Rapid diagnoses of mycobacterial diseases, and their implication on clinical management

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This review has been accepted as a thesis together with seven previously published papers, by the University of Copenhagen, September 22, 2005, and defended on January 22, 2006.

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Dan Med Bull 2006;53:28-45

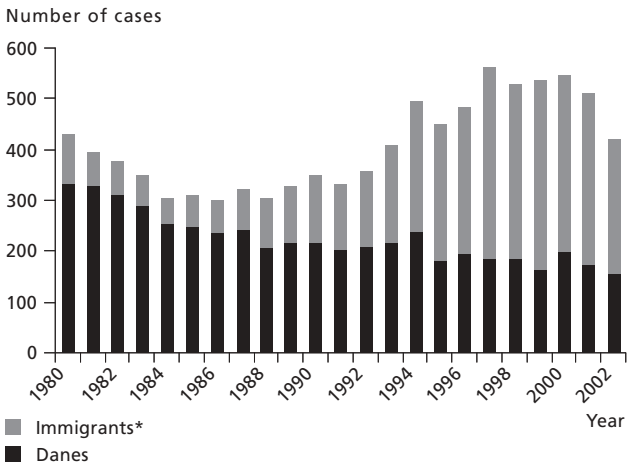
1. INTRODUCTION

1.1. TUBERCULOSIS EPIDEMIOLOGY WORLDWIDE AND IN DENMARK

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* complex. Currently, more than one third of the world's population is infected with the bacteria, and about 8 million new TB cases and 2 million deaths from TB are estimated each year. Over 95% of the estimated annual new cases and deaths occur in the developing world. In 1993, the World Health Organization (WHO) declared TB a global emergency. Through this unprecedented step WHO showed the extent of the concern about the modern TB epidemic. WHO estimated that between the year 2002 and 2020, nearly one billion people will be newly infected, 150 million people will get ill, and 36 million will die from TB, if control is not strengthened (212).

TB and HIV/AIDS are the world's most common causes of death from infectious diseases. TB accounts for about 11% of AIDS deaths worldwide. In Africa, HIV is the single most important factor determining the increased incidence of TB in the past 10 years (41).

The incidence of TB in many industrialised nations has also risen over the last two decades and this trend seems to continue. The increase was initially attributed to the HIV epidemic and the growth of poverty, but soon attention was drawn upon the recent waves of immigration from TB high-burden countries (49, 137). For instance, in Denmark, only 299 new cases were notified in 1986, the lowest number ever registered (135). In 2000, 548 new cases were notified – an increase of 83% (**Figure 1**). Although this increase through the last decade was mainly due to immigration from high-prevalence countries, an increase was also observed among socially marginalized Danish males due to recent infection (16). In 2002 a slight decrease in TB has been observed in parallel with the simultaneous decrease in immigration. A major high incidence group in Denmark are the Somalis that continue to have a high incidence of TB even after several years in Denmark (91). Furthermore, in Denmark, there has been no decline in TB incidence among the native population, which is in contrast to the decrease in TB incidence among the native population of Sweden, Norway and the Netherlands (16, 90, 204).



*) Immigrants are persons born abroad and their children <25 years.

Figure 1. Annually notified TB cases in Denmark 1980-2003.

1.2. RESISTANT TUBERCULOSIS

The emergence of drug resistant tuberculosis has made the current epidemic worse. Multidrug-resistant (MDR)-TB is defined as TB caused by bacteria resistant to at least rifampin and isoniazid. In two worldwide surveys covering 1994 to 1999, it has been documented that MDR-TB is a rapidly increasing health problem, with major socio-economic and individual consequences (52, 118). WHO has estimated that in the year 2000, MDR-TB accounted for 3.5% of all new cases of TB (48). Although MDR-TB is not currently an emerging problem in Denmark, there are reports of a high prevalence in Eastern Europe including the Baltic States which makes it an essential and imposing issue also in Denmark (81, 106, 111).

Evaluations of several outbreaks have shown that late identification and recognition of drug resistance contributed considerably to the mortality and spread of MDR-TB particularly among immunocompromised patients (35, 36, 55). The spread of MDR-TB can only be prevented if patients with drug-resistant TB are identified rapidly and treated with a combination of effective drugs. The first important step in achieving this goal is that microbiological laboratories are able to perform reliable and rapid drug susceptibility testing to both first- and second-line drugs (V, VI, VII).

1.3. NONTUBERCULOUS MYCOBACTERIA: EPIDEMIOLOGY AND CLINICAL MANAGEMENT

With the introduction of new laboratory techniques, almost 100 different mycobacteral species have been recognised of which some are potential pathogens whereas others are non-pathogenic to humans. However, an increase in infections caused by nontuberculous mycobacteria (NTM) has been observed mainly due to the AIDS epidemic (54). NTM may also cause severe disease in immunocompetent persons (30, 100, 113, 132). Therefore, there is a need for rapid diagnosis since increased morbidity and mortality is associated with NTM infections (IV). As few clinical and radiological findings differentiate NTM infections from TB, microbiological identification to the species level is necessary. The distinction between species has not only epidemiological implications but is also relevant for the management of patients in regards to appropriate treatment, isolation and contact tracing. Antibiotic treatment may vary according to the species encountered (7).

The objectives of this review were: 1) To present an overview of the microbiological diagnostic of TB and other mycobacteriosis with special reference to the new and rapid molecular biological diagnostic methods (I, II, III, IV) 2) To discuss the methodological problems associated with the diagnosis of resistant TB with special reference to the molecular biological diagnostic methods (V, VI, VII).

2. CURRENT METHODS IN DIAGNOSTIC MYCOBACTERIOLOGY

2.1. MICROSCOPY

Robert Koch discovered the tuberculous bacterium, and introduced a microscopic staining technique for it in 1882. Currently, two procedures are commonly used for acid-fast staining: the carbol-

fuchsin stains (Ziehl-Neelsen and Kinyoun procedure), examined by light microscopy using oil immersion (**Figure 2a**), and the fluorochrome stains (auramine-rhodamine or auramine O), examined by fluorescence microscopy (**Figure 2b**). The fluorochrome stains have now become routine procedure in laboratories because they allow rapid screening of specimens and because the procedure is more sensitive than the traditional Ziehl-Neelsen stain.

In spite of modern advances, microscopy remains an important diagnostic tool in tuberculosis control since it identifies the most infectious cases; it is rapid, inexpensive, technically simple, and specific for acid-fast bacilli (AFB). Microscopy can, however, not discriminate between *M. tuberculosis* and other mycobacteria, lacks sensitivity, and cannot be applied to monitoring of treatment as it does not discriminate between live and dead bacilli. While 10^6 AFB/ml of specimens usually result in a positive smear, only 60% of the smears are positive if 10^4 AFB/ml are present (126). The overall sensitivity of the microscopy has been reported to range from 22 to 78% (43), however, the sensitivity appears to be lower in nonrespiratory specimens due to the lower bacterial load and lower for some NTM species due to poorer staining of the cell wall (93, I, II).

2.2. CULTURE

Mycobacterial culture is the microbiological gold standard for diagnosis of mycobacterial infection as culture and possible identification of species is the only way to establish a definitive TB diagnosis. Traditional mycobacterial culture media include egg-based (Löwenstein-Jensen, Petragnani, American Trudeau Society, and Ogawa), agar-based (Middlebrook 7H10 and 7H11), and liquid media (Middlebrook 7H9 and Dubos). All media should be incubated for 8 weeks. The time to detection of growth of mycobacteria can be shortened significantly (mean 10-12 d) with the use of semi-automated (BACTEC 460TB system) or automated (BACTEC MGIT 960, ESP Culture System II, and MB/BacT ALERT 3D system) liquid culture systems (62, 130, 208). The highest recovery rate has been obtained using a combination of solid and liquid media. Reliable information about the limit of the detection of culture is difficult to obtain due to the mycobacterial tendency to clump and the impact of harsh sample decontamination, but it is estimated to be about 100.

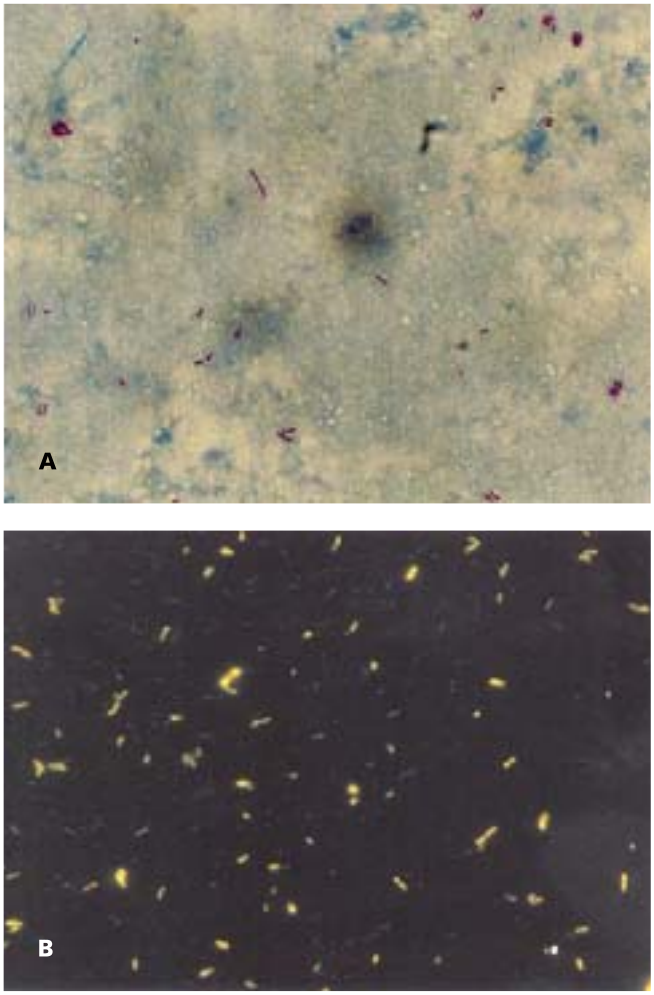


Figure 2. Acid-fast staining of mycobacteria. **A:** The carbol-fuchsin stains. **B:** The fluorochrome stains.

