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REVIEW

Molecular diagnosis of tuberculosis: current clinical validity and future perspectives

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ABSTRACT: The rapid development and availability of a variety of new molecular genetic technologies present the clinician with an array of options for the accurate diagnosis of infectious diseases. This is particularly the case for tuberculosis, since molecular methods have been rapidly introduced into all working areas of the mycobacteriology laboratory.

Nucleic acid amplification methods to detect *Mycobacterium tuberculosis* in clinical specimens are increasingly used as a tool to diagnose tuberculosis. The bulk of recently available data from clinical evaluations performed under routine laboratory conditions indicate that these molecular methods are rapid and sensitive, but yet inferior, to culture with regard to sensitivity and specificity. Therefore, until gene amplification tests have proved to be reliable and quality control procedures exist, their clinical validity remains controversial. Consequently, definition of selected clinical applications of gene amplification to routine diagnosis of tuberculosis is important and need to be discussed.

This review will focus on the clinical role of molecular methods in the direct detection and diagnosis of *M. tuberculosis* in clinical samples. In addition, molecular genetic approaches designed to determine drug susceptibility and to discriminate strains below the species level will be outlined and discussed in terms of their current and future clinical applicability.

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The pathogen causing tuberculosis, *Mycobacterium tuberculosis*, has a rather long generation time of approximately 18 h. It is well known that, as a result of this fact, all microbiological reports (from isolation by culture to susceptibility testing) are delayed for weeks, although improved techniques, such as the radiometric culture system, are available. Moreover, tuberculosis continues to be, as it has been for centuries, one of the most prevalent infectious diseases of humans and is the leading cause of mortality from a single infectious disease worldwide [1]. Laboratory methods play a crucial role in establishing the diagnosis, monitoring therapy, and preventing transmission of tuberculosis. In addition, the importance of the mycobacteriologist has grown, in view of a changing epidemiology (*e.g.* social factors, the acquired immune deficiency syndrome (AIDS) pandemic) and an increasing resistance of *M. tuberculosis* to drugs [2, 3].

To meet the aim of managing tuberculosis, laboratory services have to be provided in a shorter time and with the aid of more accurate methods [4, 5]. However, with the development of new techniques, such as the detection of microorganisms by hybridization with probes introduced in the 1970s [6], and immunological procedures [7–9], limitations in the sensitivity and/or specificity of established techniques have become apparent. The greatest breakthrough, as for the entire field of infectious diseases, came from biotechnology, with the introduction of nucleic acid amplification techniques (NATs) [10]. Gene amplification can achieve the goal of reducing the generation time of microorganisms to minutes, and of replacing biological growth on artificial media by enzymatic reproduction of nucleic acids *in vitro* [11]. The importance of nucleic acid amplification methods lies in their wide applicability in the life sciences, and their potential to revolutionize the practice of medicine. Examples are nucleic acid sequence analysis and genetic fingerprinting [12–14].

Direct detection of *M. tuberculosis* by amplification of nucleic acids

Variety of methods

Since its first application to the diagnosis of tuberculosis in 1989 by Brisson-Noël *et al.* [15], the polymerase chain reaction (PCR) has become the most widely-used technique for amplifying nucleic acids from Mycobacteria. Nevertheless, almost the complete panel of alternative amplification methods available has already been used, including strand displacement amplification (SDA), transcription-mediated amplification (TMA),

Table 1. – Overview of *in vitro* nucleic acid amplification techniques (NATs) for the detection of *Mycobacterium tuberculosis*

NAT	Principle of the method and representative references	Target sequence	Manufacturer and detection format of amplified product
Target amplification			
PCR	Thermal cyclic synthesis of dsDNA by hybridization of specific oligonucleotides to ssDNA target, extension to dsDNA by a thermostable polymerase and denaturation of ssDNA, which serves as a new target for the next cycle [19–23].	IS6110 65 kDa protein gene 16S rDNA gene MPB64 gene 35 kDa protein gene	Roche Molecular Systems (16S rRNA, Amplicor™ Testkit). Colorimetric, automated sandwich-hybridization assay using horseradish peroxidase (Cobas Analyzer).
TMA	Autocatalytic, isothermal synthesis of RNA. Reverse transcriptase copies the RNA target into a transcription complex, which is then amplified by RNA polymerase produced transcripts [24].	16S rRNA	Gen-Probe (MTD, Testkit). Homogeneous solution hybridization using an acridinium ester-labelled DNA probe and a luminometer.
SDA	Isothermal synthesis of ss- and dsDNA. Targets with *HincII* sites are generated following a cascade of primer annealing, extension and displacement of extended strands by the aid of an exonuclease-deficient Klenow fragment of polymerase I. These targets are then amplified in cycles of nicking with *HincII* binding of primers and extension/displacement reactions, whereas sense and antisense reactions are coupled. Strands displaced from the sense reaction serve as targets for antisense reactions and vice versa [25].	IS6110	Beckton Dickinson*. Microtitre plate sandwich-hybridization assay using an alkaline phosphatase-labelled detector probe and chemiluminescence.
NASBA	Autocatalytic, isothermal synthesis of RNA. Hybridization of a specific probe with a T7-promoter sequence to the RNA target, cDNA synthesis (reverse transcriptase, AMV-RT) and subsequent hydrolysis of RNA from the RNA-cDNA hybrid (RNAase H). AMV-RT produces dsDNA, from which multiple copies of RNA transcripts are generated by T7 RNA polymerase. This RNA is reintroduced into the cycle of amplification [26].	16S rRNA	Organon Teknika*. Automated homogeneous quantitative reading system using electrochemiluminescence (QR System Analyzer).
Probe/primer amplification			
LCR	Thermal cyclic synthesis of dsDNA by hybridization of specific oligonucleotides to ssDNA target, extension to dsDNA by filling the gap between the bound probes using a thermostable polymerase and ligation by a thermostable ligase. Like PCR, the sequences generated serve as new targets for the next cycle after denaturation to ssDNA [27].	Protein antigen b gene	Abbott. Automated system (LCx Analyzer) using microparticle enzyme immunoassay and fluorometric detection of catalyzed substrate.
Q-Beta	Autocatalytic, isothermal replication of bacteriophage Q-Beta RNA coupled to a detector probe. Hybridization of specific capture and detector probes to the RNA target and amplification of the captured, washed and then released detector probe by Q-Beta replicase [28].	23S rRNA	GeneTrak*. Sandwich-hybridization dual-capture reversible paramagnetic target capture and direct fluorometric detection of RNA produced (binding of propidium iodide).
Signal amplification			
bDNA	Capture probes on a solid phase and target probes hybridize with target nucleic acids, bDNA (amplimer) is added and hybridizes with the target probe-target nucleic acid hybrids. Multiple alkaline phosphatase-labelled probes hybridize with the amplimer, followed by incubation with a chemiluminescent substrate [29].	As for PCR	Chiron. Microtitre plate assay using chemiluminescence detection.
Infection with mycobacteriophages			
LRM	Mycobacteriophage (*e.g.* mutant of pHAE40 derived from phage TM4) carries the gene for firefly luciferase and infects viable Mycobacteria. The replicated phage products are detected by simply adding luciferin as a substrate and by measuring light emission [30].	Injected phage gene for luciferase	Microtitre plate assay using a luminometer. This assay is currently used for research purposes to screen antimicrobial substances against *M. tuberculosis*.

