. tuberculosis* isolates from the treatment outcome studies were evaluated. Rifampin being a surrogate marker of MDR-TB, the GT*plus* assay would have identified all the MDR-TB cases in DK. Mutations in *rpoB* and *katG*/*inhA* genes correctly determined resistance in 100% and 82% of all isolates tested. In my opinion this emphasizes the importance of strengthening the NTP with the inclusion of rapid methods to detect MDR-TB resistance mutations to ensure effective treatment, improve the standard of care and prevent the emergence of more cases. Overall, the GT*plus* analysis identified 94% of H resistance in 111 strains. The *katG* S315T1 and *inhA* C15T mutations correctly identified high- and low-level H resistance in 84% and 84% of the strains, respectively. Isoniazid-resistant TB may be treated with modified standard treatment effectively, therefore the impact of rapid mutation analysis on treatment outcome remains to be determined. In the future, the mutation analysis may assist to establish whether the therapy recommendations by the WHO and CDC may be shortened and improved, and whether H DST at high- and low-levels as recommended by the CLSI is necessary. Globally, rapid detection of mutations conferring H resistance and continued treatment outcome monitoring may prevent transmission and MDR-TB development.

Overall, the sensitivity and specificity of the GT*plus* assay for R is sufficiently high such that both resistant and susceptible results can be used. However, for H resistance the sensitivity is not sufficient to exclude H resistance based on a negative result. Mutations that cause H resistance may often occur in sequences that have not been commonly associated with H resistance, possibly in yet uncharacterized genes, and there are new H targets yet to be discovered [134]. Inclusion of mutations in other genomic regions, such as the *ahpC*, *kasA*, and *ndh* genes either in the same reaction or in separate reactions may improve the sensitivity of the GT assays.

The GT*plus* is reliable when used with AFB smear-positive specimens, but is less reliable when used on AFB smear-negative specimens. In order for the GT*plus* assay to be offered universally to all patients, studies on optimization of the DNA extraction and testing of different specimen inoculum sizes is necessary such that smear-negative samples that both are NAA positive and negative may yield results. Only 5-10% of the specimens were available for these studies. Most likely, a larger specimen sample or using the whole specimen would increase sensitivity. Future validations of molecular DR tests against each other are warranted.

The GT technique has recently been further developed to detect XDR-TB including mutations coding for the first-line drug E and the second-line drugs; the Q´s, Am/cyclic peptides, termed the GenoType® MTBDR*sl* (GT*sl*) assay. This assay has shown promising results detecting Q resistance in 91% of 32, Am in 85% of 46 and capreomycin in 87% of 45 resistant strains, and found applicable directly in smear-positive and culture-positive smear-negative sputum specimens enabling the detection of XDR-TB within 1-2 days [135]. However, the sensitivity for E resistance detection was unacceptably low, pinpointing discussions on the test concentrations for E [135]. Recently, the GT*sl* has been found to efficiently detect second-line drug mutations, however as not all mutations are covered by the assay, particularly DNA gyrase gene subunit B *gyrB* mutations, it was in that study concluded that conventional DST is still recommended [136].

In order for molecular methods such as the GT assays to replace conventional DST in the future, sensitivities must be increased to the level of *M. tuberculosis* NAA detection tests. It is possible that the GT*plus* may be combined with a *M. tuberculosis* NAA test. The tests must be expanded to include all genes that lead to phenotypic resistance for all drugs tested. Mixed populations of resistant and susceptible organisms may occur even when the apparent phenotype is susceptible. As phenotypic DR detection relies on >1% (for Z >10%) of the bacterial population to be called resistant in the antibiotic containing medium compared with drug-free medium, further analyses to assess whether the GT assays have the ability to detect these levels of resistance are needed. More extensive geographical characterization of resistance mutations, and studies on treatment outcomes related to mutations present and the presence of heteroresistance are necessary for the molecular DR tests to replace conventional DST in the future. The growing international WHO/IUATLD laboratory networks and better access to high throughput sequencing facilities will beyond doubt in the near future throw more light on the global diversity of TB resistance mutations and their clinical impact.

Treatment of NTM is mostly empirical as there is no correlation between in vitro susceptibility testing and treatment response. Potential development of rapid molecular tests to detect mutations causing resistance for the NTM may improve treatment of these organisms including newly described species in the future [137,138].

Finally, this is the first time that the GT assay has been employed to study resistance directly in clinical specimens. Reliable results can be obtained faster than with any other current phenotypic methods and the assay could be applied to smear-positive clinical specimens. The earlier detection of resistance and initiation of effective therapy, the use of the rapid GT*plus* assay for detecting R and H resistance may reduce periods of infectiousness of MDR-TB cases by as much as 6 weeks, reduce further spread and development of MDR-TB, and may improve treatment outcomes. The increase in migration from countries with endemic and epidemic high levels of resistance emphasizes the need for rapid methods to curtail drug-resistant TB. Our findings have proved highly reproducible even in low-income countries and I believe that implementation of the GT*plus* assay may be an important step towards eliminating drug-resistant TB worldwide.