2.3. SPECIES IDENTIFICATION OF MYCOBACTERIA

Whenever possible, mycobacteria should be identified at the species level. Numerical analysis of biochemical features and growth characteristics have traditionally been used to differentiate between the species (189). Properties such as growth rate, pigmentation, colonial morphology etc. are used in the differentiating process and the procedures are relatively inexpensive but they are time-consuming (2 to 6 weeks to complete) and delay identification. In addition, since several recently described mycobacteria species are not sufficiently phenotypically characterised, biochemical tests often yield erroneous identification (164).

An alternative tool in species identification is mycolic acid analysis, which is the one of the minimal criteria for the description of new mycobacterial species. High-pressure liquid chromatography of mycolic acid esters has been demonstrated to be a rapid and reliable method for identification of many *Mycobacterium* species in 1-2 days (29). The method is standardised, highly sensitive and specific but it requires considerable biomass in growth, expertise and expensive equipment.

DNA sequencing of the gene encoding the 16S rRNA has become the gold standard for identification of mycobacterial species. The method and primers are described in detail by Kirschner and coworkers (80). Sequencing of hypervariable regions A and B of the gene allows for identification of the majority of mycobacterial species. However, no polymorphisms are present within *M. tuberculosis* complex (i.e. *M. tuberculosis, M. bovis, M. africanum, M. microti, M. canettii*) and some of the species cannot be discriminated by this method. Several other target genes have been investigated for this purpose. These include the gene encoding the 32-kDa protein (161), the *dnaJ* gene (170), the superoxide dismutase (*sod*) gene (218), the *gyrB* gene (78, 114), the *rpoB* gene (79), the internal transcribed spacer 16S-23S sequence (144, 145), and the widely used the *hsp65* gene (139). Recent improvements in automation of target amplification and sequence analysis have led to practical implementation of the method but it is relatively labour intensive, requires high technology and expensive equipment.

PCR restriction enzyme analysis of the *hsp65* gene was developed by Plikaytis et al. (133) and later modified by Telenti et al. (174) for rapid identification of the most mycobacterial isolates. However, members of *M. tuberculosis* complex are not discriminated by this method. The technique is time consuming, is not commercialised, and requires extensive in-house validation.

Commercially available identification probes such as AccuProbe (GenProbe Inc. Calif.) targeting rRNA can only identify *M. tuberculosis* complex, *M. avium, M. intracellulare, M. avium* complex, *M. kansasii,* and *M. gordonae* (86). The assay is rapid and easy to perform but has certain limitations. It requires large amounts of cultured bacteria, and works in a "trial and error" manner as one probe is tested at a time. Furthermore, mixed infections are difficult to detect unless isolates are routinely tested with all probes.

Recently, two new DNA line probe technologies have been developed for simultaneous identification of mycobacteria. The Inno-LiPA Mycobacteria (Innogenetics, Ghent, Belgium) is based on PCR amplification of the 16-23S rRNA spacer region of *Mycobacterium* species, and has been designed for identification of mycobacteria grown in culture media and discrimination between *M. tuberculosis* complex, *M. avium, M. intracellulare, M. kansasii, M. gordonae, M.*

xenopi, M. scrofulaceum, and *M. chelonae* group including *M. abscessus* with reverse hybridisation of the amplified product to probes immobilised as parallel lines on a membrane strip. The first generation of the assay was evaluated on clinical isolates with an excellent specificity (107, 152, 167, 194, IV). More recently, the second generation of the assay has become commercially available and it includes the following mycobacteria: *M. avium* complex, *M. malmoense, M. celatum, M. genavense, M. simiae, M. marinum, M.ulcerans, M. haemophilum, M. fortuitum* complex, and *M. smegmatis,* in addition (192).

The GenoType Mycobacterium (Hein Lifescience, Germany) assay is based on PCR amplification of 23S rDNA and hybridisation to the 13 probes in addition to first generation of Inno-LiPA Mycobacteria, *M. celatum, M. malmoense, M. peregrinum, M. phlei,* and two subgroups of *M. fortuitum* (99, 146).

Both DNA arrays are easy and rapid to perform (5-6 h), several species can be identified by a single PCR assay, hybridisation can be carried out in an automated manner. None of the assays differentiate within the *M. tuberculosis* complex.

2.4. APPLICATION OF NUCLEIC ACID AMPLIFICATION (NAA) METHODS IN DIAGNOSING TB

Molecular methods used directly on clinical specimens have created new opportunities for the clinical mycobacteriology laboratories to influence patient management in the areas of initial diagnosis, rapid determination of the drug-resistance pattern and epidemiological information. Current molecular tests are mainly used to identify *M. tuberculosis* complex, which can be transmitted from person to person (I, VI). Four different amplifications methods are currently available for this purpose. These methods are only standardised for respiratory specimens, but are applied to nonrespiratory specimens.

2.4.1. NAA methods in respiratory specimens

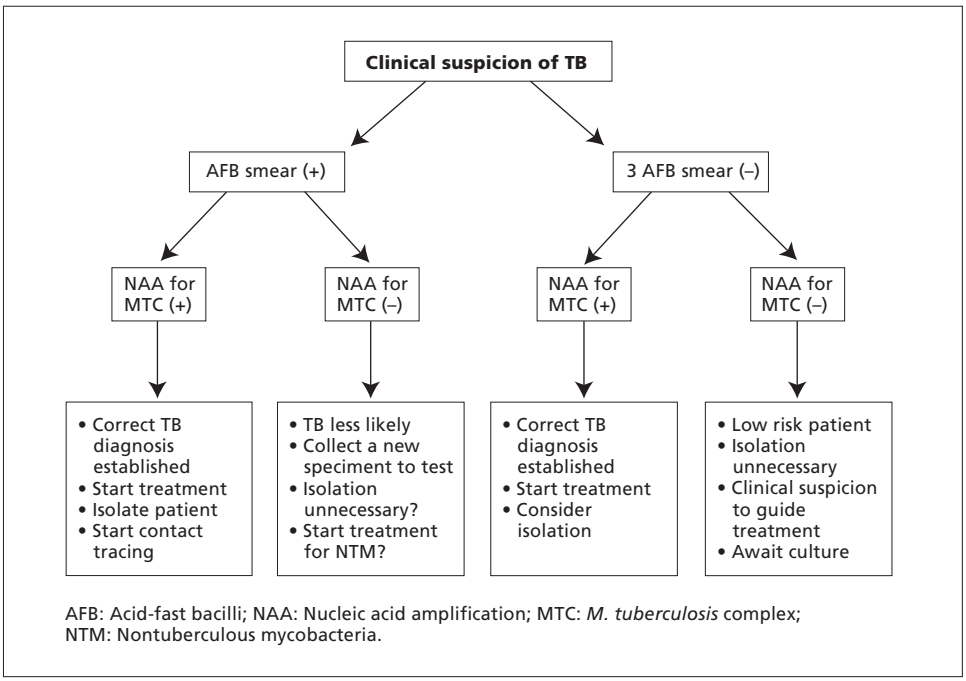
Early diagnosis of TB and determination of drug resistance is crucial for the initiation of treatment and interruption of the chain of transmission. The role of the NAA test results in the initial evaluation of patient with suspected respiratory TB compared to microscopy is summarised in **Figure 3**. A positive NAA result in microscopy positive patient indicates TB and subsequently leads to isolation of the patient and initiation of treatment. A negative NAA result in a microscopy positive patient indicates the presence of nontuberculous mycobacteria. In this case, the patient could be discharged, investigation of contact tracing is unnecessary and the treatment could be directed toward the NTM at an early stage.

The major impact of applying rapid diagnostic tests to patient care would be in the case where the patient is suspected of having TB but has a smear-negative specimen. Approximately up to half of the patients with pulmonary TB are smear negative (49). The proportion is even higher among immunocompromised patients due to the paucibacillary nature of the disease and neither chest radiograph nor infection parameters are specific for TB (28, 121, 123, 160). NAA analysis can confirm the diagnosis in a few days as opposed to several weeks by culture. The diagnostic delay causes some of the patients to convert to smear positivity (65), and poor treatment outcomes in smear-negative pulmonary TB patients co-infected with HIV are reported (64, 66). These patients account for about 17% of the TB transmission (17).

In order to minimise the false results, additional specimens (not to exceed three) should be tested (34), and laboratories should establish adequate quality controls (115, 116)

The performance of NAA assays for detection of *M. tuberculosis* complex relies on several factors such as species-specific primers, sample volume used for amplification and inclusion of internal control for validation of results. The four different NAA methods and their performances are summarised in **Table 1**. All techniques consist of three steps: specimen preparation, amplification and detection.

Polymerase Chain Reaction (**PCR**) amplification is an *in vitro* method for the amplification of specific nucleic sequences through repetitive cycles of thermal denaturation, oligonucleotide primer annealing, and primer extension by a thermostable DNA polymerase (150). The technique has been applied targeting different genes using labour intensive procedures for sample preparation, detection of amplicon by radioisotopic methods (26, 45, 51). These early attempts generally lacked standardisation. In order to by-pass these limitations, kit PCR method for detection of *M. tuberculosis* complex in respiratory clinical specimens (Amplicor *M. tuberculosis* PCR test, Roche Diagnostic Systems, NJ) has been developed. An internal amplification control has been included in the later version (Cobas Amplicor). The sensitivity was 99-100% in smear positive specimens in most studies but it varied from 45.4% to 75% in smear negative respiratory specimens (50, 138, 154, 181).