PCR: polymerase chain reaction; TMA: transcription-mediated amplification; SDA: strand displacement amplification; NASBA: nucleic acid sequence-based amplification; AMV: avian myoblastosis virus; dsDNA: double strand deoxyribonucleic acid; ssDNA: single strand DNA; RNA: ribonucleic acid; cDNA: complementary DNA; rRNA: ribosomal RNA; LCR: ligase chain reaction; Q-Beta: Q-Beta replicase amplification; bDNA: branched DNA signal amplification; LRM: luciferase reporter mycobacteriophage assay. *: assays are under development (commercially available, presumably by 1997).

nucleic acid sequence-based amplification (NASBA), Q-Beta replicase amplification, ligase chain reaction (LCR), branched deoxyribonucleic acid (DNA) signal amplification (bDNA), and reporter mycobacteriophage methods (firefly luciferase assay). Good reviews are available, to which the reader is referred for further methodological details [16–18]. All of these methods are summarized briefly in table 1. In order to provide a framework for understanding NATs, they are subdivided into three categories: 1) target amplification systems; 2) probe or primer amplification systems, which exploit hybridization of short probes (primers) to the target and various enzyme activities to modify or synthesize DNA or ribonucleic acid (RNA); and 3) signal amplification, in which the signal generated from probes is enhanced by means of compound probes or branched-probe technology, without the aid of the above-mentioned enzymes.

The common objective of all technologies underlying *in vitro* amplification of mycobacterial nucleic acids is: to reduce the time necessary to detect the pathogen in clinical specimens; to increase the sensitivity and specificity; and to simplify the test by automation and incorporation of nonisotopic detection formats. The diagnostic techniques are diverse and are presently at various stages of development. PCR, TMA and LCR have recently been made available in a kit-based, user friendly format, and the other techniques will follow in the near future (table 1). Each method has certain advantages, but the impact of any single method on clinical sensitivity has not yet been convincingly demonstrated, as will be discussed in the following sections. In general, it is to be expected that any methodological advantage will be felt more on a merely technical level. For example, isothermal assays are more rapid and do not require special equipment, such as thermal cyclers. In contrast, PCR is simple and the most widely-used method, with good availability of reagents. PCR is most flexible when different applications are requested in a research laboratory, because any in-house protocol can more easily be introduced and the largest supply of standardized kits for different pathogens is commercially available as a PCR format.

Applications that target RNA, including NASBA, TMA (which strongly resembles NASBA) or Q-Beta, are expected to be more sensitive, because RNA already occurs in high copy numbers in bacterial cells. However, it is often the case that a higher analytical sensitivity does not necessarily improve clinical sensitivity [17]. Rather, the performance of NATs in the detection of *M. tuberculosis* depends largely on factors such as collection, volume and preparation of samples [28, 31, 32]. Higher analytical sensitivity may even translate into a loss of specificity. Nested PCR, a popular modification of PCR using nested sets of primers and reamplification of the amplified nucleic acids from a first round of PCR [33], is very sensitive, but also extremely prone to carry-over contamination [17]. Ultimately, concrete descriptions of specific applications will have to be formulated for each of the different approaches. These will have to meet the criteria (discussed below) that define the indications for NATs in the routine clinical laboratory. The limiting factors will be the same for all of these techniques: how to avoid contaminations; and how best to prepare and process the sample.

Genus-specific targets

The basic inherent step necessary for all of these methods to work is the hybridization of nucleic acid probes to a specific target in the genome or RNA of *M. tuberculosis*. Nucleic acid probes are selected segments of DNA or RNA sequences that are chemically easy to synthesize, and that have been labelled with enzymes, antigenic substrates, chemiluminescent moieties, or radioisotopes available as commercially prepared kits. Under stringent conditions, they bind (hybridize) rapidly and specifically to target nucleotide complementary sequences. A current example of how efficient hybridization can be is presented by the probe technology used to identify the species of Mycobacteria from cultures (GeneProbe Accuprobe method). This method is now considered the state of the art for the rapid culture confirmation of *M. tuberculosis* or the *Mycobacterium avium* complex [5, 6, 34]. The target used by this method is the 16S ribosomal ribonucleic acid (rRNA); the attractive feature of this molecule is that it contains conserved genus-specific as well as various species-specific regions. Therefore, it was straightforward to use this molecule or the gene coding for the 16S rRNA, namely the 16S ribosomal deoxyribonucleic acid (rDNA), as a powerful target for amplification of Mycobacteria both on the genus and the species level [19, 24, 26, 35, 36]. Similarly, this strategy is applicable to other targets in the RNA operon, particularly the 23S RNA [28] or the more variable 23S-16S rDNA spacer [37, 38], or in genes coding for proteins common to all Mycobacteria [21, 23, 39, 40]. Species identification in these assays is performed by probing, restriction enzyme analysis or direct sequencing. Three potential concerns with genus-specific assays have to be discussed: 1) competitive amplification of undesired contaminants (*e.g. Mycobacterium gordonae*) may lead to reduced amplification of the relevant pathogen, which may, thus, escape detection by the species-specific probe; 2) the clinical utility of a purely qualitative assay to diagnose nontuberculous Mycobacteria in respiratory specimens using gene amplification remains rather questionable, with some exceptions, such as patients with AIDS; 3) all of the targets used are single-copy genes in slowly growing Mycobacteria, which, theoretically, results in an inferior sensitivity compared to repetitive targets [20].

IS6110: a repetitive target

The only repetitive target useful for a NAT in tuberculosis, which is so far available, is an insertion sequence (IS) designated IS6110 [20, 41]. The latter is specific for the *M. tuberculosis* complex and generally occurs in 1–20 copies per cell, making it an ideal target for amplification. Thus, IS6110-based NATs are the systems applied most frequently, generally as in-house PCR protocols [42, 43] (table 2). Although the question of an added sensitivity using the repetitive IS6110 has not yet been studied systematically, the high detection rate of IS6110 sequences in blood specimens [45, 46] and results from one study comparing IS6110-based PCR with 16S rDNA-based PCR [47] (table 3) point in this direction. Of course, one single study performed under restricted research conditions does not reflect the situation that will

Table 2. – Results of different studies for the detection of *Mycobacterium tuberculosis* deoxyribonucleic acid (DNA) using in-house polymerase chain reaction (PCR) methods compared with culture results and clinical data

	Methods					Results							
In-house PCR Reference	IC	Compared with	Specimens	Specimens (patients)	Positive cultures	Sensitivity microscopy*	Parameters of the NAT compared to culture %				Sensitivity (PPV) after discrepant analysis %‡		False-positive results NAT	
Target				n	%	%	Sen	Spe	PPV	NPV	NAT	Culture	n	%
1. Clarridge *et al.* [42] 1993 IS6110	Partially	FC, LJ, 7H11, BACTEC and clinical data	Mixed (58% respiratory)	1166#	19	67	83	99	94	96	83 (98)	99	9	(0.8)
2. Kirschner *et al.* [44] 1996 16S rDNA	Yes	FC, ZN, LJ, BACTEC and clinical data	Mixed (58% respiratory)	729#	15	51	80	94	89	96	83 (98)	84$	3	(0.4)
3. Own study (unpublished) IS6110, MPB 64 and 65 kDa protein genes	Yes	FC, LJ Septi-Check and clinical data	Mixed (65% respiratory)	502 (480)	6	32	88	99	81	99	90 (94)	84+	3	(0.6)
4. Nolte *et al.* [43] 1993 IS6110	Yes	FC, LJ and 7H11	Sputum	313#	39	88	91	99	99	94	No clinical data available, sensitivity of NAT inferior in culture (PPV NAT 100)			

*: reflects the occurrence of paucibacillary specimens in the selected patient group studied; ‡: the values were calculated following the data available; positive results from patients under therapy were excluded for comparison of NAT with culture; #: numbers of patients are not specified, but apparently more than one specimen was submitted for most of the patients; $: genus-specific PCR for all Mycobacteria (sequencing); specimens from patients under therapy with discrepant results and with *Mycobacterium genavense* infections were excluded (130 true-positives); +: all PCR positive specimens missed by culture were from extrapulmonary sites. IS: insertion sequence; IC: internal control for detection of inhibitors; FC: fluorochrome stain; ZN: Ziehl-Neelsen stain; LJ, 7H11: Löwenstein-Jensen or Middlebrook solid media; BACTEC and Septi-Check: culture in broth; NAT: nucleic acid amplification technique; Sen: sensitivity; Spe: specificity; PPV: and NPV: positive and negative predictive values, respectively.

