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Molecular genetic methods for diagnosis and antibiotic resistance detection of mycobacteria from clinical specimens

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Mycobacteria comprise a diverse group of bacteria that are widespread in nature, some of which cause significant disease in humans. Members of the *Mycobacterium tuberculosis* complex (MTBC) are the most important human pathogens of the genus *Mycobacterium.* Traditional methods for detection and identification of mycobacteria include microscopy, culture and phenotypic tests. These methods either lack sensitivity, specificity, or are time consuming. Advances in the field of molecular biology have provided rapid diagnostic tools that have reduced the turnaround times for detecting MTBC and drug resistance in cultures and directly in clinical specimens from weeks to days. This review discusses the molecular genetic techniques for detecting and identifying MTBC as well as drug resistance of mycobacteria in clinical specimens.

Key words: Mycobacteria; molecular techniques; detection; drug resistance.

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The genus *Mycobacterium* comprises more than 100 species and includes obligate parasites, saprophytes and intermediate forms. *Mycobacterium species* are ubiquitous and can be detected from a diversity of clinical samples and environmental sources. They cause a variety of illnesses in humans and animals that differ in severity and public health importance. The genus comprises bacteria belonging to the *Mycobacterium tuberculosis* complex (*M. tuberculosis, M. bovis, M. africanum*, *M. microti*, *M. canetti,* and *M. tuberculosis* subsp. *caprae*) and a group of species collectively referred to as the atypical, nontuberculous mycobacteria (NTM) or mycobacteria other than *M. tuberculosis* (MOTT). *M. tuberculosis* complex (MTBC),the causative agent of tuberculosis (TB), is by far the most important of the genus *Mycobacterium* (1). *M. tuberculosis* is the leading cause of human death from a single infectious agent; it kills more than 2 million people and is responsible for 8 million new cases annually (2). Most of the TB cases (95%) occur in the developing countries (3). The control of TB has been compounded by the emergence of multidrug resistance (MDR) (4, 5). A recent survey conducted in 77 countries comprising one fifth of the global total of new smear-positive cases reported MDR prevalence rates of up to 14% in certain settings (6). The burden of TB has been further exacerbated by the synergistic effect of human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) (7). In the recent past disease due to mycobacteria belonging to the *M. avium* complex (*M. avium* subps. *avium*, *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *lepraemurium,* and *M. avium* subsp. *silvaticum*) has

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become clinically important, especially in association with AIDS (8, 9). The introduction of highly active antiretroviral therapy has resulted in a remarkable reduction of opportunistic infections in HIV-infected individuals (10). However, other NTM – especially those causing nosocomial infections – still cause concern (11).

Active TB and mycobacterioses in general are currently diagnosed by traditional laboratory procedures, including microscopic examination of samples for the presence of acid-fast bacilli (AFB) and/or isolation by culture followed by identification and drug susceptibility testing (DST) of the isolates. Although microscopy is fairly rapid, it suffers from low sensitivity, especially in extrapulmonary tuberculosis and paucibacillary cases common among HIV-infected individuals, and is not species specific (12). The culture-dependent laboratory procedures may take 7 to 10 days on most recently developed liquid media and 4 to 6 weeks on solid media. Early detection and identification of *M. tuberculosis* is particularly important because it can be transmitted from one person to another, and because diseases due to NTM require adapted treatment regimens.

The above-mentioned limitations of traditional laboratory diagnosis of TB have resulted in many laboratories turning towards novel approaches for rapid and reliable detection and identification of mycobacteria. The discovery of the polymerase chain reaction (PCR) led to the development of nucleic acid amplification tests (NAATs) for rapid detection and identification of MTBC in clinical samples, sometimes with limited determination of drug resistance. Furthermore, detection and identification of some clinically important NTM became possible as well as determination of drug resistance in MTBC isolates. This review will discuss the application of currently available molecular tools for the diagnosis of TB with emphasis on the detection of drug-resistant MTBC in clinical specimens.

MOLECULAR DETECTION OF MTBC

Several commercial and 'in-house' NAATs for detecting MTBC in clinical specimens have been described. The methods use various targets including DNA or RNA sequences that are genus or species specific, followed by detection on agarose or acrylamide gels, or after hybridisation using various formats.

Commercial diagnostic assays

There are currently four commercial tests for direct detection of MTBC in clinical specimens: (i) the Enhanced Amplified Mycobacterium Tuberculosis Direct Test (E-AMTDT), (ii) the Amplicor® *Mycobacterium tuberculosis* Test (Amplicor MTB test) and its automated version the Cobas Amplicor MTB test, (iii) the BDProbe Tec ET test, and (iv) the INNO-LiPA-Rif. TB test (Table 1). Of these, the E-AMTDT and the Amplicor tests have been approved by the Food and Drug Administration (FDA) for direct detection of MTBC in acid-fast bacilli (AFB) smear-positive respiratory specimens (13).

Enhanced Amplified *M. tuberculosis* Direct Test (E-AMTDT)

The E-AMTDT (Gen-Probe®, Inc., San Diego, CA) is approved by the FDA for the direct detection of MTBC in both smear-positive and smear-negative respiratory specimens from patients suspected of having TB (14). It is an RNA-based technique that relies on an isothermal transcription-mediated amplification system first described by Kwoh (15). In this test, the mycobacterial rRNA released from target cells by sonication is reverse transcribed to a cDNA-RNA intermediate. After degrading the initial RNA strand, a second primer binds to the cDNA, resulting in the formation of double-stranded DNA, which is transcribed by DNA-directed RNA polymerase to produce more rRNA molecules. The new transcripts then serve as targets for amplification. RNA amplicons are detected with an acridinium ester-labelled DNA probe in a hybridisation assay. The esterified acridinium on the hybridised probes is hydrolysed by the addition of alkaline hydrogen peroxide, resulting in the production of visible light, which is measured in a luminometer (16). The test is autocatalytic and is performed at 42 °C in a single tube. Results are available within 3.5 h after specimen decontamination. The performance of the E-AMTDT has been extensively evaluated by several investigators in both respiratory and nonrespiratory specimens. As shown in Table 1, the overall sensitivity of the test in