AFB: Acid-fast bacilli; NAA: Nucleic acid amplification; MTC: *M. tuberculosis* complex; NTM: Nontuberculous mycobacteria.

Figure 3. Role of nucleic acid amplification tests for *M. tuberculosis* complex in the initial evaluation of patient with suspected pulmonary tuberculosis.

Table 1. Available different nucleic amplification tests and their performances in diagnosing respiratory tuberculosis.

NAA-tests	Methods/ target gene	Volume (µl)	IAC/automation	Assay time (h)	Sensitivity (%)	Specificity (%)	References
Amplicor	PCR/16S DNA	100	+/+	6-7	66.3-94.2	98.8-100	50, 154
AMTD-2	TMA/16S RNA	450	–/–	2.5	85.7-97.8	99.1-100	117, 154
LCx	LCR/38-kDa	500	–/+	6	75.7-90.8	92.4-100	12, 128
ProbeTec	SDA/ IS6110	500	+/+	3	82.7-97.1	96-99.8	I, 14

IAC: Internal amplification control.

Transcription Mediated Amplification (TMA) is an amplification system based on reverse transcription of RNA into cDNA using RNA polymerase and reverse transcriptase enzymes to drive the reaction (82). This isothermal method has been applied to detection of *M. tuberculosis* complex targeting 16S rRNA (Gen-Probe amplified *Mycobacterium tuberculosis* Direct Test, AMTDT-1). Modifications of the procedures made the newer version of the assay (AMTDT-2) more sensitive and rapid (39,57,117,128,154). On the other hand, the hereby increased specimen volume makes the system more susceptible to inhibitory substances in the amplification reaction and the lack of an internal amplification control makes this problematic. The assay may, however, yield false positive results for *M. tuberculosis* in specimens containing *M. celatum* as a cross-reaction was demonstrated (188).

Ligase Chain Reaction (LCR) is a probe-based amplification system (The Abbott LCx system) and differs from PCR as it amplifies the probe molecule rather than producing amplicon through polymerisation of nucleotides. Evaluations of the assay on respiratory specimens revealed a lack of sensitivity in microscopy-negative specimens (12, 58, 92, 128, 195).

Strand Displacement Amplification (SDA) is an isothermal in vitro amplification technique consisting of two phases. The first phase is target generation, where two primers and two bumpers will generate a defined target length from the native DNA. The second phase is an exponential amplification of the target sequences by repeated nicking, strand displacement and priming of displaced strands (163, 202) (**Figure 4**). The method is based on the simultaneous amplification and detection of the *M. tuberculosis* complex specific IS*6110* target DNA with real-time detection using fluorescence resonance energy transfer (BDProbeTec ET Direct Detection assay, USA). It has been shown that some *M. tuberculosis* complex strains lack the IS*6110* sequence (199) which should be taken into consideration when interpreting results.

SDA has only recently been introduced as a tool for TB diagnostics. We assessed the performance of SDA on 351 respiratory specimens and compared the results to those obtained by microscopy and culture. The assay yielded 100% sensitivity in 85 microscopy-positive specimens and 60% sensitivity in 65 microscopy-negative specimens, culture positive for *M. tuberculosis* complex. The sensitivity for microscopy negative specimens improved upon retesting of those false negative specimens yielding initial results just below the cut-off value. The specificity was 99% compared to culture and clinical data. Laboratory contamination was not considered the possible reason for the two false positive results. One patient had been treated for TB for 10 days when the sample was taken and the test may have detected DNA from dead bacteria. The other patient lived in an high incidence area and it has been shown that the lung tissue from patients without active TB can be colonised with a low number of *M. tuberculosis* detectable by PCR (69). The primers were specific as we observed no cross-reaction to the 26 most frequently encountered NTM species tested (I). The assay could be used for rapid screening of smear positive specimens with a confirmation of TB diagnosis in a few hours as the prevalence of NTM infection is high in Denmark (178). This could have major implications in clinical practice and on cost-effectiveness.

SDA has been evaluated by others and performances reported listed out in **Table 2.** Some authors reported higher sensitivity in smear negative specimens ranging from 33.3 to 87.1%. The reason

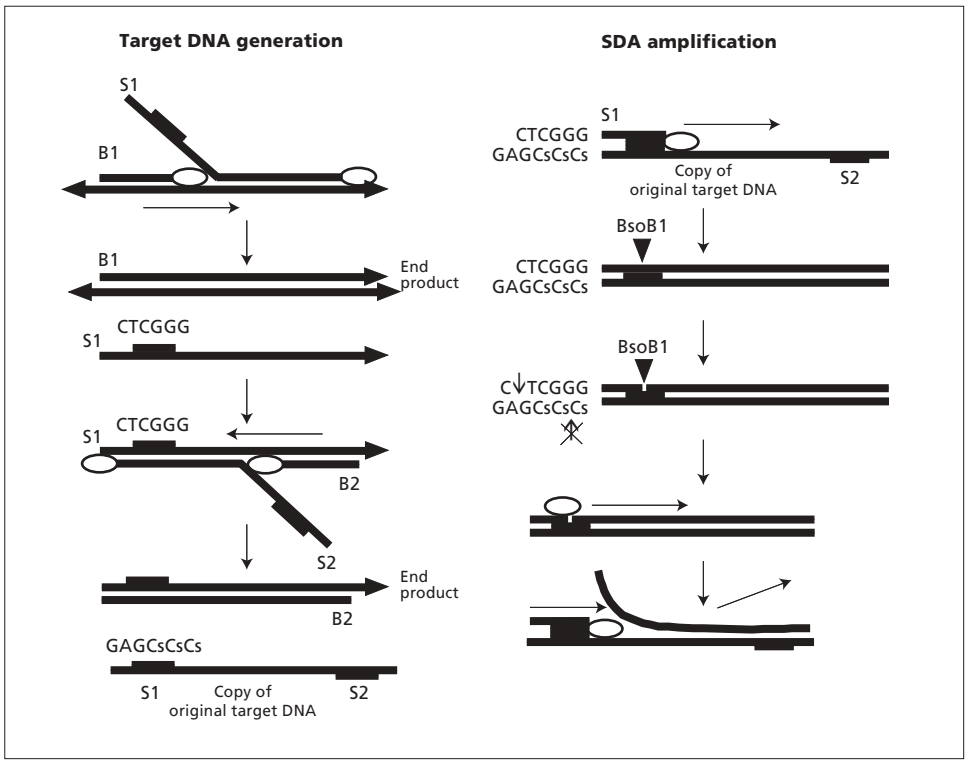


Figure 4. Strand displacement amplification.

S1 and S2: Primers.
B1 and B2: Bumpers.
BsoB1: Restriction enzyme.

for these varying sensitivities is not known, but could be explained by the proportion of specimens with a negative smear and positive culture.

In conclusion, compared to other NAA methods, SDA appears to be highly sensitive and specific for direct detection of *M. tuberculosis* complex in respiratory specimens. No cross-reaction between *M. tuberculosis* complex and NTM was reported. The method offers the distinct advantage of including an internal amplification control to validate negative results. It offers an easy diagnostic procedure for routine laboratories providing shorter assay time.

2.4.2. NAA methods in nonrespiratory specimens

Nonrespiratory TB increases and is primarily seen in HIV-infected patients where it may be lethal (20, 159, 215). In Denmark, nonrespiratory TB constitutes approximately one third of all new TB cases with tuberculous lymphadenitis being the most frequent manifestation (66%) (8, 9). Diagnosis of nonrespiratory TB is usually more difficult than respiratory TB due to its varying localisation, appearance and symptoms. Due to the involvement of relatively inaccessible sites, and the characteristics of sites involved, fewer bacilli may cause severe damage. In addition to the increased incidence of nonrespiratory TB in HIV-seropositive patients, these patients are more prone to infections with NTM, making diagnosis an even harder challenge and clinical outcome highly dependent on the rapid onset of treatment.

Microscopy is often negative for AFB in nonrespiratory specimens (I, II, 93), especially in cerebrospinal fluid (CSF) ranging from 0 to 20% (83, 134, 183). However, in a recent study in a high incidence setting, higher sensitivity of microscopy in CSF was demonstrated using a larger volume of CSF and was examined over a longer period of time (184, 185). Although culture on broth media has somewhat shortened the diagnostic delay, it is still often 3-5 weeks, if it becomes positive at all. Recently, assays that measure the release of interferon gamma in the blood have shown promising results compared to the tuberculin skin test, but their utility in a clinical setting remains to be elucidated (27, 53, 102, 109). Histopathological examination of the nonrespiratory specimens could help with providing the diagnosis but histological differentiation between TB and NTM infections and other granulomatous diseases may be very difficult.

Biochemical analysis of CSF is used worldwide as the initial examination. Pleocytosis with lymphocytosis, increased protein and reduced glucose level combined with the symptoms of the patient may support the suspicion of tuberculous meningitis (TBM) but the diagnosis remains uncertain as other pathological affections of the central nervous system may reveal similar features (186). Antibody detection in CSF is a quick test. This approach, however, is not standardised and lacks both sensitivity and specificity (108, 203). Detection of anti-BCG secreting cells in CSF is sensitive and specific for early diagnosis of TBM, but the technique is laborious (95).

Although none of the NAA tests are approved for the detection of *M. tuberculosis* complex in nonrespiratory specimens, and no standardised procedures for processing such specimens exist, they are often applied with varying performances (I, II, 129). The main causes of the varying performance are paucibacillarity, sampling error, and presence of inhibitory substances.