Table 3. – Results of different studies for the detection of *Mycobacterium tuberculosis* deoxyribonucleic acid (DNA) using a commercially available polymerase chain reaction (PCR) test kit (Amplicor™; Roche Molecular Systems) compared with culture results and clinical data

	Methods					Results							
Amplicor reference	IC	Compared with	Specimens	Specimens (patients)	Positive cultures	Sensitivity microscopy*	Parameters of the NAT compared to culture %				Sensitivity (PPV) after discrepant analysis %‡		False-positive results NAT	
				n	%	%	Sen	Spe	PPV	NPV	NAT	Culture	n	%
1. Bennedsen *et al.* [48] 1996	Partially	FC, 7H11, BACTEC and clinical data	Respiratory	7194 (3738)	9	69	82	96	68	98	88 (95)§	100	30	(0.4)
2. Carpentier *et al.* [49] 1995	No	FC, LJ and partially BACTEC	Mixed† (84% respiratory)	2073 (1125)	9	58	86	98	82	99	No clinical data available, sensitivity of PCR inferior to culture (PPV NAT 95)		9	(0.4)
3. Stauffer *et al.* [50] 1995	No	FC, LJ, BACTEC and clinical data	Respiratory	722 (456)°	9	Not known	87	99	91	99	88 (97)	95	2	(0.3)
4. Schirm *et al.* [47] 1995 a) Amplicor b) In-house (IS6110)	In-house PCR only	ZN, LJ In-house PCR and clinical data	Respiratory	485 (340)	5	52	a) 75 b) 92	93 92	72 73	98 98	70 (76) 92 (83)	89	6	(1.2)

*: reflects the occurrence of paucibacillary specimens in the selected patient group studied; ‡: the values were calculated following the data available; positive results from patients under therapy were excluded for comparison of NAT with culture; †: the Amplicor Testkit is not recommended for samples other than specimens from the respiratory tract by the manufacturer; the results of Study No. 1 were not differentiated according to the source of the specimen; °: from some of these patients, other specimens, such as sputum or bronchoalveolar lavage, were examined (not shown); §: only valid when considering analysis of specimens with discrepancies in duplicate using two independent DNA preparations. For further abbreviations see legend to table 2.

be encountered when these methods are applied in a routine laboratory setting. It is evident that the detection of *M. tuberculosis* DNA in peripheral mononucleolar cells from immunocompetent patients remains debatable, until this finding is unequivocally confirmed in further studies. In our laboratory, we could obtain confirmation of IS6110 PCR-positive results from microscopy-negative and culture-positive respiratory specimens in only 81 and 93% of cases using single-copy genes, namely PCR with the 65 kDa heat shock protein gene [21], and the MPB64 protein gene [22] as targets, respectively (unpublished data) (table 2). These results underline the importance of comparing different methods, targets and the host range of specific sequences. Unfortunately, such studies have so far been performed too rarely [51]. The observation that the IS6110 could be amplified in *Mycobacterium ulcerans* and *Mycobacterium gilvum*, reported in 1996, 6 yrs after its first diagnostic application [52], may set a precedent for the need to correctly assess the specificity of targets of any NAT.

Sample preparation

The choice of specimen preparation strongly determines the subsequent performance of nucleic acid amplification assays. It is important to bear in mind that Mycobacteria: are unevenly distributed in the sample; often occur at an extremely low density in the sample; are detected in specimens that frequently contain inhibitors of the amplification reaction; and are not efficiently processed by classical lysis protocols, due to their resistant cell wall composition. Therefore, several prerequisites should be fulfilled. The method has to: concentrate larger amounts of specimen, without any significant loss of bacteria; remove substances inhibiting the amplification reaction; efficiently release the nucleic acids from the target cells; and offer a simpler protocol compatible with the routine clinical flow of work. Although effective disruption methods for Mycobacteria, such as sonication with glass beads and alkaline lysis, and simplified protocols have been described [32, 51, 53, 54], little attention has been paid to questions related to sample concentration [28]. Apparently, the most serious drawback is imposed by the fact that only very small amounts of the sample can currently be processed by molecular methods [28, 32], and this only after a laborious multi-step process of washing in several centrifugation steps. Large amounts of sample are easily processed by culture, for example, 20 mL of purulent sputum after decontamination and a single centrifugation step. The same decontamination protocols that are applied to culture are, typically, also used to prepare specimens for mo-lecular testing [31, 32, 44], but it must be kept in mind that these and other protocols do not sufficiently remove inhibitors and unwanted cell debris. Therefore, the presence of inhibitors affecting the efficiency of amplification is a problem that is frequently encountered when applying NATs as routine laboratory tests [42–44, 47]. This disturbing interference occurs more frequently when simplified methods of specimen preparation are used [42–44]. Since such unwanted substances can bind to the nucleic acids immediately after cell lysis, even improved nucleic acid purification methods cannot completely prevent this phenomenon [15].

In view of these issues, it can be stated that nucleic acid-based techniques are, at present, clearly inferior to culture methods. The future success of NATs will depend on how these intriguing problems of specimen preparation and input volume are solved. Ideally, sample concentration should accumulate bacteria or bacterial nucleic acids, while at the same time removing undesired substances to a significant extent. Improvements in the sensitivity of NATs will be achievable mostly through the introduction of new methods of specimen preparation, using, for example, magnetic enrichment of cells or nucleic acids [55, 56]. The higher cost of more sophisticated methods will be compensated for by a more efficient and sensitive detection that, ultimately, avoids unnecessary repetition of the assay and false-negative results. Research should be reorientated toward basic studies addressing these issues.

In summary, the specimens of most interest for molecular testing in tuberculosis are specimens from the respiratory tract, biopsies and cerebrospinal fluid [31]. The commercial tests have not yet been cleared for application to extrapulmonary specimens. A variety of different protocols for all types of specimens have been tested using in-house methods, but a standardized general method remains to be established. In particular, preparation of samples, such as lymph node tissue, remains unsolved. Due to problems with inhibitors, analysis of stool and blood samples is not yet recommended [32, 44]. It is worth noting that molecular techniques (similar to culture) detect bacteria, or particles of the bacteria, after centrifugation. Therefore, plasma, serum or swabs are inadequate specimens. Moreover, it is important to know that culture is always performed in parallel to the molecular method. For this reason, a sufficiently large volume of the sample must be submitted, especially in the case of cerebrospinal fluid, where a specimen of at least 5 mL is recommended.