TABLE 1. *Comparison of commercially available molecular techniques for detection of* M. tuberculosis *complex from clinical specimens*

Test	Amplicor	E-AMTDT	BDProbeTec ET	INNO-LiPA Rif. TB
Manufacturer	Roche	Gene-Probe	Beckton Dickinson	Innogenetics
Amplification technique	PCR	TMA	SDA	PCR
Target	16S rRNA	rRNA	IS*6110*	*rpoB* gene
Detection	hybridisation	In situ hybridisation	Fluorescent energy transfer	Reverse hybridisation
Overall sensitivity	79.4–91.6%[a]	85.5–97.8%[c]	77.8–100%[d]	58.8%[f]
Sensitivity for smear-positive respiratory specimens	97.1–97.9%[a]	91.7–100%[c]	90–100%[e]	92.1%[g]
Sensitivity for smear-negative specimens	40.0–73.1%[b]	40.0–92.9%[c]	33.3–100%[e]	89.01[g]
Non-respiratory specimens	27.3–98.6%	NA	NA	NA
Inclusion of internal amplification control	Yes	No	Yes	No
Carry-over contamination prevention	Yes	No	Yes	No
Turnaround time after specimen decontamination	6.5–7 h	3.5 h	3.5–4 h	<48 h
Equipment for amplification	Thermocycler	Heat block	Thermocycler	Thermocycler
Equipment for detection	Photometer	Luminometer	Luminometer	Visual
Approved use	AFB smear-positive respiratory specimens	AFB smear-positive and negative respiratory specimens	Respiratory specimens	NA
Mycobacterium spp. detected	MTBC	MTBC	MTBC	MTBC
Additional information	RMP resistance profile			

[a] Based on Refs. (1, 35–39); [b] Based on Refs. (51, 32); [c] Based on Refs. (17–27); [d] Based on Refs. (56–61); [e] Based on Refs. (56–64); [f] Based on Ref. (66); [g] Based on Ref. (Traore et al., in preparation); NA: Not available.

respiratory specimens compared with culture ranged from 85.7% to 97.8% and was higher in smear-positive specimens (91.7% to 100%) compared to between 40.0% and 92.9% for smear-negative samples (17–27). The main features that make this test attractive are its rapidity, the minimised risk of cross-contamination since the amplification takes place in a single tube, and the fact that the isothermal reaction does not require a thermocycler. The major disadvantage of the E-AMTDT is its inability to detect inhibitors as it does not contain an internal amplification control (IAC). Reports on its use for nonrespiratory specimens are not available.

Amplicor and COBAS Amplicor MTB test

The Amplicor test (Roche® Diagnostic Systems Inc., Branchburg, NJ) is a PCR technique in which a 584-bp segment of the gene encoding the 16S rRNA present in all mycobacterial species is amplified. The amplicons are denatured and applied to a microtitre plate onto which a MTBC-specific oligonucleotide probe has been immobilised. Detection is achieved with the help of avidin-horseradish peroxidase conjugate and 3,3′,5,5′-tetramethyl benzidine in dimethylformamide and measured with a spectrophotometer. The test has an IAC for the detection of inhibition and a uracil-N-glycosylase (UNG) carry-over prevention system to avoid contamination by DNA possibly present from previous runs. The Cobas Amplicor MTB test is an automated version of the assay available in Europe (28). The results of the assay are available after 6 to 7 h, according to the manufacturer. In addition, the test does not require the use of purified chromosomal DNA, thereby eliminating the need for elaborate DNA-extraction procedures.

The manual Amplicor MTB test has undergone several evaluations since its introduction. This test performs very well with smear-positive respiratory specimens. However, the assay was rather disappointing when tested by different investigators with nonrespiratory specimens such as pleural effusions, cerebral spinal fluids and gastric aspirates (29–34), and smear-negative respiratory samples. The reported sensitivity and specificity values after resolving discordant results (discrepant analysis) ranged from 79.4–91.9% and 85.7–100% for respiratory specimens and 27.3–98.6% and 85.7–100% for nonrespiratory specimens, respectively (1, 35–50).

Two studies recently evaluated the automated method (Cobas Amplicor MTB test) in both respiratory and nonrespiratory specimens (32, 51). The sensitivity of the test was similar in both cases for smear-positive respiratory specimens (97.1 and 97.9%, respectively) with a specificity of 100% after discrepant analysis. Meanwhile, a marked difference was observed for sensitivity of smear-negative specimens (48.6 and 68.8%) with a specificity of 99.8 and 99.2%, respectively. The presence of inhibitory substances, found in percentages ranging from 1 to 20, may in part explain these results (42, 43, 48, 49, 51–54). However, this remains intriguing since both groups applied a decontamination and centrifugation step prior to DNA preparation and PCR according to the manufacturer's instructions. As for nonrespiratory specimens the sensitivity and specificity values obtained by the two studies were similar (50 and 57.8%, and 100 and 98.6%, respectively). The strengths of this assay lie in its rapidity and in the inclusion of an IAC and the UNG carry-over prevention system to detect inhibition and minimise the risk of carry-over contamination, respectively. Its disadvantages are the disappointing results for paucibacillary and nonrespiratory specimens. A more extended DNA-extraction procedure might overcome the problem of lower sensitivity, especially for nonrespiratory specimens. This option would, however, most likely have an impact on the rapidity of the assay.

BDProbe Tec ET

The BDProbeTec ET test was developed by Becton Dickinson (Becton Dickinson, Sparks, MD) for the detection of MTBC directly in respiratory specimens. The technique is a semi-automated isothermal enzymatic process, and includes an IAC. It is based on strand displacement amplification (SDA), which is an isothermal DNA-amplification method based upon the combined action of a DNA polymerase and a restriction enzyme. It allows co-amplification and detection of different segments, in this case segments of the MTBC-specific IS*6110* and the 16S rRNA common to most mycobacterial species. Amplicons are detected using a fluorescent energy transfer detection system (55).