We evaluated the reliability of the standard SDA on varying sources of 372 nonrespiratory specimens (160 body fluid specimens, 103 tissue specimens, 76 pus samples, 31 wound swabs, and 2 stool specimens) from 283 patients. The results were compared to results of microscopy and culture, in case of discrepancy the patients chart history was examined. The assay yielded 98.5% sensitivity in the 67 microscopy-positive specimens and no cross-reaction with NTM species was observed (I). This implies that the assay may be used for rapid screening of the microscopy positive nonrespiratory specimens for TB diagnosis. The assay was negative in one microscopy positive specimen but the value was just below the cut-off. The sensitivity was solely 40.3% in the 124 microscopy-negative specimens, culture positive for *M. tuberculosis* complex. However, rapid diagnosis of microscopy negative TB is of great importance since it allows early treatment, and avoids unnecessary and potentially harmful diagnostic procedures as well as minimizes the deterioration of the disease due to diagnostic delay. The assay provides little additional information in this category. The performance of the assay was much better in urine, synovial fluid, pus and gastric lavages, especially when compared to the performance of microscopy. The overall performance of the standard assay was much lower in nonrespiratory specimens than in respiratory specimens (60.7% vs. 82.7%) (I).

Overall specificity was 96.7% when compared to culture, but increased to 98.9% upon re-evaluation of the six false-positive samples containing either dead bacteria or originating from patients fulfilling both clinical and classical pathological features of TB. DNA based assays do not assess the viability of the organism, and are thus inappropriate for the monitoring and follow-up of the patient during treatment and after cessation (68, 181). Two samples were truly false-positive.

The method has been evaluated for diagnosing nonrespiratory TB in a clinical setting by others and performances are summarised in Table 2. The overall sensitivities were generally higher ranging from

Table 2. Detection of *M. tuberculosis* complex by strand displacement amplification in respiratory and nonrespiratory specimens.

Study (reference)	No. of specimens/ specimens type	No. of MTC cultures/smear-positive specimens	Sensitivity (%)			Specificity (%)
			smear –	smear +	overall	
Bergmann et al. (21)	600/R	16/12	75	100	93.8	99.8
Johansen et al. (I)	351/R	150/85	60	100	82.7	99
	372/NR	192/67	40.3	98.5	60.7	98.9
Barrett et al. (14)	200/R	104/101	33.3	99	97.1	96
Maugein et al. (101)	547/R	69/43	76.4	100	89.5	98.2
	74/NR	8/4	85.7	100	NA	NA
Piersimoni et al. (131)	331/R	91/76	75	98.7	94.5	99.6
	184/NR	30/22	82.3	100	92.3	100
Mazzarelli et al. (103)	537/R	184/135	70.6	99.2	91.3	98.1
	294/NR	68/28	69	90	77.8	97.7
Iinuma et al. (73)	411/R	93/83	50	98.7	93.5	99.7
de la Calle et al. (75)	502/R	32/21	81.8	100	93.5	99.5
Rüsch-Gerdes and Richter (149)	735/R	98/36	87.1	100	93.5	98.2
	396/NR	27/3	83.3	100	91.6	98.7

R: respiratory; NR: nonrespiratory; MTC: *M. tuberculosis* complex; NA: not available.

77.8 to 92.3%. This huge variation is attributable to the varying proportion of the different (selective) nonrespiratory specimens included in each study, to the different types of specimens and especially to the varying distribution of smear and culture results in included specimens. The assay yielded only 0.3% of inhibition despite testing a broad variation of specimens and our inhibition rate was much lower than reported by others (1 to 14%).

SDA is reliable in providing the diagnosis of nonrespiratory TB in microscopy positive specimens, although sample processing prior to amplification may need improvement in order to increase the sensitivity in microscopy negative specimens.

2.4.3. Improved performance of NAA in tuberculous meningitis

Tuberculous meningitis (TBM) is a medical emergency and rapid diagnosis is essential for a positive clinical outcome. Delaying or interrupting the treatment of TBM has been shown to be important predictors of mortality (72, 200). Although the incidence of TBM is low in industrialised countries, it remains an important health threat in connection with HIV, immunosuppression, senescence and immigration from countries with a high incidence of tuberculosis (24, 37, 134, 200).

The microbiological diagnosis remains a true challenge, mainly because of the low count of mycobacteria in CSF (4). A recent meta-analysis by Pai and co-workers reviewed 49 studies evaluating both commercial and in-house nucleic amplification tests for TBM. The estimated values for sensitivity and specificity were 56% and 98%, respectively for the 14 studies using commercial tests (120). Sensitivity and specificity values could not be estimated for in-house PCR tests due to wide variability in performances.

We studied 101 CSF specimens collected from 94 patients in Denmark and Turkey with microscopy, culture for mycobacteria and SDA with and without modification for detection of *M. tuberculosis* complex DNA. Thirteen specimens (12.8%; seven Danish, six Turkish specimens) were *M. tuberculosis* complex culture positive, and three of these (23%; one Danish, two Turkish specimens) were positive for AFB by microscopy. The results in both procedures are shown in **Table 3**. The 13 culture positive CSF specimens were from 12 different patients and the time needed for culture to become positive ranged from two to five weeks (II).

Applied directly to CSF, the standard method yielded a 61.5% sensitivity compared to culture. In order to obtain better performance and to diminish loss of DNA during the sample processing, the washing buffer step was omitted in the modified procedure. This step is intended to remove inhibitors from the specimens. We demonstrated a higher sensitivity (76.9%), even though the modified test was generally carried out on less volume of CSF from each sample. The assay yielded one false positive result on the same specimen by both procedures. The specimen was from a patient who was treated for culture verified TBM as confirmed by another specimen demonstrating the importance of multiple sampling. The cut-off value for a positive test may be too high when the system is applied to certain nonrespiratory specimens like CSF. If the cut-off value obtained by the modified method was decreased from 3,400 to 1,000, the sensitivity would increase to 84.6% and the specificity would remain 100%, thereby obtaining a higher performance than any of previous studies (120). Omitting the washing step did not influence the rate of inhibition; all negative results were valid.

SDA provides reliable results in cases with TBM where conventional methods may be insufficient. The modified sample preparation step yields promising features and, it could be considered to lower the cut-off value.

2.4.4. Detection of *Mycobacterium tuberculosis* complex in formalin-fixed, paraffin-embedded tissue specimens

Despite the fact that TB is a highly prevalent infectious disease, the clinical diagnosis remains a true challenge due to its varying localisation, appearance and symptoms (10, 44, 56, 94, 96, 142, 216). Malignancy is a differential diagnosis to TB and samples may only have been sent for histopathologic examination. Such samples are formalin-fixed and paraffin-embedded (FFPE) making microbiological examination, apart from low sensitive, non-specific microscopy, impossible. If histopathology raises suspicion of mycobacterial infection e.g. due to the presence of granulomatous inflammation, it may warrant repeated sample collection. Repeated invasive procedures are both inconvenient, expensive and can be impossible, and it is thus desirable to optimise diagnostic methods applicable on FFPE samples.

New molecular methods are currently being used for diagnostic purposes in order to detect e.g. malignancy and infectious diseases (87, 88, 105) in such specimens. Furthermore, Rish and co-workers used a PCR method on FFPE tissue from mice experimentally infected with the H37Rv strain of *M. tuberculosis* complex, and were able to detect as few as nine bacteria in a 5 μm section of tissue (140). Previous studies have described the use of PCR on human FFPE specimens for detection of *M. tuberculosis* DNA with promising results, but almost all studies applied in-house PCR methods (46, 63, 89, 124, 151, 156).

We investigated 66 FFPE samples from various locations from 60 patients with necrotizing granulomatous inflammation collected in Denmark and Sweden by SDA after paraffin extraction with xylene. Results were compared to TB notifications from the national TB registers in both countries (**Table 4**) (III).

The prospective part of the study involved 47 FFPE tissue specimens from 43 patients. Of these, 20 specimens were from patients who were notified as having clinical TB upon receipt of the histopathologic results. SDA was positive for *M. tuberculosis* complex DNA in 18 providing the TB diagnosis in one working day where no fresh material was left or available for mycobacterial culture. The false negative results in two patients may be due to paucibacillarity in this type of specimens or loss of DNA during the paraffin extraction and sample preparation for amplification. Culture was performed on specimens collected at a later date from 27 of these patients. Culture revealed *M. tuberculosis* complex in 14 patients and SDA was positive in 13 of these 14 patients. The assay was negative in the specimens from patients with culture positive for NTM and in all the remaining patients. The overall sensitivity and specificity of the SDA in FFPE tissues were 90% and 100% respectively, when compared to national TB notification data, and 92.8% and 100% respectively, when compared to culture alone (III).

By using the SDA method, our results extend the findings reported by Salian and co-workers, who demonstrated the feasibility of in-house PCR using IS*6110* target DNA on 60 FFPE tissue samples prospectively collected in various geographical areas yielding a 73.6% sensitivity and 100% specificity compared to final TB diagnosis (**Table 5**) (151).

The retrospective aspect of our study involved 19 FFPE tissue samples from 17 patients (Table 5). The overall sensitivity and spe-

Table 3. Results of culture and strand displacement amplification with different lysis procedures for cerebrospinal fluid.

M. tuberculosis **culture (n = 101)**	**Standard SDA**			**Modified SDA**		
	positive	**borderline**	**negative**	**positive**	**borderline**	**negative**
Positive (13)	8	0	5	10	1	2
Negative (88)	1[a]	0	87	1[a]	1[b]	86

a) The specimen was from a patient from whom another specimen revealed culture verified tuberculous meningitis.
b) The specimen was from a patient with culture-verified tuberculosis, as determined with a previously collected specimen. This specimen was taken during the third month of the antituberculous treatment.
MOTA: Metric other than acceleration; Positive: MOTA >3,400; Borderline: 1,000 <MOTA <3,400; Negative: MOTA <3,400.