Trends in clinical evaluation of NATs for diagnosing tuberculosis

Since PCR was introduced, there have been several phases of evaluation of the clinical utility of NATs for detecting *M. tuberculosis* in patient specimens. Early studies focused on testing different targets. These were followed by initial clinical investigations into the sensitivity and specificity, using a wide range of different sample preparation and amplification protocols [20–24, 42, 43, 46]. The overall result of these studies was that various difficulties arose in obtaining sensitivities and specificities comparable to those of conventional culture methods. It was soon recognized that NATs may be less sensitive compared to culture using respiratory specimens, and that positive results are obtained in patients with no clinically apparent signs of disease [42]. The problems encountered and conclusions drawn from the application of amplification chemistry to the detection of *M. tuberculosis* in clinical specimens can be summarized as follows:

1. One of the most significant problems that must be solved before NATs become clinically useful is false-positivity due to contaminating nucleic acids. Such contaminations come from two sources, namely amplification

Table 4. – Results of different studies for the detection of *Mycobacterium tuberculosis* rRNA (AMTD, Gen-Probe) compared with culture results and clinical data

AMTD reference	Methods				Results								
	IC	Compared with	Specimens	Specimens (patients) n	Positive cultures %	Sensitivity microscopy* %	Parameters of the NAT compared to culture %				Sensitivity (PPV) after discrepant analysis %‡		False-positive results NAT	
							Sen	Spe	PPV	NPV	NAT	Culture	n	%
1. Pfyffer *et al.* [31] 1996	No	FC, LJ, 7H11 BACTEC and clinical data	1117 respiratory and 322 nonrespiratory	1117 (998)#	9	40	85	95	67	98	85$ (90)	100+	11	(1)
2. Rocco *et al.* [60] 1994	No	FC, LJ BACTEC (only Lab H) and clinical data	Sputum	760 (246) Lab H 312 Lab L 448	8 33	54 88	65 93	99 99	94 99	97 97	89 (99)	100	2	(0.3)
3. Jonas *et al.* [24] 1993	No	FC, LJ, clinical data and IS6110 PCR	Sputum	758 (235)	16	56	80	97	82	99	82 (97)	88	4	(0.5)
4. Ehlers *et al.* [32] 1996	Partially	FC, LJ Septi-Check and clinical data	261 respiratory and 294 nonrespiratory	261 (242)	18	34	83	95	78	96	85$ (94)	85	3	(1.1)

*: reflects the occurrence of paucibacillary specimens in the selected patient group studied; ‡: the values were calculated following the data available; positive results from patients under therapy were excluded for comparison of NAT with culture; #: number of patients for all types of specimens; $: no significant difference comparing respiratory with nonrespiratory specimens; +: 15 of 50 discrepant cases (AMTD-positive only) were excluded because the patients were under therapy; of the remaining 35 specimens, 11 were considered false-positive and 24 true-negative (negative upon retesting, cut-off of the test defined as a zone). AMTD: Amplified *Mycobacterium tuberculosis* Direct Test. For further abbreviations see legend to table 2.

products generated daily in the laboratory, and genomic DNA or RNA from other specimens or cultures containing large amounts of target cells [17, 18]. Sterile bronchoscopes contaminated with residual DNA deserve mention as a potential source of false-positive results [57]. Therefore, setting quality standards to combat laboratory contamination is of the highest priority. NATs should only be used by experienced referral laboratories, that possess appropriate expertise, personnel, laboratory infrastructure and contamination control measures. The current uncritical neglect of these stringent requirements in many laboratories has led to the widely held, but mistaken, belief that clinical nucleic acid-based diagnostic tests are simple to perform and can easily be introduced in any normally equipped microbiology laboratory.

2. A second concern related to specificity is associated with the inherent ability of NATs to detect molecules without any information on the viability of the pathogen. Moreover, the tests used so far provide only qualitative results. Many open questions have arisen, including curious speculations on dormant infection as a cause of false-positive PCR results [51, 58], or the possibility of monitoring therapy by detecting nucleic acids of *M. tuberculosis* [56]. Conversion of acid-fast smear results in patients on antimicrobial therapy for tuberculosis is a well-documented and cost-saving rapid test. It is firmly established that nucleic acids are detectable long after effective therapy has been started and after conversion of culture and microscopy occurs [32, 59]. Studies are being performed to corroborate the limited data available on this issue at present. As long as these studies are still under way, warnings must be given not to use qualitative detection of DNA or rRNA as a marker for relapse or therapy failure, since it gives no reliable feedback to clinicians.

3. The sensitivity of nucleic acid-based assays clearly depends on the number of bacilli in a specimen, since it has been shown that sensitivity decreases significantly when microscopy-negative specimens are investigated (see references in tables 2–4). Further reasons for this are threefold: i) due to a loss of bacteria during sample preparation and other reasons discussed in the section above, the detection limit of the molecular methods rarely reaches a level of less than 10 bacteria·mL^{-1} of specimen; ii) Mycobacteria form clumps and are not distributed uniformly throughout the specimen. Sample inhomogeneity leads to discrepant results when paucibacillary specimens are processed. Obviously, this represents a mere methodological problem that can be overcome either by improving methods of sample preparation [56], or, in a more costly and unrealistic manner, by analysing more than one specimen from each patient; and iii) inhibitors strongly influence the sensitivity of nucleic acid amplification assays. However, it is noteworthy that this represents a problem for more or less every enzyme-based amplification method [24, 31, 32, 42, 43].

Sensitivity and specificity of NATs using respiratory specimens

The addition of NAT-based molecular methods to the clinical diagnosis of tuberculosis makes it necessary to distinguish between the "diagnostic" and the "clinical"

validity of these new approaches. On an analytical level, NATs perform well and are comparable to culture, with an excellent sensitivity and specificity in solutions of predetermined composition. Although application of NATs is now widespread, it is important to note that their clinical validity is still controversial. However, a consensus is emerging as results from trials performed with larger numbers of respiratory specimens under routine laboratory conditions have become available. In contrast, diagnosis of extrapulmonary tuberculosis, using specimens such as lymph node biopsy or urine, remains an aspect which has so far been poorly investigated [31, 32, 44]. Therefore, this issue is not considered in the following section. A selection of representative studies investigating respiratory specimens from defined patient populations has been compiled in tables 2–4. These tables will assist the reader to gain a broad overview of the performance of NATs in the diagnosis of tuberculosis using respiratory specimens. Three major results emerge from these data:

Firstly, before any conclusions can be drawn from the results of a given study, it is important to formulate variables that influence the determination of sensitivity. In general, it can be stated that sensitivity of NATs compared to culture ranges 65–95%. Due to problems in defining the gold standard and often missing clinical data or longer follow-up of patients, the sensitivities obtained in the studies shown in tables 2–4 are represented after comparison with the results of microscopy and culture. However, comparison with routine mycobacterial culture has certain limitations, because a portion of patients with tuberculosis will have negative cultures, and another portion of the patients will have received treatment before the specimens are submitted. Therefore, comparison of sensitivities for culture and NATs are shown in a further column, after resolution of discrepancies using clinical data and after exclusion of cases from patients under therapy, as far as this information was provided. In contrast to NATs, culture is often performed with more than one specimen. In some cases, culture is performed using only solid media, which are less sensitive than liquid culture procedures [24, 43, 47, 60]. Moreover, the studies have not all been performed in a blinded fashion, and it will be difficult to circumvent bias when "discrepancy resolution" is undertaken.