Evaluation studies have reported overall sensitivities and specificities ranging from 77.8 to 100% and 96 to 99.8%, respectively, after discrepant analysis. The range of the same parameters reported for smear-positive specimens alone was narrower (90 to 100%) compared to those of smear-negative specimens (33.3 to 100%) (56–61). The main features that make this test attractive are the inclusion of an IAC, the minimised risk of cross-contamination since the amplification takes place in sealed microwells, and that the procedure has to be performed on a bench top (62–64) since the bacteria are heat killed early in the specimen preparation process. Reports on its use for nonrespiratory specimens are not available.

Line Probe Assay (INNO-LiPA Rif.TB)

The INNO-LiPA Rif.TB (Innogenetics N. V., Zwijndrecht, Belgium) is an assay for rapid detection of MTBC and simultaneous determination of the rifampicin (RMP) resistance profile. It is based on amplification of the *rpoB* gene by PCR using biotinylated primers followed by hybridisation to oligonucleotide probes immobilised in parallel on membrane strips. Detection is done by addition of streptavidin labelled with alkaline phosphatase and a chromogenic substrate resulting in a purple-brown precipitate (65). The probes include an MTBC-specific probe, five wild-type probes covering the entire amplified region and showing no mutations, as well as four probes representing the most frequently encountered mutations causing RMP resistance (65). Reports on its use in clinical specimens are rare. Besides, the few available reports used a nested-PCR, whereas the original protocol does not include a secondary amplification step. One study that tested 75 predominantly nonrespiratory clinical specimens found the assay to be more sensitive (58.8%) than culture (35.3%) for detecting MTBC when compared with final diagnoses. Sequencing data of the *rpo*B gene and LiPA patterns agreed in 29 of 30 MTBC-positive specimens (96.7%) (66). Another study performed on respiratory specimens found a sensitivity of 78.3% and all results were concordant with those obtained by culture using the BACTEC 460 system (67). In our laboratory the assay detected 92.1% (291/316) of ZN-positive and 89% (107/109) of ZN-negative sputum specimens, whereas culture achieved a sensitivity of only 73.3%, probably due to long-term storage of the specimens (Traore et al., in preparation). The lower sensitivity reported in the first study compared to the one by Traore and colleagues may be due to the paucibacillary nature of the predominantly nonrespiratory specimens 88% (66/75) tested by Marttila et al. (66). There is no obvious explanation for the differences reported by Johansen compared to Traore et al.

In general, the INNO-LiPA-Rif.TB is rapid (<48 h), seems to be sensitive if a nested PCR is used and is easy to perform in routine PCR laboratories, but no IAC is included. The main advantage of this assay is the rapid information on RMP resistance, a good marker for MDRTB in regions with a high prevalence of drug resistance (6, 68). However, although the assay can detect any mutation within the amplified region of the *rpo*B gene, it only gives detailed information on four mutations and the high cost of the kit may limit its use.

Noncommercial diagnostic assays

Apart from the commercial diagnostic TB assays, many researchers report on the use of 'in-house' PCR-based systems. The parameters used, such as targets, primers, amplification, detection and specimen-pretreatment systems, vary considerably, resulting in a wide range of assays. Comparing the accuracy of the various systems is difficult because none of the studies includes a direct comparison of all the tests, and samples used differ considerably in ratios of respiratory to nonrespiratory and smear-positive to smear-negative specimens.

Real-time PCR

Real-time PCR is based on hybridisation of amplified nucleic acids with fluorescent-labelled probes spanning DNA regions of interest, and monitored by cameras in thermal cyclers. The range of probes used for detecting MTBC and drug-resistant mutations includes the TaqMan probe (69–71), molecular beacons (72, 73), Fluorescence Resonance Energy Transfer (FRET) probes (69, 70, 73), and biprobes (74). A detailed explanation of the principle behind each of these probes is available in a recent review (75). Suffice it to state that all real-time PCR systems depend on the detection of a fluorescent reporter whose signal increases in direct proportion to the amount of PCR product in a reaction. The results of real-time PCR are generally available within 1.5 h after DNA extraction.

Of the several protocols developed to detect MTBC, the majority have been performed in cultures and only a few have been applied directly to clinical specimens. The reported sensitivity of real-time PCR in clinical specimens has ranged from 71.6 to 98.1% (71, 76, 77), thereby not differing from classical NAATs. The specificity in all the three studies was 100% (Table 2). Similar to other NAATs, real-time PCR appears to perform better in respiratory specimens than in nonrespiratory specimens (71, 76, 77).

The main advantage of real-time PCR is the rapidity of the test, mainly due to the fact that post-PCR detection by electrophoresis or hy-

TABLE 2. *Evaluation of real-time PCR for detection of mycobacteria in clinical specimens*

Ref.	Target/Species detected	Fluorescent dye	Specimen No.	Specimen Type	Sensitivity	Specificity
76	IS*6110*/MTBC-specific	FAM-TAMRA	366	Smear-positive (R)	98.3	100
77	ITS/genus-specific	FAM-TAMRA	102	Lymph-node (NR)	71.6	100
71	ITS/MTBC-specific	FAM-LC640	135	Smear-positive (R)	98.1	100

ITS=Internal transcribed spacer; R=respiratory; NR=non-respiratory.

bridisation can be omitted. In addition, since the risk of carry-over and cross-contamination is minimised as both amplification and detection are performed in a single tube, the absence of an IAC and a UNG system is not a disadvantage. The drawbacks of the assays are the need for expensive and sensitive equipment as well as expensive reagents, in addition to the high technical skill of the staff demanded.