Table 4. Detection of *M. tuberculosis* complex in various sources of 66 formalin-fixed, paraffin-embedded tissue specimens by strand displacement amplification compared to tuberculosis notifications.

Type of specimens n=66	Notified with TB n=35	SDA positive n=24 (68.5%)
Lymph node (32)	17	12 (70.5%)
Lung (10)	3	2 (66.7%)
Gastrointestinal tissues (5)	5	3 (60%)
Liver (5)	2	2 (100%)
Peritoneum (5)	5	2 (40%)
Subcutaneous tissues (3)	0	0
Bone (2)	0	0
Genital (2)	1	1 (100%)
Kidney (1)	1	1 (100%)
Spleen (1)	1	1 (100%)

cificity of SDA in the archival samples were 40% and 100%, respectively, when compared to national TB notification data (III). However, the sensitivity was considerably lower in the three to five years old archival samples, which might be explained by DNA damage due to long storage. It is well known that old archival FFPE samples are not as suitable as fresh samples as the time-dependent physical degradation of DNA in FFPE tissue affects the success rate of the amplification (60, 136). This problem should be taken into account, when the assay is used in archival samples stored more than two years. In order to increase the sensitivity particularly in archival specimens, it should be considered to include more tissue sections (>3) in each run. The performances of the different NAA tests to detect *M. tuberculosis* complex in FFPE tissues are summarised in Table 5. Higher sensitivities ranging from 52.6 % to 100% in the retrospective studies are demonstrated by others and proteinase K digestion, which is considered the best known extraction method, was used in all the studies.

SDA provides rapid, reliable results when applied to prospectively collected FFPE tissue samples with necrotizing granulomatous inflammation. Thus the assay provides an additional opportunity to identify *M. tuberculosis* where TB is not initially suspected and no fresh specimen is available for routine microbiological TB diagnostics.

2.5. APPLICATION OF NAA METHODS IN DIAGNOSING NONTUBERCULOUS MYCOBACTERIOSIS

Nontuberculous mycobacteria (NTM) include those *Mycobacterium* species that are not members of the *M. tuberculosis* complex or *M. lepra*. This group of bacteria is ubiquitous in the environment and was recognized as human pathogens much later than *M. tuberculosis* bacteria. The first classification of NTM was developed in the 1950s by Timpe and Runyon, who classified according to the rate of growth, the color and appearance of colonies (187). Early reports indicated that patients with NTM lung diseases were epidemiologically distinct from patients with TB having coincidental lung diseases such as chronic obstructive lung diseases and being older. Family members of these patients were not infected in contrast to TB (42, 207). The incidence and natural-history of NTM disease has changed radically worldwide due to the emergence of the AIDS epidemic (54, 97). These and other immunocomprimised patients tend to have disseminated disease (25, 74), are at a higher risk and suffer from increased morbidity and shortened survival (19, 38, 71). NTM may also cause lymphadenitis, skin and soft tissue infection and osteoarticular infections (207).

In contrast to TB, NTM disease is not subject to notification and the value of estimates of its incidence and prevalence has thus been limited. Laboratory surveillance data in Denmark showed that NTM comprised approximately one-third of the mycobacterial isolates cultured annually in line with previous reports (61, 178). Diagnosing respiratory NTM infection is a real challenge for the clinician. Nontuberculous mycobacteriosis may present with a wide variety of clinical and radiological manifestations that allows no differentiation from TB. A single culture positive specimen is sufficient to confirm the TB diagnosis in contras to NTM diagnosis where collection of at least three respiratory specimens is required (7). In case of a microscopy positive specimen for AFB combined with symptoms treatment should be commenced. In countries with a high incidence of NTM infections interpretation of microscopy positive specimens can be dubious and the need for tools to enable rapid distinction between *M. tuberculosis* and NTM to the species level is obvious. In a Dutch study it was reported that 63% of the patients with NTM infections were treated unnecessarily or inappropriate due to a positive microscopy for AFB (198). This could be avoided by using fast techniques to differentiate *M. tuberculosis* complex from NTM and to perform identification at species level.

In a routine setting identification to the species level has so far depended upon positive culture postponing the results up to several weeks (IV). It has been demonstrated that HPLC with fluorescence detection can be used directly on smear positive sputum specimens for detection of *M. tuberculosis* and *M. avium* complex with 56.8

Table 5. The performance of different nucleic amplification methods for detection of *M. tuberculosis* complex in prospectively and retrospectively collected formalin-fixed, paraffin-embedded tissues.

Study (reference)	Amplification method/target	No. of specimens/ patients	No. of positive specimens by the NAA method/ by the gold standard	Sensitivity (%)	Specificity (%)
Prospective					
Salian et al. (151)	In-house PCR/IS6110	60/60	14/19	73.6	100
Johansen et al. (III)	SDA/IS6110	47/47	18/20	90	100
Retrospective					
Johansen et al. (III)	SDA/IS6110	19/17	6/15	40	100
Perosio and Frank (124)	Nested PCR/65-kDa	25/25	16/25	64	100
Berk et al. (23)	In-house PCR/IS6110	25/25	18/19	94.7	83.3
Diaz et al. (46)	In-house PCR/IS6110	43/43	9/17	58	96
Hardman et al. (63)	In-house PCR/16S rRNA	42/42	11/11	100	NA
Li et al. (89)	In-house PCR/IS6110	131/115	38/53	72	73
Ruiz-Manzano et al. (148)	AMTD and LCR	74/74	30/57 AMTD 36/57 LCR	52.6 63.2	100 100
Selva et al. (156)	In-house PCR/IS6110	49/49	45/49	92	100

and 33.3% sensitivity, respectively (77). Drawbacks of the system have been discussed earlier.

NAA tests have primarily been used for the detection of *M. tuberculosis* complex and scarcely for NTM. We assessed the feasibility of the DNA array technique for this purpose. The first generation of line probe assay (Inno-LiPA Mycobacteria) which was designed for identification of mycobacteria grown in culture media was for the first time used directly on 14 microscopy positive specimens (11 sputa, 1 gastric fluid, 1 tissue, 1 wound swab). Specimens were selected so all levels of microscopy quantitation (1+ to 4+) were approximately equally present. All specimens were identified correctly under the limitations of the assay (**Table 6**) (IV). Three specimens were identified as *M.* species due to lack of species-specific probe, however, the proportion of this group would be less by using the second generation of the assay. The other advantage with the new version is the addition of *M. genavense* which is difficult to isolate due to the slow growth and the need for selective media. Rapid diagnosis is, however, crucial for the survival of these patients (25, 180).

In conclusion, DNA array technique should be use as a rapid tool in cases with microscopy positive specimens in order to establish a correct diagnosis.

3. DRUG SUSCEPTIBILITY TESTING (DST) OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX

The standard regimen for the treatment of TB is the so-called short course antituberculous chemotherapy, which must be continued for at least 6 months. This regimen consists of an initial phase of 2-months treatment with isoniazid (INH), rifampin (RMP), ethambutol (EMB), and pyrazinamide (PZA) followed by a 4 months continuation phase with INH and RMP. These drugs represent the most potent drug combination and therefore constitute the mainstay of the treatment. The initial phase is to be continued at least until DST results are available (213).

Drug resistance is defined as a decrease in the *in vitro* susceptibility of *M. tuberculosis* of sufficient degree to be reasonably certain that the strain concerned is different from a wild type strain that has never come into contact with the drug. The term monoresistance is used when a strain is resistant to only one of the first-line antituberculous drugs; whereas polyresistance is used when resistance to two or more drugs are found.

DST is crucial in the appropriate management of the patient (1), and should be performed on the first isolate of *M. tuberculosis* complex obtained from each patient. If culture fails to convert to negative after three months of therapy or if reduced clinical response to treatment is found, DST should be repeated on a new isolate (175).

Table 6. Clinical specimens tested by the DNA array and the conventional methods.

Specimens n = 14	Microscopic quantitation (1+ to 4+)	Results by the DNA array	Results by 16S rDNA analysis and selected biochemical tests
3	2+(n=1) 3+(n=2)	*M. tuberculosis* complex	*M. tuberculosis* complex
3	1+(n=1) 2+(n=1) 3+(n=1)	*M. avium*	*M. avium*
3	1+(n=1) 2+(n=1) 3+(n=1)	MAIS	*M. malmoense*
2	1+(n=1) 2+(n=1)	*M. intracellulare*	*M. intracellulare*
3	1+(n=1) 2+(n=1) 4+(n=1)	*M.* species	1 *M. marinum*, 1 *M. genavense*, 1 *M. szulgai*

MAIS probe for *M. avium* complex and *M. malmoense*.

3.1. MECHANISMS OF RESISTANCE

Drug resistance to *M. tuberculosis* is a man-made amplification of a natural phenomenon. The problem was recognised soon after the introduction of streptomycin and INH for the treatment of TB (31, 40). Even in the absence of antituberculous therapy, resistance develops through spontaneous mutations during bacterial multiplication. This natural phenomenon is linked to large initial bacterial populations and the frequency at which it occurs has been determined. Mutations resulting in resistance to RMP occur at a rate of 10^{-10} per cell division and lead to an estimated resistance prevalence of 1 in 10^8 bacilli. The rate for INH is approximately 10^{-7} to 10^{-9}, resulting in resistance in 1 in 10^6 bacilli (209). Bacillary populations greater than 10^7 often prevail in lung cavities (31). Though diluted within the majority of the drug susceptible bacterial population, the mutant organisms do occur even in the absence of antimicrobial agents. Exposure to antimicrobials, however, provides the selective pressure favouring resistant cells, which may multiply and become predominant. Exposure to a single drug thus suppresses the growth of bacilli susceptible to that drug but permits the selection of mutant organisms, especially in those patients carrying a large load of bacilli (209). This is the foremost reason for the use of multidrug regimens in the treatment of TB.