Estimation of sensitivity of the molecular method is influenced by factors determined by the patient population under investigation, mostly by the percentage of smear-positive samples. Therefore, the best way to measure the benefit of a NAT is to calculate the increment in sensitivity compared to the sensitivity of microscopy. Indeed, looking at studies Nos. 2 and 4 in table 2 reveals that study No. 4 seems more sensitive than study No. 2 when compared to culture (91 *versus* 80%), but a much higher increment in sensitivity over microscopy was obtained in study No. 2 (+29%) than in study No. 4 (+3%). The average of this value for all studies listed in tables 2–4 is approximately +25%. Due to the significance of this calculation, any laboratory performing molecular methods to diagnose tuberculosis should be able to provide these data by performing conventional and molecular methods in parallel, and this information should be requested by clincians.

Secondly, two issues related to predictive values of the NAT involved can be raised. When estimating the validity of a diagnostic tool in clinical practice, the knowledge about predictive values is of even greater significance than the knowledge about sensitivity and specificity of the assay. Therefore, it is important to remember that predictive values of an assay are strongly influenced by the prevalence of the disease in the patient population investigated. In the setting of low disease prevalence, in which the new tests are challenged, a small proportion of false-positive results will be meaningful in relation to a small proportion of true-positive results (positive predictive value (PPV) = true-positive results/all positive results obtained by test). On the other hand, false-negative results will not represent a significant relationship to a large number of true-negative results (negative predictive value (NPV) = true-negative results/all negative results obtained with the test). Therefore, PPVs of the molecular genetic methods rarely reach a value higher than 90% (see tables 2–4), although we must bear in mind that comparison with culture alone is of limited value, as stated above. Furthermore, the NPV of NATs is explicitly very high, irrespective of the prevalence of positive cultures. Thus, NPVs higher than 96% have been reported in most studies. The first conclusion that can be drawn is that screening samples to diagnose tuberculosis using gene amplification is unreasonable. This can be exemplified as follows: screening 200 specimens with an expected 2% prevalence of tuberculosis would result in four positive samples, 2 out of 4 of which are detected by microscopy, 3 out of 4 by NATs. Assuming a specificity of 99% for the amplification method, these results are faced by two false-positive results (PPV 60%). The second conclusion that deserves mention is that taking stringent measures to establish an indication for performing nucleic acid detection in clinical specimens will increase the prevalence, thereby leading to a higher PPV of the molecular genetic assay.

Thirdly, it is abundantly clear from the results shown in tables 2–4 that, although rigorous procedural and quality control practices are in use, false-positive results frequently occur. The rate of false-positive results can be estimated to lie between 0.2 and 1.5%, but we can assume that this figure reported under investigative conditions underestimates the true contamination rate in normal routine practice [31, 61].

Progress toward fulfilling the goal: standardization and quality control

Although the success of the gene amplification methods suggests that they are rapid, sensitive and lead to improved clinical management, it is recognized that the results have to be interpreted with extreme caution. Much of the preliminary optimism was dampened when the results from a frequently cited interlaboratory study were published by NOORDHOEK and co-workers in 1994 [61]. This study aimed to test the reliability of PCR to detect *M. bovis* in a panel of negative and positive experimental samples. Sensitivity and specificity for detecting 10^3 bacteria ranged 0.02–0.90 and 0.03–0.77, respectively. This finding clearly showed that effective measures for monitoring sensitivity and specificity of amplification

techniques are required before the assays are used in clinical diagnosis. Although some retained the belief that the reliability of NATs would increase rapidly, the result of a second study undertaken 2 yrs later was very similar [62]. In this second interlaboratory study, performance of amplification tests was investigated with a smaller panel of samples in order to facilitate introduction into the working routine of the participating laboratories. Of 30 participants, 18 declared they were using NATs as an adjunct method for routine diagnosis of tuberculosis, and eight of them used commercially available test kits. Only five laboratories correctly reported the presence or absence of mycobacterial nucleic acids in all samples. Considering a sum of 298 negative samples tested in all laboratories, 17 laboratories reported false-positive results with a sum of 69 wrong results (23%). Of greatest interest, in contrast to the suggestions of the companies that sell test kits for gene amplification, no difference was seen in the reliability of in-house or commercial tests. Rather, the outcome of the study emphasizes that general elements of quality assurance are needed. These must include: 1) structures, such as the adequacy of the workplace and provisions to carry out the task; 2) processes, *i.e.* the proficiency with which NATs are performed; and 3) outcome, *i.e.* the consequences of and adjustments to the service.

The increasing experience gained in recent years led to a replacement of the initial enthusiasm by a more realistic view of the limitations and the practical value of molecular diagnostics for tuberculosis. This was the start of an era in which suitable controls, quality assurance and proficiency testing were the predominant topics discussed as a solution to overcome the problems. Various national and international initiatives have led to elaboration of guidelines that describe essential measures for the internal quality control of NATs [63–65], and progress is being made to establish proficiency testing programmes. The main conclusions these efforts have come to are: 1) NATs are not suitable for screening; 2) NATs cannot replace culture; 3) controls that monitor the whole process from sample preparation to detection, have to be defined, so that they provide a control over efficiency of nucleic acid extraction, contamination, sensitivity, inhibition and specificity of amplification; and 4) for a correct interpretation of results, it is necessary to confirm positive results. Therefore, three categories for reporting an amplification-based result are proposed: a positive (reproducible), a negative, and an unreproducible result, whereby reasons for the latter have to be stated. Of note, only 7 out of 12 (58%) of the studies shown in tables 2–4 routinely used internal controls for all samples to detect inhibition. Single-sample NAT-negative results must be considered carefully, because of potential false-negatives.

How to diagnose tuberculosis with inclusion of NAT

At present, microscopy for acid-fast bacilli continues to be the mainstay of routine clinical laboratories for any rapid diagnostic approach to a patient under clinical suspicion of tuberculosis. In view of current knowledge, only three clearly defined indications for performing NATs can be pointed out, investigation of: 1) previously untreated patients with a positive smear for acid-fast bacteria in areas with a high prevalence of nontuberculous mycobacterial infections or in patients at high risk for such infections, for example patients with AIDS; 2) children with tuberculosis of the lymph nodes, because of the high incidence of *M. avium* complex infections in these cases; and 3) material obtained by biopsies, surgery or other invasive procedures, because these specimens are often difficult to obtain and, therefore, particularly valuable. The studies so far published on the use of NATs for examining nonrespiratory specimens suggest that nucleic acid-based diagnostic tests provide a sensitive and specific means to increase the number of tuberculosis infections detected long before culture results are available [31, 32]. All other indications for NAT within the diagnostic work-up of patients under suspicion of tuberculosis must be discussed within a wider clinical context.

Clinical settings that define an indication for performing NAT

The clinical value of NATs and their role in diagnostic work-ups depend on the strength of the clinical evidence for active tuberculosis and the severity of the current disease. In our opinion, three different settings can be defined in which NATs might be indicated, as illustrated in figure 1.