Classical PCR using IS*6110* as target

This MTBC-specific insertion sequence is commonly used as a target in NAATs for detecting MTBC. The IS*6110* (IS*986*) segments that have frequently been used include the 123-bp (78–83, 95), 158-bp (84), 181-bp (85, 86), 200-bp (87), 215-bp (88), 245-bp (89), 263-bp (90, 93), 325-bp (91) and 541-bp (16, 92) fragment. Both single- and nested-PCR systems have been used, and detection was done by simple agarose electrophoresis, autoradiograms or hybridisation assays. The overall sensitivity and specificity of the assays range from 84.2–100% and 83–100%, respectively, for respiratory specimens (16, 78, 79, 89, 91, 93, 94) (Table 3). In nonrespiratory samples lower sensitivities were recorded in most studies (84, 89, 95), with the exception of successful detection in pleural biopsy specimens in one study (83), and even in blood samples in another study (95). Wilson et al. used a one-tube nested PCR, in which the two stages of amplification are distinguished by different annealing temperatures with sensitivities of up to 92% (86). The main advantage of the IS*6110* targeted NAATs is the fact that most MTBC isolates carry more than five copies of this transposon, thereby increasing the sensitivity of the test. However, in some Asian regions MTBC isolates with no or few IS*6110* copies are more prevalent (96).

PCR directed at other targets

Examples of these targets include the MPB64 that was used to detect MTBC in 393 AFB-smear-negative samples comprising 169 sputa, 134 gastric aspirates and 90 urine samples from 109 patients suspected of having active PTB. The assay was found to be highly sensitive (97.8%) but lacked specificity (27.0%) for sputum samples, and showed a lower sensitivity (63.4%) but higher specificity (76.7%) for gastric aspirates. The authors attributed the low specificity to positive PCR results obtained in the 30 patients with nonactive PTB (97). In a study of nonrespiratory specimens conducted by Martins and colleagues, the MPB64 exhibited a sensitivity and specificity of 70% and 88% (98). The p27 gene has also been shown to be highly specific for MTBC, detecting MTBC that do not contain the IS*6110* element (99). An IS*1081*-based PCR has been used to detect MTBC in 7 of 16 (43.75%) peripheral blood samples (100). The genes coding for the 32 kDa antigen (102), the 85A antigen (102) and the 16S rRNA gene (103) have also been used to detect MTBC in respiratory and nonrespiratory clinical specimens with high sensitivity and specificity values. Most of these assays have been described in single reports only, and it is not clear to what extent they are used in the field.

Studies comparing two detection techniques, interlaboratory studies and quality control studies of MTBC

A number of studies comparing different detection techniques and a few interlaboratory and quality control studies for detection of MTBC in clinical samples have been conducted. In most of the comparative studies reported in the literature, 'in-house' IS*6110*-PCR assays, in which each laboratory used its specific specimen

TABLE 3. *Evaluation of 'in house' NAA for detecting MTBC in clinical specimens*

Ref.	Target	Fragment size (bp)	Amplification system	IAC	Detection system	Specimens No.	Specimens Type	Sensitivity (%)	Specificity (%)
91	IS*6110*	325	Single PCR	No	Agarose gel	75	NR+R	97.3[a]	100
78	IS*6110*	123	Single PCR	No	Auto-radiography	162	R	98[a]	99
84	IS*6110*	158	Single PCR	No	Dot blot/ digoxygenin	17	NR+R	(64.7[a]/100[c])	(100)
89	IS*6110*	245	Single PCR	No	Agarose gel	227	NR+R	(35.6[a]/100[c])	(100)
93	IS*6110*	263	Single PCR	No	Agarose gel	145	R	95[a+c]	99
16	IS*6110*	541	Single PCR	No	Agarose gel	135	R	84.2[b]	94.2[a]/ 100[b]
94	IS*6110*	123/325	Single PCR	No	Auto-radiography	59	NR	25[a]	97
86	IS*6110*	181	One-tube nested PCR	No	Microtitre plate/ digoxygenin	171	R	75[d] 92[e]	99 99
92	IS*6110*	541	Single PCR	No	Agarose gel	218	R+NR	(100[c])	(100[c])
79	IS*6110*	123	Single PCR	No	Agarose gel	70	R	85.7[a]	98
90	IS*6110*	263	Single PCR	No	Agarose gel	33	NR	54[a]/83[c]	94
88	IS*6110*	215	One-tube nested PCR	yes	Agarose gel	1497 536	R NR	89[a] 42[a]	99.7 99.7
95	IS*6110*	NA	Nested PCR	No	Agarose gel	41	NR	95[a]	89
80	IS*6110*	123	Single PCR	No	Agarose gel	65	R+NR	40[a]	80
85	IS*6110*	181	Nested PCR	No	Agarose gel	131	NR	75[c]	94
81	IS*6110*	123	Single PCR	No	Agarose gel	80	NR	20–100[a]	93
87	IS*6110*	200	Single PCR	No	Agarose gel	286	NR	94[a]	100
82	IS*6110*	123	Single PCR	No	Agarose gel	45	NR	46.6[a]	
83	IS*6110*	123	Single PCR	No	Agarose gel	45	NR	90[a]	100
100	IS*1081*	306	Single PCR	No	Auto-radiography	16	NR	43.75[a]	
99	p27	410	Single PCR	No	Agarose gel	64	NR+R	31.25[a]	100
97	MPB64	240	Single PCR	No	Agarose gel	393	R NR	97.8[a] 63.4[a]	27.0 76.6
98	MPB64	240	Single PCR	No	Agarose gel	19	NR	70[a]	88
102	Ag85A	162	Single PCR	No	Auto-radiography	206	NR+R	93.9[a]	94.3
101	38 kDa	322	Nested PCR	No	Agarose gel	417	R+NR	97[a]	92
103	16S rRNA	NA	Single PCR	yes	Agarose gel	729	R+NR	84.5[b]	99.5

NA=not available; R=respiratory; NR=non-respiratory; [a] overall sensitivity; [b] after discrepant analyses; [c] compared to culture; [d] using the chaotrope-silica method for DNA extraction; [e] using the chloroform DNA-extraction method; Sensitivities and specificities in parentheses were calculated based on raw data available in the paper.

pretreatment procedures, scored slightly better (Table 4).