"Acquired drug resistance" is a term applied when the bacilli develop resistance during treatment. Transmission of such resistant bacilli to other persons is the basis for "primary drug resistant" TB (32). Due to confusion with regard to these definitions, the WHO has now introduced the more simple term "drug resistance among previously treated cases" and "drug resistance among new cases" (209).

The proportion of drug resistance among new patients reflects the transmission of drug resistant bacteria in the society. From 1995 to 1998 this proportion among Danes was 5.9% in Denmark and therefore close to the rate of <5% estimated in good national programmes by WHO (179). However, the proportion did increase to 8% in 2002 (177). Drug resistance among previously treated cases is an indicator of the quality of the TB management in the country in the past as it reflects the combination of doctors' ability to initiate standard treatment and patients' compliance to such treatment. The proportion of acquired drug resistance has decreased in Denmark, and was not seen at all among Danes in 2002 (177, 179).

3.2. PHENOTYPIC METHODS

Currently used phenotypic tests are classified as direct or indirect test depending on whether they are applied to primary specimens or to isolates. Direct tests can however be performed on microscopy positive specimens only if sufficient bacteria are present. Although, direct tests can provide rapid results, it is neither widely performed nor considered reliable due to the lack of standardisation.

In order to assess the global magnitude of the resistance problem, WHO and the International Union Against Tuberculosis and Lung Disease began the Global Project on Anti-tuberculosis Drug Resistance Surveillance in 1994 focusing on standardised sampling and bacteriological methods (84, 85). Currently four growth-based (indirect) methods have been approved for DST by WHO: 1. The absolute concentration method, 2. The resistance ratio method, 3a. The proportion method and 3b. Its variant in liquid media (the radiometric BACTEC 460 method). The first two methods are no longer commonly used and are described in detail elsewhere (33).

The proportion method is currently considered the standard reference method worldwide (33). The ratio between the number of colonies growing on drug-containing medium and the number of colonies growing on drug-free medium indicates the proportion of drug resistant bacilli present in the bacterial population. This is the only method in which the validity of critical drug concentrations and drug resistance proportions has been correlated to clinical effectiveness in patients with TB (176). The proportion method defines resistance as growth of greater than 1% of an inoculum of bacterial

cell in the presence of a critical concentration of drug. The critical concentration of a drug represents the lowest concentration of the drug that inhibit 95% of wild-strains of *M. tuberculosis* without inhibiting strains that have been isolated from patients not responding to the treatment. The method and the drug concentrations used are described in detail by the National Committee for Clinical Laboratory Standards (176).

The methods 1, 2, and 3a are based on solid media and require 3 to 4 weeks for completion after the isolation of sufficient and pure bacterial growth and therefore, the final results may not be available until 2 months from receipt of the primary specimens.

The radiometric Bactec 460 method is based on the principles of the proportion method, developed in the 1980s (158), and is now used on a routine basis in many industrialised countries. It is a variant of the proportion method in which production of 14C-labeled CO_2, a metabolic breakdown of palmitic acid, is measured. The production in a standard bacterial inoculum in the presence of drug is compared to the labelled CO_2 produced by a 1:100 dilution of the original inoculum in the absence of drugs. Results are available within 4 to 12 days following inoculation, but the technique suffers from certain disadvantages primarily the need for radioactive isotopes and the use of needles.

In association with the outbreaks in the early 1990s it was demonstrated that delays in laboratory detection of drug resistant TB contributed to the magnitude of the outbreaks, therefore CDC now encourages laboratories to report susceptibility test results within 30 days of specimen collection thereby necessitating the use of rapid methods (175). During the last decade, three fully automated liquid based culture systems with continuously monitoring were developed for drug susceptibility testing of *M. tuberculosis* to the first-line antituberculous drugs: ESP II (Trek Diagnostics System, Westlake, OH), BACTEC MGIT 960 (Becton Dickinson, Sparks, MD), and MB/BacT ALERT 3D system (Organon Teknika, Belgium). The main advantages of these systems are the elimination of radioactivity, hands-on-work and use of needles. None of these methods are yet approved by the WHO.

Studies comparing the performance of the ESP II and MB/BacT Alert systems to reference proportion method and radiometric method found that they were reliable especially for the determination of INH and RMP resistance with comparable turnaround time (22, 47, 147, 193).

We found that the performance of the MGIT 960 system for DST on 222 clinical *M. tuberculosis* strains was excellent with a overall agreement of 98.9% for RMP, INH and EMB and 100% for PZA (V). The system correctly detected all 23 MDR strains indicating that the system is reliable in detecting such strains. The same observations were made in other studies carried out in Europe (11, 18, 190).

Susceptibility testing to EMB remains a challenge for the diagnostic laboratories. The low reproducibility and efficiency has been documented in two worldwide studies (84, 85). Recently, the low agreement between Bactec 460 and reference agar proportion method was addressed in a multicenter study (98). In our setting EMB test revealed a sensitivity of 88.2% and a specificity of 99.0% compared to the radiometric and the agar proportion method. Low agreement was also described in previous studies (2, 18, 190). As suggested in the multicenter study, EMB monoresistance should not be reported and resistant results should be verified by retesting including testing with the high concentration.

In Denmark, susceptibility testing for PZA has been performed routinely on all isolates by Bactec 460 since 1990. Acquired PZA resistance is not a problem in Denmark (76), but natural PZA resistance of *M. bovis* should be identified as the treatment strategy differs. PZA is active only at acidic pHs, therefore in order to obtain correct measurements of the in vitro activity of the drug the pH of the growth medium must be adjusted from 6.8 to 5.5. However, many isolates either fail to grow or grow poorly in agar-based methods. Until recently the Bactec 460 system has been the only reference method for PZA susceptibility testing. We assessed performance of MGIT PZA test medium on 57 clinical *M. tuberculosis* complex (6 *M. bovis* BCG and 2 *M. bovis*) isolates and found a 100% agreement between the two systems (V). The pH of test media is 5.9 and sufficient growth is achieved for all isolates. MGIT PZA test medium was initially evaluated by Pfyffer at al., who reported an overall agreement of 96.6% on 58 clinical isolates (127).

There are factors interfering with the DST results that warrant special attention. These are the purity of the culture, the homogeneity and the size of the inoculum. The studies previously evaluating the new broth systems for DST reported predominantly false resistant results (2, 11, 18), and Tortoli et al. pointed out that this could be due to contamination (190). In order to address this problem we performed purity checks on all resistant cases on a routine basis, and found that 2.3% of the isolates were contaminated. This could be explained by the content of the media, which supports the growth of the organism (62, 153, 162, 191). To avoid false registration of resistant strains, the laboratory should be aware of the contamination risk and might consider routine purity check of resistant isolates.

Although, turnaround times were comparable, MGIT does provide an advantage as inoculation can be done on any weekday as the growth is monitored automatically. Furthermore, in the majority of cases primary isolation and DST can be performed within a maximum of 30 days.

3.3. GENOTYPIC METHODS

Considerable progress has been made towards understanding the molecular basis of antimicrobial resistance, subsequently leading to the development of methods for rapid identification of resistance patterns. There are several reasons to apply genotypic methods for the identification of antimicrobial resistance. The major benefit is in the guidance of early antimicrobial treatment i.e. before time-consuming, growth based phenotypic susceptibility test results are available.

To date, resistance mechanisms and responsible genes are known for all four first-line drugs and for some of the frequently used second-line drugs. The genes and the associated minimal inhibitory concentration (MIC) values are shown for susceptible as well as resistant strains in **Table 7**.

When applying genotypic methods for rapid drug susceptibility testing, the major concern is the correlation to susceptibilities determined by phenotypic methods as these are still considered the gold standard.

3.3.1. Rapid molecular determination of the MDR-TB

Time-consuming drug susceptibility testing postpones effective treatment of patients suffering from MDR-TB as treatment is normally initiated with standard first-line antituberculosis drugs. Additionally, the risk of spreading MDR-TB in the community persist as patients with pulmonary smear positive tuberculosis are routinely isolated during the first two weeks of treatment only unless resistance or non-compliance is suspected. It is reported that rapid initiation of appropriate therapy significantly affected survival among HIV-positive patients (122, 197).

Since 1991, only 19 patients with MDR-TB (0.5%) have been identified in Denmark (177, 179, 182). However, countries close to Denmark have reported much higher prevalence. TB notification rates and drug resistance magnitude in selected European countries is listed out in **Table 8** according to the European TB surveillance data (204). Countries with low MDR-TB prevalence at the moment may experience an increase due to the immigration from MDR-high prevalence countries and due to increased travelling activity in the future.

RMP resistance is an internationally accepted marker for MDR-TB, as RMP and INH resistance almost always occurs simultaneously. It is caused by mutations in the *rpoB* gene that encodes the

Table 7. Genes with mutation, attributed percentage of resistance and the associated minimal inhibitory concentration values in *M. tuberculosis* complex.