The first setting involves a severely ill patient suffering from a life-threatening disease, who is, for example, at a high risk for active pulmonary or extrapulmonary

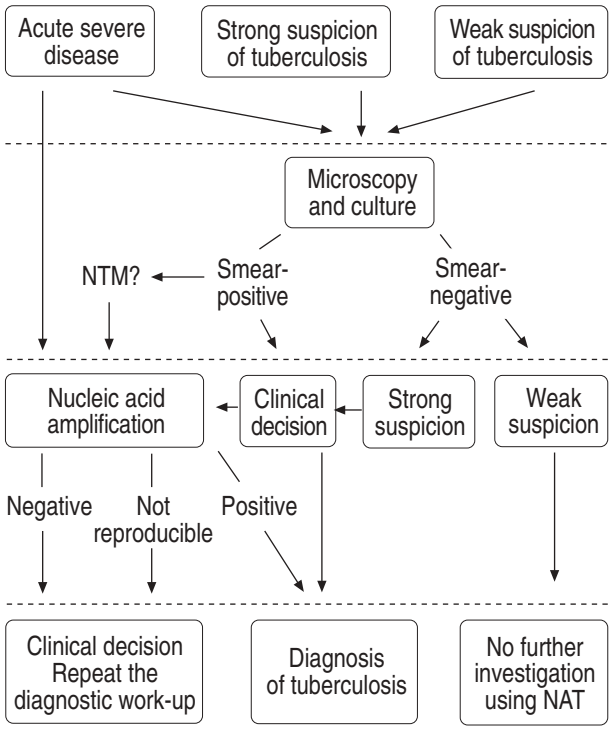

Fig. 1. – Flow chart of clinical conditions (upper rank) leading to diagnostic approaches, with priorities in performing microscopy and nucleic acid amplification techniques (NATS) as rapid tools for diagnosing tuberculosis (lower ranks). The figure emphasizes that clinical findings and decisions lead to specific indications for the application of NATS. However, it is even more important that the final decision to initiate therapy or not is governed both by the test result and the clinical data. NTM: nontuberculous Mycobacteria.

tuberculosis because of any kind of severe immunosuppression, such as human immunodeficiency virus (HIV) infection or immunosuppressive therapy following organ transplantation, or any patient with nonpurulent meningitis confirmed by cytology and chemistry of spinal fluid [66]. Under these circumstances, an immediate diagnosis of tuberculosis is essential. Therefore, any possibility of obtaining a diagnosis as fast and as reliably as possible has to be exploited. Besides microscopy of any available specimen using fluorochrome stains, the primary inclusion of NATs as a rapid diagnostic test, performed in accordance with the above-discussed quality standards, will be helpful because they are more sensitive than microscopy.

Therefore, a positive and reproducible NAT result in these patients will make it possible to start antituberculosis treatment, regardless of the results of microscopy (fig. 1). However, it must be mentioned again that NATs will yield false-negative results in some patients with paucibacillary disease. In such cases, it might be useful to repeat the NAT test with new specimens and/or with material obtained from fluid culture systems after 1 or 2 weeks of incubation. In all cases of negative NAT results, the decision to treat a severely ill patient for tuberculosis must be based on clinical data.

The second setting is that of a patient with a strong clinical suspicion of pulmonary tuberculosis. As illustrated in figure 1, it is crucial to define the term "strong suspicion", because the decision to request NATs is governed by delimitation of patients with strong suspicion from those with a low suspicion of tuberculosis. A high degree of suspicion is given when the following criteria are present: 1) known risk factors for tuberculosis (alcohol or drug abuse, homelessness, origin from a country with a high incidence of tuberculosis); 2) history of tuberculosis with or without appropriate therapy; 3) history of contact with smear-positive patients; 4) cavitary disease or upper lobe infiltrations [67]; 5) history of chronic pulmonary infectious disease with cough, night-sweat and weight lost; and 6) positive tuberculin skin test using low purified protein derivative (PPD) concentrations or proven skin test conversion within 2 yrs [68, 69]. In these patients, the first diagnostic approach should be microscopy of three sputum samples, or of material obtained by bronchoscopy in severely ill patients and patients unable to produce sputum. If the results of microscopy continue to be negative, NATs are indicated to confirm the diagnosis. However, it is essential to note that NATs can likewise give false-negative results. Until this problem is completely solved, a negative NAT result should not be used to withdraw antituberculosis therapy in patients with strong clinical evidence of pulmonary tuberculosis [70, 71]. In patients with a positive acid-fast smear result, molecular testing might be helpful to differentiate between *M. tuberculosis* and nontuberculous Mycobacteria. This approach will be helpful in areas with a high incidence of nontuberculous Mycobacteria or in patients at risk for nontuberculous mycobacteriosis [72, 73].

The third setting involves a patient with pulmonary disease of unknown origin, in whom the diagnosis of tuberculosis should be excluded. All specimens obtained from patients with a weak suspicion for tuberculosis should be investigated by conventional methods only. Given the current performance of gene amplification techniques in directly detecting *M. tuberculosis* in clinical smear-negative specimens, it is inappropriate to use these costly methods for the exclusion of tuberculosis. However, it remains to be determined whether or not NATs are indicated in patients with an otherwise undiagnosed pulmonary disease. In these patients, NAT might be helpful as a second approach following negative diagnostic procedures, including bronchofibroscopy, whereby more than one sample should be submitted in these cases. Regarding differential diagnosis of other granulomatous diseases, it must be mentioned that nucleic acids from *M. tuberculosis* have been detected in tissue biopsies from patients with sarcoidosis [74]. However, this issue remains debatable until confirmed by reliable figures obtained from further studies.

In conclusion, with the exception of meningitis and severely ill patients, in whom we can expect that nucleic acid-based tests will improve the diagnostic possibilities, application of NATs as a diagnostic tool must follow conventional test results and their indications should be based on a careful clinical judgement.

Impact on patient management

A further aspect to be considered in assessing the utility of NATs in the diagnosis of tuberculosis is how NAT results may affect treatment decisions. At a 1996 workshop of the American Thoracic Society, six working groups attempted to outline the impact of gene amplification compared to the impact of sputum smear microscopy on the clinician's decision-making behaviour [75]. Here, two different settings can be distinguished.

The first setting includes all patients with a strong clinical suspicion of tuberculosis, in whom treatment will be started regardless of smear results (table 5). In patients with a positive smear test, negative *M. tuberculosis* specific NAT results might affect the decision about treatment, because of the high probability of nontuberculous Mycobacteria. It must be remembered, however, that even if *M. avium* complex predominates because a patient population includes a large number of HIV-infected individuals, the high predictive value of acid-fast positive smears for tuberculosis remains almost unchanged [76]. In contrast, the impact of a NAT result on the treatment decision in smear-negative patients with a strong clinical suspicion of tuberculosis is low.

The second setting includes all patients with a weak clinical suspicion of tuberculosis. Again, in this group of patients, a possible impact of negative NAT results on treatment decisions is given only in smear-positive patients because there is a high probability of nontuberculous Mycobacteria. In a patient with a weak suspicion and with negative smear results, application of NATs is not recommended because of the low PPV of these tests.

In conclusion, an impact of nucleic acid amplification chemistry on the treatment decision can be expected only in smear-positive patients with a negative *M. tuberculosis* specific NAT result. It must be mentioned that this applies only if the NAT results are reproducible and obtained by a test system with high sensitivity and specificity.