Studies comparing two 'in-house' detection techniques

Brisson-Noel et al. (104) compared the performance of IS*6110*-PCR and 65kD-PCR on 489 samples. The sensitivity of the tests was reported to be 80% and 67% by PCR and culture, respectively, for clinically confirmed TB-patients. In an earlier smaller study, the same targets were assessed for the detection of MTBC DNA from 15 microscopy- and culture-positive pleural effusions. IS*6110*-PCR detected 9/15 samples, while the 65kD antigen-PCR only identified 3/15 (105). However, another study that used blood samples to detect MTBC from 162 suspected PTB patients using an 'in-house' IS*6110*-PCR, MBP64-PCR and pPH7301 clone-PCR reported very low positivity rates: 20% for the first two methods and 15% for pPH7301 clone-PCR (106). The low sensitivity for peripheral blood samples was attributed to the nature of TB disease.

Studies comparing commercial and 'in-house' methods

In a comparative study between an 'in-house' IS*6110*-PCR and the Amplicor MTB test on 504 clinical specimens, the sensitivities of the tests were 92.6 and 70.4%, respectively, compared to 88.9% for culture and 52.4% for microscopy (36). Similar results were obtained for nonrespiratory specimens. When the performance of the IS*6110*-PCR and the Cobas Amplicor MTB test was evaluated for the detection of MTBC in 43 human lymph node specimens, the 'in-house' IS*6110*-PCR was found to be more sensitive (87.5%) compared to the Cobas Amplicor MTB test (45.5%). The specificities were 100% and 91.3%, respectively. Inhibition rates were also found to be higher in the latter (19.5%) than in the former test (4.5%) (31). In another comparative study of the above two tests in a total of 1, 681 clinical specimens, the Cobas Amplicor MTB test was again found to have a lower sensitivity of 66.33% compared to 91.08% for the 'in-house' IS*6110*-PCR. The specificities were comparable (44). Observed differences in sensitivity between the Cobas Amplicor assay and the 'in-house' PCRs may have two explanations. All 'in-house' PCRs used IS*6110* as a target, which is present in multiple copies in most *M. tuberculosis* isolates, whereas the 16S rRNA gene used in the Cobas Amplicor assay is present in only a single copy. Further, the DNA-extraction and purification procedure of the Cobas Amplicor assay is restricted to a single centrifugation step followed by alkaline lysis and neutralisation, whereas the 'in-house' PCR systems applied here included at least three washing and centrifugation steps, and a more intense extraction step in procedures used by different laboratories. Moreover, the bigger volume (50 μl) of pretreated specimen used in the Cobas Amplicor assay could contain more inhibitors, compared to the smaller volumes (between 5 and 25 μl) used in the 'in-house' PCRs.

Comparison of the IS*6110*-based PCR with another commercial test (AMTDT) revealed similar findings with positivity rates of 84.2% and 91.9% for IS*986*-PCR and AMTDT, respectively, compared with 71.9% by smear and 96.9% by culture. Again, different procedures for sample preparation were used and differences were not statistically significant (16). Gomez-Pastrana et al. compared an 'in-house PCR' based on the 65 kD antigen of MTBC, the Amplicor MTB test and culture on 251 clinical specimens, and reported sensitivity and specificity of the 'in-house PCR' of 60% and 96.8%, respectively. The corresponding values for the Amplicor MTB assay and culture were 44% and 93.7% and 44% and 100%, respectively (34).

Studies comparing two commercial assays

Six studies have compared the performance of commercial assays for detecting MTBC (17, 27, 50, 52, 54, 64,) (Table 4). Most of them compared an assay with and without an IAC. The results revealed that all but one study found no significant difference between the respective techniques tested. The only comparative study in which a significant difference (p=.02) between the assays tested (BDProbeTec ET and Cobas Amplicor MTB Test) was observed analysed pleural fluid specimens (50). Overall inhibition rates were reported in three studies ranging from 0% to 5% (17, 54, 64), and were higher in nonrespiratory specimens (54). The best NAAT for detecting MTBC in clinical specimens remains unknown because no single study has compared all the available commercial assays.

TABLE 4. *Results of direct comparative studies using 'in-house' and/or commercial molecular diagnostic assays for detecting* M. tuberculosis *in clinical specimens*

		All clinical specimens combined			Respiratory specimens			Nonrespiratory specimens			
Assays used	Ref.	No.	Sensitivities (%/%)[a]	Specificities (%/%)	No.	Sensitivities (%/%)[a]	Specificities (%/%)	No.	Specimens	Sensitivities (%/%)[a]	Specificities (%/%)
Two 'in-house' methods											
IS*6110*-PCR/ 65 kDa PCR	95							15	Pleural effusions	60/20	NR
IS*6110* PCR/ MBP64 PCR	96							162	Blood	20/20	NR
Commercial versus 'in-house' method											
IS*986* PCR/ AMTDT	16				135	84.2/ 91.9	94.2/ 95.1				
IS*6110* PCR/ Amplicor MTB Test	36				504	**92.6/ 70.4**	98.9/ 98.7				
IS*6110* PCR/ Cobas Amplicor MTB Test	31							43	Lymph nodes	**87.5/ 45.5**	100/ 91.3
IS*6110* PCR/ Cobas Amplicor MTB Test	44	1, 681	*91.1/ 66.3*	99.8/ 99.7							
65 kD PCR/ Amplicor MTB test	34	251	*60/ 44*	96.8/ 93.7							
Real-time/ Amplicor MTB Test	76				366	99.5/ 96	98.2/ 100				
Two commercial methods											
Amplicor/ Cobas Amplicor MTB Test	17				755	90.8/ 92.4	100/ 100				
E-AMTDT/ Amplicor MTB Test	52				1385/ (1380)	97.1/ (96.7)	99.5/ (100)				
E-AMTDT/ Cobas Amplicor MTB Test	54				296	85.7/ 94.2	100/ 100	190	Gastric aspirates, urine, sterile body fluids, pus biopsy samples	82.9/ 85.0	100/ 100
E-AMTDT/ BDProbeTec MTB Test /	27	94	96.1/ 96.1	97.1/ 100							
E-AMTDT/ BDProbeTec ET MTB Test	64				331	88/ 94.5	99.2/ 99.6	184	Gastric aspirates, urine, sterile body fluids, pus biopsy samples	74.3/ 92.3	100/ 100
BDProbeTec ET/ Cobas Amplicor MTB test	50				152	69.2/ 76.9	100/ 93.7	101	Pleural fluid	**33.3/ 88.9**	92.4/ 100

[a] Sensitivities and specificities were obtained after discrepancy analyses with clinical data; bold numbers for sensitivities indicate a significant difference between the two PCR assays for those types of specimens, non-bold numbers are not significantly different, and for italic numbers no statistical data are available.