ABBREVIATIONS

AFB	Acid-fast bacilli
ATS	American Thoracic Society
Am	Amikacin
BACTEC	BACTEC 460
CDC	Centers for Disease Control and Prevention
CI	Confidence interval
CLSI	Clinical and Laboratory Standards Institute
DK	Denmark
DOT	Directly observed therapy
DR	Drug resistance
DST	Drug-susceptibility testing
E	Ethambutol
Q	Fluoroquinolone
GT	GenoType® MTBDR
G*tplus*	GenoType® MTBDR*plus*
GT*sl*	GenoType® MTBDR*sl*
H	Isoniazid
HIV	Human immunodeficiency virus
inhA	NADH-dependent enoyl ACP reductase gene
IUATLD	International Union Against Tuberculosis and Lung Disease
katG	Catalase-peroxidase gene
LPA	Line probe assay
MDR	Multidrug-resistant
MGIT	Mycobacterial Growth Indicator Tube 960 system
M	*Mycobacterium*
NPV	Negative predictive value
NTP	National TB control programme
NTM	Nontuberculous mycobacteria
NAA	Nucleic acid amplification
PCR	Polymerase chain reaction
PPV	Positive predictive value
R	Rifampin
rpoB	B-subunit RNA polymerase gene
RFLP	Restriction fragment length polymorphism
SSCP	Single-stranded conformation polymorphism
TB	Tuberculosis
WHO	World Health Organization
WT	Wild-type
XDR	Extensively drug-resistant
Z	Pyrazinamide

SUMMARY

This PhD thesis is based on 5 studies conducted in the period 2006-2010 during my employment at the International Reference Laboratory of Mycobacteriology, Statens Serum Institut.

The overall aim was to assess tuberculosis (TB) treatment in Denmark with specific focus on the risk of relapse of TB disease, and to analyse treatment outcome of patients with multidrug-resistant (MDR) or isoniazid-resistant TB. The project established the need for rapid methods to detect resistance and follow-up of treatment. A rapid method to detect DR was optimised and evaluated for use directly in clinical specimens.

The studies were based on data from the Mycobacterial registry in the period 1992-2007, which included the results from microscopy, culture, drug-susceptibility and restriction fragment length polymorphism (RFLP). Information on dates of death/emigration were taken from the CPR-registry and treatment from surveillance data and patient records.

The rate of recurrent TB was found to be low in Denmark, during 13.5 years of follow-up. Relapse accounted for 1.3% of the recurrent cases and reinfection was rare, only in 0.5% cases. The relapse hazard increased up to four-years after diagnosis. Cavitary disease was associated with relapse as opposed to reinfection and may need prolonged treatment and closer monitoring. The incidence of MDR-TB and isoniazid resistance was confirmed to be low. Successful short- and long-term treatment outcome of MDR-TB and isoniazid-resistant TB was high. High- and low-level isoniazid resistance did not affect treatment outcome.

A multiplex PCR hybridization mutation analysis, that simultaneously detects the most frequent *rpoB* and *katG* gene mutations conferring rifampin and high-level isoniazid resistance, was optimized for direct use and evaluated in smear-positive specimens as opposed to slow conventional drug-susceptibility testing (DST). The second-generation rifampin and isoniazid resistance mutation assay, additionally included detection of mutations within the *inhA* gene conferring low-level isoniazid resistance. This assay was found to be rapid (<48 h) and easy to perform in isolates and clinical specimens. A high concordance between mutation and conventional DST results was found for rifampin, while for isoniazid results varied. The mutation analysis identified all MDR-TB cases and the majority of isoniazid-resistant cases in Denmark.

Standard 6 months multiple anti-TB drug therapy is necessary to treat drug-susceptible TB. Drug-resistant TB often requires therapy adjustments and extended treatment. MDR-TB particularly poses therapeutic challenges. Rapid detection of resistance mutations directly in smear-positive patient specimens may improve MDR-TB patient treatment, although the impact on isoniazid-resistant TB treatment outcome remains to be determined. The mutation assay is a rapid supplement to the gold standard conventional DST in high- income countries such as Denmark, while in low-income countries it can be used for preliminary DST. The assay may be applied to smear-positive samples from patients suspected of treatment failure, recurrent TB, drug-resistant TB exposure or originating from countries with high levels of DR. The new extended mutation assay has proved to be a useful tool, which has now been included in the World Health Organizations policy to combat and prevent new cases of MDR and extensively drug-resistant TB.

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