Table 5. – Possible impact of results obtained by smear microscopy and by nucleic acid amplification techniques (NATs) on the treatment decision in patients with either a strong or a weak suspicion of tuberculosis

Clinical suspicion of tuberculosis	Result of		Treatment with availability of		Impact of NAT
	Microscopy	NAT‡	Smear results only	Smear and NAT results	
Strong	Positive	Positive	Yes	Yes	No
	Positive	Negative	Yes	No	Yes#
	Negative	Positive	Yes	Yes	No
	Negative	Negative	Yes	Yes	No
Weak	Positive	Positive	Yes	Yes	No
	Positive	Negative	Yes	No	Yes#
	Negative	NR	No	-	-
	Negative	NR	No	-	-

NR: not recommended; ‡: *Mycobacterium tuberculosis* specific NATs only; #: provided the NAT results are reproducible (confirmed) and obtained by using a standardized method (quality control, clinical evaluation with high sensitivity, specificity and positive and negative predictive values; for details see text).

Rapid molecular drug susceptibility testing

Background

One of the most alarming aspects of the recent increase in the incidence of tuberculosis are outbreaks involving drug-resistant strains [2, 77]. Therefore, rapid assessment of drug susceptibility testing of *M. tuberculosis* represents the main objective of current research worldwide. Unfortunately, there is still too long a delay between improved early diagnosis, for example by means of the BACTEC 460 TB system or amplification of nucleic acids, and the reporting of drug susceptibility results. As a consequence, weeks of empirical therapy pass before a scientific rationale for treatment becomes available. Present and future trends in antimycobacterial susceptibility testing have recently been reviewed by INDERLIED [78]. Two approaches are currently under investigation: 1) search for molecular tests that detect changes in genes that confer resistance; and 2) development of assays for the rapid detection of antimicrobial resistance on the basis of quantification of growth, metabolism or viability, by means of highly sensitive molecular methods.

Direct approach: molecular analysis of changes in gene sequences

The current state of our knowledge on the molecular genetic basis of antimicrobial resistance in Mycobacteria has been summarized and discussed in an excellent review by MUSSER [13]. Readers are referred to this article for more detailed information on a sequence or gene level. Most molecular data are available for rifampicin (RIF) and streptomycin, and, partially, for isoniazid (INH) [13]. However, no detailed genetic data known to be associated with resistance to pyrazinamide, ethambutol and second-line drugs have been described. Mutations identified in the gene encoding the RNA polymerase β-subunit (*rpoB*) directly confer RIF resistance to *M. tuberculosis* [79]. Mutations of a short 81 base pair (bp) core region of the *rpoB* gene, comprising 35 distinct allelic variants, have so far been found in >97% of RIF-resistant strains [13]. The absence of mutations in approximately 3% of strains suggests that other mechanisms exist, which mediate RIF resistance in *M. tuberculosis* [13]. Irrespective of this drawback, detection of RIF resistance by means of molecular genetic methods is a reasonable endeavour, because resistance can be demonstrated in a substantial percentage of strains and the methods are suitable for routine molecular laboratories that use automated DNA sequencing [80] or other methods for the identification of sequence variants within amplified DNA, *e.g.* single strand conformation polymorphism (SSCP) [81]. Unfortunately, the situation is somewhat more complicated for the genetic resistance mechanism of INH. Resistance to INH in *M. tuberculosis* has been associated with various perturbations found scattered over the gene encoding catalase-peroxidase (*katG*), and to some extent in an *InhA* protein gene locus [82]. Complete deletions of the *katG* gene also occur but are rare [83]. Identification of *katG* and *inhA* as drug targets is not sufficient to explain the complex mode of action of INH, and mutations in these genes do not account for approximately 15% of INH-resistant strains [82, 83].

Methods that target genes directly promise to provide the fastest and most unambiguous assays. Ongoing efforts to further elucidate genetic causes for the acquisition of resistance will certainly provide important new insights into primary resistance mechanisms. To the extent that resistance to single therapeutic agents is not based on only one molecular variant, and as long as the genes conferring resistance are not known for all first-line drugs, we can safely state that none of the molecular genetic approaches to determine drug susceptibility are at present amenable to utilization in general mycobacteriology laboratories [13]. Firstly, molecular methods will probably never completely replace growth-dependent methods, and it will take years of experience to evaluate their reliability in treatment monitoring. A further problem lies in their dependence on separate sophisticated methods for each individual drug. Finally, methods to detect multiple potential mutations throughout a locus, such as sequencing or PCR-SSCP, lack sensitivity because more than 15% of resistant colonies in a mixed growth are required to reveal the presence of the altered gene in the population [84].

Functional approach: detection of viability or growth

The BACTEC system utilizes a liquid medium and radiometric detection of growth. However, growth and identification still take about 2 weeks, and another 5–7 days for drug susceptibility testing initiated at a growth index (GI) of 500 [5] (fig. 2). In view of the many drawbacks of gene-based methods discussed above, growth- or viability-dependent approaches that yield results more

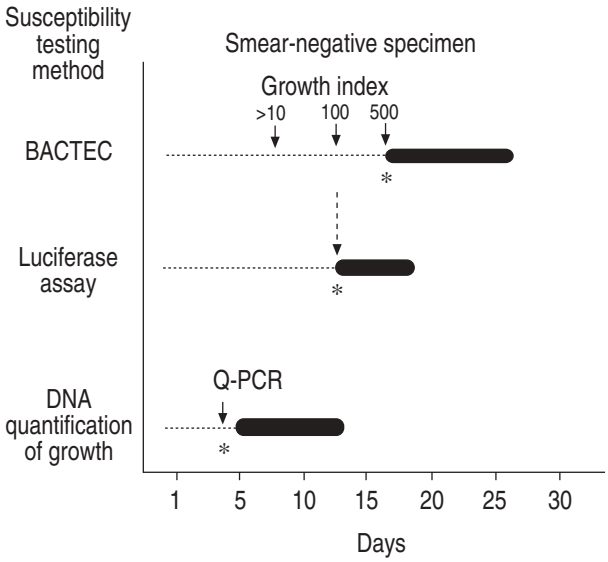

Fig. 2. – Schematic graph of the mean time required to obtain results from drug susceptibility testing of *Mycobacterium tuberculosis* using different growth- or viability-dependent rapid methods: BACTEC [5]; the luciferase reporter mycobacteriophage assay [85]; and quantitative polymerase chain reaction (Q-PCR) to measure growth by deoxyribonucleic acid (DNA) quantification [86]. *: determination of bacterial density in the culture as a standardized source of inoculum for the susceptibility test; dotted line: culture of the specimen in broth; bold line: mean time range of susceptibility testing.

rapidly than the BACTEC radiometric system should prove more promising in the near future. These include assays that exploit either biochemical measures to recognize viability, for example by staining with fluorescein diacetate and flow cytometry [87], or molecular genetic methods, such as the firefly luciferase assay [30, 88] or hybridization assays to detect rRNA [89].

The technique using a luciferase reporter mycobacteriophage to measure the viability of Mycobacteria has met with wide acclaim, but remains to be perfected. If infection by the phage is supported in viable Mycobacteria, the luciferase gene is expressed and detected by addition of luciferin (table 1). The success of this method will depend on the availability of the mycobacteriophage, its host range and efficiency of infection. The main drawback, currently, seems to be that the phage assay is not specific for *M. tuberculosis* because some Mycobacteria other than tuberculosis are also infected [85]. At present, the firefly luciferase assay seems to be of primary value in drug discovery studies [78], but in due course it will probably become available for clinical mycobacteriology laboratories.