Interlaboratory studies

The first interlaboratory study conducted in 1994 included 7 laboratories and analysed 200 sputum samples with and without spiked *M. bovis* DNA. Each laboratory used its own specimen pretreatment, DNA-extraction, and detection procedures by 'in-house' PCR assay or commercial assay. The results were rather disappointing with several laboratories reporting a wide range of false-positive PCR results (3 to 20%) and sensitivities varying among the different participants (108).

Two studies were conducted 2 years later. The first study comprised 20 samples (including MTBC, NTMs, and no mycobacteria) and 9 French laboratories using the same IS*6110*-based PCR and hybridisation protocols in all laboratories (109). Eight out of the nine laboratories reported positive PCR results for samples containing 10 colony-forming units per ml or more. The rate of false-positive results was 7%. The authors concluded that the technique was neither very sensitive nor specific for the detection of MTBC. The second study included 30 laboratories in 18 countries with each laboratory using its own PCR detection system on 20 sputum specimens with no, 100 and 1000 mycobacterial cells. The most common target used in detecting mycobacteria was IS*6110* (19/30). Other targets included the 16S rRNA (4/30), 16S rDNA (5/30), MPB64 (1/30) and the 32kDA protein (1/30). Only 5 laboratories scored correctly for all specimens, 7 detected mycobacterial DNA in all positives, and 13/30 correctly identified the negative samples. All laboratories used negative and positive controls to monitor the amplification process, whereas only 11/30 used controls to monitor the complete procedure, including decontamination, pretreatment and DNA amplification. In addition, 17/30 laboratories used the dUTP/UNG to avoid carry-over contamination with amplicons. Yet the sensitivity was acceptable but the specificity was less desirable, and this was not related to the use of either 'in-house' or commercial tests (110).

In the most recent evaluation reported in 2004, 82 laboratories in 23 countries were each given 8 sputum specimens and 4 phosphate-buffered saline (PBS) samples spiked with dilutions of *M. bovis* BCG from 0 to 50,000 CFU. Overall, 586 (86.3%) of the 679 test results for positive sputum samples were correct, whereas only 61.2% (104/170) of the sputum specimens considered as 'smear-negatives' were reported as positive. There were 4.3% false-positive results. Correct results for all sputum specimens were similar for 'in-house' and commercial assays (35.3% and 37.8%, respectively) and comparable for PBS samples (45.8% and 41.5%, respectively). The results were generally better than earlier studies and showed a substantial decrease in the percentage of false-positives; the sensitivity for smear-negative samples was still insufficient (111).

These interlaboratory studies show that more and more laboratories can perform PCR analyses under proper conditions and produce reliable results. Most probably the technical skills of the staff as well as the PCR facilities have improved over time.

MOLECULAR IDENTIFICATION OF MTBC AND NTM

Identification of mycobacteria in a clinical laboratory is traditionally achieved using a number of phenotypic tests such as growth on solid media in the presence of inhibitors, temperature, growth rate, colonial morphology, pigmentation, niacin accumulation, semiquantative catalase or catalase activity, tween hydrolysis, and arylsulphatase activity. However, it takes a long time to obtain results and difficulties arise in precise identification of closely related species apart from some variability in the results (112). The identification of mycobacteria responsible for disease has important consequences for the proper selection of therapy. For prompt identification of mycobacteria, molecular methods have become increasing important in recent years due to their rapidity and, in most cases, production of unequivocal results. The methods are mainly based on 16S rRNA, 16S rDNA, the internal transcribed spacer (ITS) between 16S-23S rDNA and 16S rDNA, and the heat shock protein *hsp*65 gene. So far, there are few evaluations of their use for direct application in clinical specimens.

Molecular detection and identification methods with limited evaluation for clinical specimens

IS-based PCRs

As for detection of MTBC, a number of insertion sequences have been used to detect and identify other *Mycobacterium* species. These include IS*2404* specific for *M. ulcerans* (113), IS*2606* for *M. ulcerans* and *M. marinum* (113), IS*900* specific for *M. paratuberculosis* (114), IS*1245* and IS*901* for *M. avium* (115, 116), and IS*1395* for *M. xenopi* (117). These assays are rapid, usually very sensitive and easy to perform. The main drawback is that the obtained result gives information on only a single species or a group of closely related species.

Oligonucleotide-specific capture plate hybridisation (OSCPH)

This is a PCR method that selectively amplifies mycobacteria with primers based on the 16S rRNA gene. A digoxigenin-11-dUTP label incorporated during PCR allows detection of the genus *Mycobacterium*, including seven species after hybridisation with species-specific biotinylated oligonucleotides in streptavidin-coated microtitre plates (118). Using this assay, De Beenhouwer et al. confirmed all culture results in 14 clinical specimens from patients suspected of harbouring NTMs, and detected *M. genavense* for which culture was negative in another sample. The assay has also been used to test 22 clinical specimens from suspected Buruli ulcer patients by including an extra probe specific for *M. ulcerans* and *M. marinum*, and detected 10 of 22 (45.5%) specimens, with no statistically significant difference from culture results (119).

The advantages of the OSCPH assay are that it simultaneously identifies most clinically important mycobacteria, and although only a limited number can be identified, the system is flexible with respect to addition or removal of probes according to needs.