Antimicrobial agent	MIC (μg/ml) for susceptible strains	Gene	Percent resistance	MIC (μg/ml) for resistant strains	References
Rifampin	0.5	rpoB	>93	≥ 2	VI, 173, 206
Rifabutin	0.06-8	rpoB	*	4-64	67, 214
Isoniazid	0.05-0.2	KatG, inhA ahpC, kasA	90	>0.2	104, 141, 172, 217
Ethionamide	0.6-2.5	InhA, ethA	ND	>2.5	13, 110
Ethambutol	1-5	EmbB	50-65	>10	6, 166
Pyrazinamide	16-100	pncA	72-97	>100	155, 165
Ofloxacin & ciprofloxacin	≤0.5-2.0	gyrA	>94	>4	VII, 171
Amikacin	0.5-1	rrs	76	>2	5, 125
Kanamycin	1-4	rrs	67.4-76		5, 168
Capreomycin	1-50	rrs	ND	>5	125

*) About 30% of rifampin resistant isolates are rifabutin sensitive;
ND: Not determined.

beta-subunit of the RNA polymerase (112, 173, 206), thereby diminishing rifampin-binding affinity for the polymerase. Detection of *rpoB* gene mutations by DNA-based assays, therefore offers alternative rapid approaches for detection of MDR-TB. There are numbers of methods available to detect these mutations (sequencing, SSCP). DNA sequencing may not be optimal, as a small fraction (e.g. 1%) of resistant bacteria may be difficult to detect. On the other hand, detection by array systems as line probe assay (e.g. Inno-LiPA Rif.TB, Innogenetics, Belgium) makes it possible to detect resistant bacteria from a mixed bacteria population. This system is based on the reverse hybridization of the *rpoB* amplicons to specific probes immobilized on membrane-based strips. Furthermore, these techniques allows for detection of *M. tuberculosis* complex in the sample simultaneously with detection of any mutation causing RMP resistance through hybridization with the corresponding amplified DNA.

Array technique has previously shown efficient performance in drug susceptibility testing when applied on isolates (3, 70, 143). This

Table 8. Tuberculosis and drug resistance in selected European countries in 2001.

Country	Notification rate/100,000	Never treated		Previously treated	
		any resistance (%)	MDR (%)	any resistance (%)	MDR (%)
Albania	18.2	11.0	1.0	67.0	40.0
Armenia	37.0	30.2	5.5	71.6	37.3
Austria	13.3	5.4	0.3	14.6	2.4
Belgium	12.9	7.5	2.3	8.2	1.4
Bosnia & Herzegovina	62.7	1.2	0.2	3.2	1.3
Bulgaria	49.1	47.1	29.5	69.9	30.1
Croatia	32.3	2.5	0.3	7.6	3.2
Czech Republic	13.2	4.1	1.2	6.7	6.7
Denmark	9.6	12.6	0.0	16.7	0.0
Estonia	59.0	33.3	14.1	65.8	51.2
Finland	9.5	6.6	0.9	11.4	3.8
France	10.6	6.3	0.9	12.7	3.9
Georgia	112.2	71.9	7.4	92.3	51.2
Germany	9.2	8.3	1.8	17.6	7.4
Greece	5.8	NA	NA	NA	NA
Hungary	31.8	12.0	1.9	25.5	6.4
Israel	9.1	23.5	5.8	56.4	21.7
Italy	7.8	14.4	0.9	45.4	30.3
Latvia	86.5	31.5	10.9	32.1	27.3
Lithuania	81	24.4	7.7	56.4	39.8
Macedonia	34.1	9.2	0.8	60.0	40.0
Moldova	89.2	36.2	13.1	51.1	24.0
The Netherlands	9.0	7.0	0.4	0.0	0.0
Norway	6.4	17.0	1.1	18.8	9.4
Poland	27.7	6.2	1.2	16.6	8.5
Portugal	43.8	12.5	1.7	20.0	8.5
Romania	136	13.3	4.5	27.1	13.7
Russian Federation	95.7	NA	NA	NA	NA
Serbia & Montenegro	27.4	2	0.6	1.1	0.0
Slovakia	19.9	2.6	0.2	9.0	4.5
Slovenia	18.7	5.4	1.1	11.5	0.0
Spain	18.7	5.4	0.8	22.8	12.4
Sweden	4.8	10.3	0.6	23.8	9.5
Turkey	27.9	NA	NA	NA	NA
Ukraine	74.9	18.6	7.5	43.9	28.0
United Kingdom	11.8	10.2	1.8	11.0	3.2

NA: Not available.

procedure is, however, still time-consuming, as bacterial growth must be awaited for 2-8 weeks. We demonstrated the ability to achieve rapid and reliable detection of rifampin resistance directly on most clinical specimens as we were able to amplify *rpoB* DNA from 78.3% of clinical specimens (VI). Therefore, it is now possible to have the diagnosis of MDR-TB just a few days after sample collection. This offers a considerable potential improvement in the management of MDR-TB as these vulnerable patients can commence treatment with second-line drugs while still in isolation. Transmission may therefore be arrested and the individual impact of MDR-TB is minimized. Due to the cost of the analysis it seems reasonable to restrict the analysis to smear-positive specimens from subgroups such as patients originating from areas with high incidence of MDR-TB, immunocompromised patients and patients previously treated for TB (55, 119, 179, 201).

Previous studies have shown regional variation in the *rpoB* gene mutations responsible for the rifampin resistance. In Denmark, the most frequent mutation was a Ser531Leu mutation followed by His526Tyr, in line with previous findings worldwide (**Table 9**) (70, 112, 173, 206).

Included in this study were also Lithuanian isolates and primary specimens. This study is the first dealing with the distribution of mutations in the *rpoB* gene in Lithuania. As seen in Denmark, the Ser531Leu mutation was the most frequent yet followed by the Asp516Val mutation (Table 9) (VI). This is in contrast to previous reports on the distribution worldwide. However, this mutation has recently been reported as the most frequent one in Hungary and Latvia (15, 196). One strain verified to be RMP resistant did not have any mutation within the *rpoB* gene, suggesting that mutations outside this gene could contribute to occurrence of rifampin resistance in agreement with previous studies (173, 206).

Correlation between the degree of rifampin resistance (as determined by MIC-values) and the type of nucleotide substitution has previously been shown. Mutations in codon 526 and 531 correlated with high-level resistance; where as mutations in codon 516 yielded low level resistance. Furthermore, the codon 526 and 531 mutations showed cross resistance between rifabutin and rifampin whereas this is not the case for the codon 516 nucleotide substitution (205, 214). Strains with the codon 516 mutations were usually susceptible to rifabutin and rapid detection of this mutation can predict rifabutin susceptibility. The five isolates in our study that carried the 516 mutations were all rifabutin susceptible (VI).

3.4. SECOND-LINE DRUGS IN TREATMENT OF MDR-TB: STANDARDISATION OF SUSCEPTIBILITY TEST METHOD

Standard methods for testing of first-line drugs have been established and an external quality assurance program has been organised by WHO (85). No such programs exist for second-line drugs, despite the fact that effective treatment of patients with MDR-TB has become increasingly important (59, 169). Various drug susceptibility testing methods for second-line drugs have been proposed (125, 157, 211), but there is no internationally accepted reference method. Standardisation of drug susceptibility testing procedures for second-line antituberculous drugs is therefore urgently needed. In order to assess the feasibility and cost-effectiveness of using second-line drugs in the management of patients with MDR-TB in middle and low-income countries, the WHO established the Directly Observed Treatment Short-course (DOTS)-Plus Working Group and the Green Light Committee. This is emphasised by the fact that expensive second-line drugs should become widely available (210).

As a first attempt to establish a quality assurance program for susceptibility testing of *Mycobacterium tuberculosis* to fluoroquinolones (FQ) (ofloxacin and ciprofloxacin), 20 strains with different FQ susceptibility patterns collected among nine mycobacterial reference laboratories of the Nordic and Baltic countries and DST was carried out. Susceptibility testing to FQ was performed according to routine procedures in each laboratory – one laboratory used the agar proportion method, the others the proportion method on Bactec 460. Results were compared to sequence analysis of the *gyrA* gene and minimal inhibitory concentration determination (MIC). Most laboratories found identical susceptibility patterns. Two isolates were resistant as they contained mutations in the *gyrA* fragment and their MICs were found to be >8 μg/ml. These resistant strains were correctly identified by all laboratories. The remaining 18 isolates were reported sensitive by six laboratories but three laboratories each reported one susceptible strain as resistant. The false results were caused by heavy inoculation and errors in the interpretation of the results. All these isolates had a wild type sequence in the analysed region of *gyrA* and the MICs were ≤1 μg/ml (VII).

These results indicate that the participating laboratories yield reliable results in detection of FQ resistant strains, although the need for external proficiency panels for drug susceptibility testing for FQ is stressed by the strains falsely reported as resistant. Such studies will improve the reproducibility of susceptibility testing as seen with first-line drugs (84, 85).

The task of standardisation of susceptibility testing of second-line drugs could initially be performed by the supranational reference laboratories, such that each laboratory would provide guidelines for certain drugs (genetic analysis, MIC value and method). Furthermore, the laboratories could provide blinded test panels, containing well-characterised strains with different susceptibility patterns, and distribute them to the national laboratories. Finally standardised stock solutions of second-line drugs should be made commercially available in order to minimise laboratory errors, as in the case of first-line drugs.

Table 9. Comparison of results obtained by the DNA array and DNA sequencing on 36 MDR-*M. tuberculosis* isolates.

DNA array pattern[a]	Mutations identified by DNA sequencing	No. of isolates[b]
R5 (Ser531Leu)	TCG → TTG (Ser531Leu)	11/11
R2 (Asp516Val)	GAC → GTC (Asp516Val)	1/4
R4a (His526Tyr)	CAC → TAC (His526Tyr)	2/0
R4b (His526Asp)	CAC → GAC (His526Asp)	1/0
ΔS1	CAA → CCA (Gln513Pro)	1/0
ΔS2	ATG → GAC (Met515Leu) ATC → TAC (Asp516Tyr)	1/0
ΔS4	CAC → CCC (His526Pro)	1/0
	CAC → CTC (His526Leu)	0/1
ΔS5	not interpretable	0/1
Wild type	no mutation	0/1

a) The DNA array provided the exact type of mutation in 85% of isolates.
b) Values are numbers of isolates from Denmark/numbers of isolates from Lithuania.