One point of particular importance is that all of these methods mentioned here require a high initial number of bacteria (at least $10^4 \cdot \mu L^{-1}$) before the susceptibility assay can be initiated, namely turbid growth or a GI of at least 100 using the BACTEC system. Moreover, determination of the inoculum, a crucial step for standardized and reliable drug susceptibility testing, is performed by the same means as is presently used for conventional methods. This means that time is saved only after *M. tuberculosis* has been accurately propagated and isolated by culture (indirect susceptibility testing). Consequently, direct susceptibility testing methods that have been shown to significantly shorten the time necessary to report results [90] are still warranted.

Replication of *M. tuberculosis* in broth during the exponential growth phase correlates with the quantity of bacterial DNA or RNA in the medium. This process is accessible to NATs from the very beginning of culture, even if microscopy-negative samples are processed. Therefore, amplification and quantification of nucleic acids to determine bacterial loads has been recognized to have a potential for developing rapid susceptibility testing assays for Mycobacteria [86, 91, 92]. Assuming that quantification methods for nucleic acids will be improved to a satisfactory level and become sufficiently reliable to be performed in a routine laboratory, we are attempting to develop a direct susceptibility test based on competitive quantitative PCR (Q-PCR) [86]. It is envisaged: to determine the density of *M. tuberculosis* in liquid media as early as possible using liquid culture and Q-PCR; to start microbroth drug susceptibility testing in a microwell format with standardized minimal inocula as soon as at least 10 bacteria·μL^{-1} broth are available; and to accurately determine growth by DNA amplification and quantitation after 4–6 days of incubation with or without drugs. This approach would be sensitive enough even for microscopy-negative samples (fig. 2), and preliminary data suggest that susceptibility results could be available for smear-negative specimens approximately 4 days before culture becomes positive.

Since this strategy is growth-dependent, it can benefit from experience gained with the radiometric macrobroth method, and it is compatible with time-honoured criteria, such as the proportion method or the 99% threshold [78]. The use of NASBA, which will be available commercially by 1997, has the advantage of detecting rRNA directly [92], and it has been shown that, in the presence of drugs, the decay of rRNA in an *in vitro* system occurs rapidly after cell death. Nevertheless, timesaving using ribosome-based NASBA, which does not require multiplication of the bacteria, will be only 2–3 days compared to the Q-PCR growth method. Preliminary results suggest that the presence of rRNA reflects viability and can replace cumbersome messenger ribonucleic acid (mRNA) detection techniques, but the accuracy and reliability of this new reading method remain to be verified in further studies.

DNA fingerprinting of *M. tuberculosis*

Methods

Subtyping *M. tuberculosis* strains used to rely mainly on testing for one or several phenotypic markers, notably unusual drug susceptibility patterns, and on phagetyping. These markers have been replaced by more powerful DNA-typing methods, since the discovery and characterization of repetitive DNA in *M. tuberculosis*, such as direct repeat (DR) sequences [93] and insertion sequences (IS6110 and IS1081), in the early 1990s [41, 94]. Alternative methods, such as arbitrarily primed PCR [14, 95], have also been applied but IS6110-associated restriction fragment length polymorphism (RFLP) is the method of choice, because of its high degree of discrimination and reproducibility. Furthermore, international consensus has been achieved on the standardization of IS6110 fingerprinting, thus enabling comparison of DNA-typing results from different laboratories [96].

The method relies on the fact that the IS element moves in the genome (transposon) and occurs in multiple copies in most *M. tuberculosis* strains (usually between 1–20), and that these sequences are scattered throughout the genome with considerable polymorphism among strains, with the exception of *M. bovis* strains, which possess only 1–5 copies of the element in a fixed position. Fragments of varying sizes generated by digestion of the circular bacterial chromosome with a restriction endonuclease, which cuts the DNA only at specific sites, are Southern blotted onto a nylon membrane after electrophoretic separation. The membrane is then probed with a fragment of the IS6110 by hybridization in order to produce an autophotograph of bands with varying positions of the IS element (fig. 3). IS6110 fingerprinting has been found to be a reliable method for typing isolates of *M. tuberculosis*. Unfortunately, the procedure is only applicable when sufficient biomass of 1–3 well-grown colonies is available.

Mixed-linker IS6110-based PCR, a method that produces the same banding results as Southern blot IS6110-based RFLP using *in vitro* amplification, may overcome this limitation [97], and is currently undergoing further evaluation. With the advent of technical improvements, this method is on its way to becoming the preferred method for IS6110-based fingerprinting [98].

Detection of laboratory contaminations

So far, application of DNA fingerprinting has focused on answering questions of short-term epidemiological concern, such as outbreak patterns, transmission in the community or spreading of resistant isolates [99–102]. In routine clinical practice, a further, more pragmatic, application of DNA fingerprinting of *M. tuberculosis* has emerged to detect specimen contamination in the mycobacteriology laboratory. Cross-contamination in the mycobacteriology laboratory has been recognized as a common problem both of conventional and radiometric methods, resulting in false-positive cultures with serious consequences [103–106]. The rate of contamination clearly increases with the number of positive specimens processed in a laboratory. Carry-over from one specimen to another during decontamination of sputum has been recognized as the main culprit. The exact rate of false-positive cultures due to laboratory contaminations is unknown, but rates of 0.2% [103] and 0.33% [104] have been reported.

We have recently found five of six suspected isolates obtained over a period of 6 months to represent false-positive cultures, corresponding to a contamination rate of 0.05% (five contaminations among approximately 9,000 specimens processed) [107]. In this study, clinical data, low counts of colony-forming units on solid media, and identical RFLP banding patterns of strains in neighbouring culture tubes suggested that contamination had occurred (fig. 3). Molecular methods are necessary for a definite confirmation of this suspicion. IS6110-based RFLP typing provides results in 3 days and, thus, represents an inexpensive and reliable method that can help to clarify such inconclusive culture results. Therefore, this method should be available for selective application in larger mycobacteriology laboratories, and be requested by clinicians in cases of unexpected positive cultures or changes in drug susceptibility patterns.

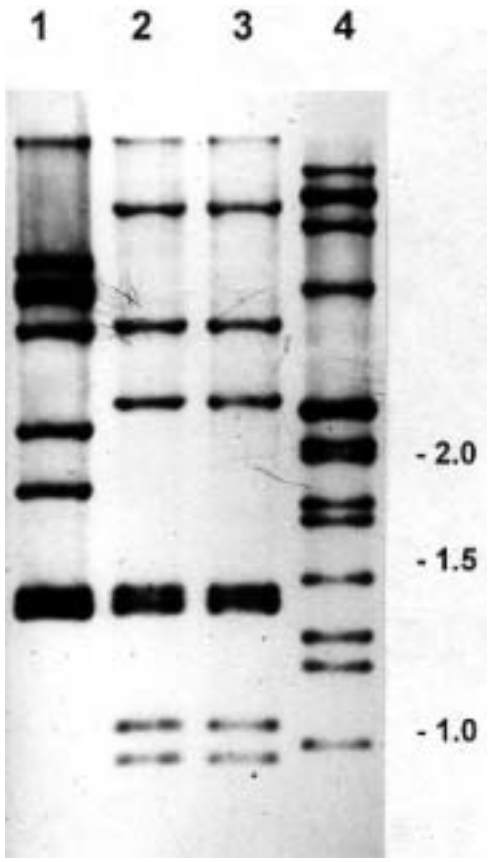

Fig. 3. – Southern blot hybridization IS6110-based restriction fragment length polymorphism (RFLP) banding patterns obtained in a group of *Mycobacterium tuberculosis* culture isolates in which cross-contamination from sample 6135 to 6136 occurred (lanes 2 and 3). Molecular weight markers indicated in kbp.

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