INNO-LiPA-Mycobacteria

INNO-LiPA-Mycobacteria (Innogenetics, Ghent, Belgium) is a commercial line probe assay developed for the simultaneous detection and identification of mycobacteria from culture using reverse hybridisation. It is based on the same technique as INNO-LiPA-Rif.TB and uses the 16S-23S ribosomal RNA spacer region (ITS) as the target (120, 121). A total of 16 mycobacterial species including MTBC can now be identified using the INNO-LiPA Mycobacteria test. The assay can be performed in about 6 h. The performance of the INNO-LiPA Mycobacteria test has been widely evaluated in mycobacterial cultures and has recently been reviewed (122, 123). Its performance in microscopy smear-positive specimens proved good in a small study in which the assay correctly identified the mycobacteria present in 13/14 specimens tested and allowed rapid discrimination of the 8 clinically most relevant mycobacteria in microscopy-positive clinical samples (124).

The advantages of the INNO-LiPA Mycobacteria test are that it contains a genus-specific probe that may be used both for genus identification and as an internal control for the amplification and hybridisation steps simultaneously; it identifies most clinically important mycobacteria. However, the test can only identify a limited number of mycobacterial species and cannot differentiate between closely related species such as *M. marinum* and *M. ulcerans*.

16S rDNA PCR

The method involves use of multiplex PCR amplification of a region of the 16S rDNA gene using species-specific primers followed by detection on agarose gel. Initially reported by Wilson et al. (125), this assay has recently been used on 103 smear-positive samples to identify *M. avium* complex (MAC) infections in HIV-1-infected patients. Diagnosis relied upon positive cultures. Thirty-four patients had TB, one had TB and MAC, and four had MAC only. The 16S rDNA-PCR identified *M. avium* from all smear-positive samples that grew MAC, suggesting its potential for prompt differential diagnosis of MAC in smear-positive specimens (126). Although MTBC and *M. avium* are the most commonly isolated mycobacteria in many clinical laboratories, extension of the technique to include more species would be helpful.

DNA microarrays

High-density oligonucleotide arrays (DNA microarrays) provide an opportunity to rapidly examine large amounts of DNA sequences in a high throughput manner. The method is based

on hybridisation of fluorochrome-labelled PCR amplicons to DNA arrays containing oligonucleotide probes based on the 16S rRNA, DNA gyrase sub-unit B (*gyrB*) or the *rpoB* genes. Bound amplicons emit a fluorescent signal that is detected with a scanner. The array includes 81 unique sequences and is able to differentiate up to 54 mycobacterial species and simultaneously detect mutations that confer RMP resistance in *M. tuberculosis* isolates (127, 128). The reported turnaround time for the technique on smear-positive specimens is 4 h.

A *gyrB* gene-based microarray has been used in PCR amplification of a 184-bp fragment common in all mycobacteria using purified DNA from clinical samples. Fluorescent-labelled RNA derived from the amplified samples was hybridised with a set of 28 oligonucleotide probes for species-specific *gyrB*-gene regions to identify 14 *Mycobacterium* species. Out of the 122 sputum samples tested by the microarray assay, 10, 6 and 5 specimens were positive for *M. tuberculosis*, *M. avium* and *M. intracellulare*, respectively. MTBC results were in agreement with the Cobas Amplicor MTB test; however, culture results were not reported. The assay additionally identified two concomitant infections with *M. avium*-*M. intracellulare* and *M. avium*-*M. kansasii* (129).

Molecular methods yet to be applied to clinical specimens

A number of methods have been developed to identify mycobacterial isolates. Although theoretically applicable directly to clinical specimens, most of these techniques still need to be evaluated for this purpose, especially to validate their sensitivity and accuracy. They include the Accuprobe® MTB test (130–139), DNA sequencing (140, 141) of various targets (142–155) with the detection of a high proportion of "novel" mycobacteria in clinical laboratories as the major impact (156–158), 16S ribosomal (r)DNA-hybridisation (159, 160), *hupB*-based PCR (161) and PCR restriction analyses (PRA, ARDRA, PCR-RFLP, PCR-REA) (162–168). Although various names have been proposed for the latter methods, they all include a NAAT followed by restriction of the amplicon by one or more endonucleases and electrophoresis of the fragments. We therefore suggest that we collectively refer to this technique as PRA, indicating PCR-restriction-analysis. As it is for PCR or other amplification techniques, a prefix could be added to specify the target used, e.g. rDNA-PRA or hsp65-PRA.

DETECTION OF DRUG RESISTANCE

The most potent first-line antituberculous drugs used for standard treatment of TB are isoniazid (INH) and rifampicin (RMP). Resistance of *M. tuberculosis* to both these drugs is termed multidrug resistance (MDRTB), and represents an important public health problem in many countries. Knowledge of the susceptibility patterns of *M. tuberculosis* isolates is important for the effective management of patients and for disease control. Second-line drugs used for treatment of MDRTB are more toxic, less effective, and more expensive.

Drug-resistance testing is traditionally performed using conventional methods in either solid or in liquid cultures (169–171). To obtain results more expeditiously molecular techniques were introduced (172, 173). These methods detect mutations in genes associated with the development of drug resistance in isolates or clinical specimens. The techniques are based on PCR in conjunction with electrophoresis, sequencing or hybridisation, and most of them have recently been reviewed (75, 123). Although most of the techniques were initially developed and evaluated to detect drug resistance in MTBC isolates, they are being explored for direct detection of MTBC and identification of mutant genes related to drug resistance in clinical specimens. However, it has to be remarked that these techniques may not detect all drug-resistant isolates: some drugs such as INH and streptomycin (SM) target different genes; other drugs such as RMP and ofloxacin are mostly restricted to a single gene; but resistance may also be due to mechanisms that remain to be elucidated.