4. CONCLUSION AND PERSPECTIVES

Early diagnosis of TB and determination of drug resistance is crucial for the initiation of treatment and interruption of the chain of transmission. We have shown that by using strand displacement amplification more than 80% of culture verified respiratory TB cases could be identified within a few hours upon arrival of the sample. Applying the rapid amplification method on smear positive specimens makes differentiation between *M. tuberculosis* and NTM possible, thereby assisting initiation of correct management of these patients. However, there is still need for optimisation of the method in order to improve its performances in smear negative respiratory specimens as poor prognosis and outcome is reported in this group of patients co-infected with HIV.

The incidence of nonrespiratory TB increases worldwide and is primarily seen in HIV-infected patients where it may be lethal and presents particular problems of diagnosis due to the paucibacillary nature of the disease. Despite that none of the NAA tests having been approved for the detection of *M. tuberculosis* complex in nonrespiratory specimens, and no standardised procedures for pro-

cessing such specimens exist, they are often applied with varying performances. We have shown that standard strand displacement amplification is a rapid and reliable tool for diagnosing TB in microscopy positive nonrespiratory specimens although it only provided little additional information regarding microscopy negative nonrespiratory TB. In order to obtain better results in microscopy negative nonrespiratory specimens, we modified the method and applied it to cerebrospinal specimens yielding a sensitivity of 85% with 100% specificity. This would have major clinical implications on patient management when tuberculous meningitis is suspected. Whether this modified procedure may also be applied to other nonrespiratory specimens as a rapid tool remains to be elucidated, but this may very well be the case.

Despite the fact that TB is a highly prevalent infectious disease worldwide, the clinical diagnosis remains a true challenge due to its varying localisation, appearance and symptoms. Malignancy is an important differential diagnosis and samples may only have been sent for histopathologic examination. Such samples are formalin-fixed and paraffin-embedded making microbiological examination impossible. We analysed both prospectively and retrospectively collected histological tissue specimens by the strand displacement amplification method and found excellent performance though further improvement is necessary in order to optimise the performance in old archival specimens.

Infections and mortality caused by nontuberculous mycobacteria has increased mainly due to the AIDS epidemic and treatment remains a clinical challenge as it varies according to the species encountered. In order to promote the identification procedure and establish rapid correct diagnosis at species level, we described a new amplification procedure used directly on microscopy positive specimens. This made it possible to identify the majority of nontuberculous mycobacteria in one day as compared to up to several weeks by the conventional methods.

Tools that generate rapid and correct drug susceptibility test results are increasingly important in the management of TB. For this purpose, we assessed the performance of an automated nonradiometric culture system to generate susceptibility tests results of four first-line antituberculous drug on a large number of drug resistant isolates and found the system to be rapid and reliable. None of the automated nonradiometric culture systems are yet standardised or approved by WHO due to lack of enough data.

Time-consuming *in vitro* drug susceptibility testing postpones effective treatment and management of patients infected with MDR-TB. We demonstrated the ability of a DNA array technique to provide rapid and reliable detection of mutations causing for rifampin resistance in 78.3% of clinical specimens, thereby establishing the MDR-TB diagnosis within a few days of sample collection. Continued genotypic studies are warranted especially for rapid identification of INH resistant *M. tuberculosis* complex strains, since the majority of drug resistant TB in Denmark contains INH resistance. Patients suffering from INH resistant TB receive INH for up to 2 months until *in vitro* drug susceptibility test results are available. These patients thus receive suboptimal treatment with the additional risk of developing further resistance and suffering from the serious side effects of INH.

The emergence of MDR-TB requires laboratories to perform reliable drug susceptibility tests including second-line drugs. Standard methods for testing of first-line drugs have been established and an external quality assurance program has been organised by WHO. No such programs exist for second-line drugs. The first attempt to establish a quality assurance program for susceptibility testing of *Mycobacterium tuberculosis* to fluoroquinolones was carried out among mycobacterial reference laboratories of the Nordic and Baltic countries. We need strongly to continue this task including other second-line drugs, since MDR-TB is a huge problem in the Baltic States.

Finally, drug susceptibility test methods and critical drug concentrations for first-line drugs are standardised for *M. tuberculosis* complex, the same is not true for NTM. Future studies aiming to clarify this subject are needed.

5. SUMMARY

This thesis is based on studies carried out during my appointment as a research fellow at the International Reference Laboratory of Mycobacteriology, Statens Serum Institute, Copenhagen, from 2000 to 2003.

About 8 million new cases of TB occur worldwide annually, leading to 2 million deaths. Mortality is particularly high in case of infection with multi-drug resistant strains and in those co-infected with HIV. Early diagnosis of TB and drug resistance improves survival and prevents spread of the bacteria. The conventional methods are hampered by low sensitivity and time consuming procedures.

This review addresses the microbiological diagnostics of TB and other mycobacteriosis with special reference to the new and rapid molecular biological diagnostic methods (I, II, III, IV), and describes key methodological problems associated with the diagnosis of resistant TB with special reference to these methods (V, VI, VII).

We worked with a new strand displacement amplification method to detect the *M. tuberculosis* complex bacteria in both respiratory and nonrespiratory specimens and found that the method provided rapid identification of the most infectious and vulnerable TB patients in a few hours although when applied to smear-negative nonrespiratory specimens improvement is necessitated (I). By modifying the method and applying it to cerebrospinal fluid specimens, we found a considerable better performance (II). This could have major clinical implications on patient management since rapid initiation of antituberculous treatment in tuberculous meningitis is proven to decrease mortality and morbidity. This protocol may prove beneficial when applied to other sterile body fluid specimens as well. Furthermore, we described a method to analyse the formalin-fixed and paraffin-embedded specimens with necrotizing granulomatous inflammation, when TB is not initially suspected. The method showed a good performance and provides an additional opportunity to identify *M. tuberculosis* bacteria, where no fresh specimen is available for routine microbiological TB diagnostic (III). Finally, we worked with a DNA array method for discrimination between clinically relevant mycobacteria in microscopy positive clinical specimens in order to provide rapid correct diagnosis (IV).

The increased prevalence of drug-resistant TB necessitates rapid and accurate susceptibility testing. We assessed the performance of a nonradiometric automated culture system for susceptibility testing of *M. tuberculosis* bacteria to four first-line antituberculous drugs and found it to be rapid and reliable especially in the detection of multi-drug resistant bacteria (V). This culture based phenotypic method is, however; still time consuming due to need for bacterial growth. As rapid initiation of appropriate therapy significantly affected survival among HIV-positive patients and reduced the transmission of the drug resistant strains in several reports, we applied a DNA array method directly in clinical specimens to detect rifampin resistance which is a marker for MDR-TB. In nearly 80% of the cases this method provided results within a few days of specimen collection (VI). Finally, due to the lack of rapid standardised methods for drug susceptibility testing for second-line drugs, the first attempt to establish a quality assurance program for susceptibility testing of *Mycobacterium tuberculosis* to fluoroquinolones was performed in Nordic Baltic collaboration. The study pinpointed current procedural flaws and listed suggestions for improvement (VII).

THE PRESENT THESIS IS BASED ON THE FOLLOWING ARTICLES:

I. Johansen IS, Thomsen VØ, Johansen A, Andersen P, Lundgren B. Evaluation of a new commercial assay for diagnosis of pulmonary and nonpulmonary tuberculosis. Eur J Clin Microbiol Infect Dis. 2002;21(6):455-60.

II. Johansen IS, Lundgren B, Tabak F, Petrini B, Hosoglu S, Saltoglu N, Thomsen VØ. Improved sensitivity of nucleic acid amplification for rapid diagnosis of tuberculous meningitis. J Clin Microbiol. 2004;42(7):3036-40.

III. Johansen IS, Thomsen VØ, Forsgren A, Hansen BF, Lundgren B. Detection of Mycobacterium tuberculosis complex in formalin-fixed, paraffin-embedded tissue specimens with necrotizing granulomatous inflammation by strand displacement amplification. J Mol Diagn. 2004;6(3):231-6.

IV. Johansen IS, Lundgren B, Thyssen JP, Thomsen VØ. Rapid differentiation between clinically relevant mycobacteria in microscopy positive clinical specimens and mycobacterial isolates by line probe assay. Diagn Microbiol Infect Dis. 2002;43(4):297-302.

V. Johansen IS, Thomsen VØ, Marjamäki M, Sosnovskaja A, Lundgren B. Rapid, automated, nonradiometric susceptibility testing of Mycobacterium tuberculosis complex to four first-line antituberculous drugs used in standard short-course chemotherapy. Diagn Microbiol Infect Dis. 2004;50(2):103-7.

VI. Johansen IS, Lundgren B, Sosnovskaja A, Thomsen VØ. Direct detection of multidrug-resistant Mycobacterium tuberculosis in clinical specimens in low- and high-incidence countries by line probe assay. J Clin Microbiol. 2003;41(9):4454-6.

VII. Johansen IS, Larsen AR, Sandven P, Petrini B, Soini H, Levina K, Sosnovskaja A, Skenders G, Hoffner S. Drug susceptibility testing of Mycobacterium tuberculosis to fluoroquinolones: first experience with a quality control panel in the Nordic-Baltic collaboration. Int J Tuberc Lung Dis. 2003;7(9):899-902.

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