PCR-sequencing

Sequencing is the gold standard and the most direct and widely used method for defining genetic resistance. The method includes PCR amplification of the DNA region of interest in clinical isolates or specimens and the detection of specific mutations after sequencing. Detection of RMP

resistance in clinical specimens is feasible by using primers designed to amplify an *rpo*B-specific PCR product. Hunt et al. (174) obtained amplicons for 7 of 16 clinical specimens, 5 of which contained MTBC-specific DNA, and sequences that correctly predicted RMP resistance. No MTBC-specific products were obtained from any of the 10 culture-negative specimens. Ohno et al. correctly predicted the RMP resistance of 26 sputum samples, 2 gastric lavages, and 1 synovial fluid sample obtained from patients with tuberculosis by direct sequencing of the *rpo*B gene (175). The assay proved to be sensitive (85%) in smears from culture-positive sputum specimens and was able to accurately identify mutations in the *rpoB* gene (176).

PCR-reverse hybridisation

INNO-LiPA Rif.TB Assay

As described above, the INNO-LiPA Rif.TB has been extensively evaluated in isolates.

Rifoligotyping

Rifampicin oligonucleotide typing (rifoligotyping) is a recently developed PCR-reverse line blot hybridisation assay in which a 437-bp fragment of the *rpo*B gene is amplified with primers, one of which is labelled with biotin. The amplicons are hybridised to a set of wild-type and mutant oligonucleotides covalently bound to a membrane by reverse line blotting (177) and detected by enzyme chemiluminescence. In an evaluation study, the assay detected 90/97 (92.8%) compared to culture results (178). More recently, the assay showed identical sensitivity (86%) with sequencing in detecting RMP resistance using DNA extracts from (ZN) stained slides (176). In general, the assay is easy to perform, can be completed in one day, and up to 43 samples can be tested in one run. In addition, the assay has the potential for increasing the number of probes on the membrane if more mutations become known.

PCR followed by differential electrophoresis

SSCP

One of the methods based on PCR-electrophoresis is Single Strand Conformational Polymorphism (SSCP). The principle of SSCP is that under nondenaturing conditions, single-stranded DNA fragments have a folded structure that is specific to the nucleotide sequence of the DNA. A difference in sequences, even a change in a single base, can affect the conformation of the single-stranded DNA. These conformational changes are detected as alterations in the electrophoretic mobility of the single-stranded DNA (179, 180). The method typically involves amplification of the target sequence by PCR, denaturation of the amplified fragments to single strands, and electrophoresis. The main advantage of PCR-SSCP is that it is simple, cheap and rapid, and suitable for analysis of a large number of samples. However, concerns about the sensitivity and specificity of the test have been raised (181, 182). By using a hemi-nested PCR system prior to SSCP, Whelen and colleagues correctly identified 21 of 24 (87.5%) culture-positive specimens, 13 of which were smear negative, in a panel of 51 (183). Furthermore, problems with reproducibility related to a number of parameters such as aspecific DNA amplification, retardation of amplimers in the presence of DNA polymerase, denaturation conditions, ionic strength, type of gel matrix, fragment length and temperature are well known.

PCR-heteroduplex formation (HDF) assay

Heteroduplex assay is another technique for detecting RMP resistance developed by Williams and co-workers (184). In this method a 305-bp fragment of the *rpo*B gene is amplified by PCR. The amplification products of a test strain are mixed with those of a reference strain and heated to denature the DNA, after which the DNA is allowed to renature on ice and then subjected to gel electrophoresis. A difference in electrophoretic mobility between the susceptible strain and a clinical isolate is indicative of the presence of a mutation. Further, Williams and co-workers used a hemi-nested PCR amplification to generate a MTBC-specific *rpo*B fragment that annealed to a universal heteroduplex generator (UHG) derived from the portion of the gene associated with RMP resistance (PCR-UHG-Rif assay) to predict genotypic resistance in five of six culture-confirmed resistant isolates (184, 185). The ability of the PCR-UHG-Rif assay to accurately determine RMP resistance directly from specimens was tested (186). Of the 1,892 culture-positive specimens, the assay detected RMP resistance correctly in 99% of the

smear-positive samples, but a positive PCR was obtained in only 49% of the smear-negative culture-positive samples. The sensitivity and specificity of the assay in comparison with culture and classical DST was 83.9 and 89.0%, respectively (186).

Real-time PCR

Real-time PCR has been employed in a number of studies to detect drug resistance in MTBC isolates (69–71, 73, 74). Van Doorn et al. recently developed a single-tube real-time PCR to detect INH susceptible or mutant codon (codon 315 of the *katG* gene) directly in ZN-positive sputum samples. The sensitivities of the wild-type and mutant probes were 70 and 94% while the specificities were 82 and 100%, respectively (187). Although both the sensitivity and specificity of the mutant probe in this study were high, the corresponding values for the wild- type probe were disappointing. Real-time PCR for detection of drug resistance has the same advantages and limitations as described above for direct detection of MTBC.

Other PCR-based resistance detection techniques

Several other molecular techniques have been suggested for detection of drug resistance, including linear signal amplification (188), dideoxy fingerprinting (189) and multiplex allele-specific PCR (190). However, these techniques are experimental and have not been extensively applied.

DETECTION AND TYPING OF MTBC

A few techniques have been developed to detect mycobacteria belonging to the MTBC coupled with typing. They are either based on variations in the number of tandem repeats (VNTR) or on variations in the presence of direct repeats (spoligotyping). In most cases the patient has no urgent need for information respecting the type of MTBC isolate, but PCR-based typing methods could be helpful in large molecular epidemiological studies.

Variable number of tandem repeat (VNTR) typing

The Mycobacterial Interspersed Repetitive Units (MIRUs), used as one of the sequences in this technique, were described in the intergenic region separating two genes encoding a mycobacterial two-component system named SenX3-RegX3, and found to be present as tandem repeats (191). These MIRUs, like other tandem repeats, vary in number of repeats among strains of the MTBC. Amplification of the loci incorporating the repeats by PCR followed by detection on agarose gel or by a DNA sequencer allows determination of the size of the amplicons and hence calculation of the number of repeats. This method was found to be 100% specific and 100% sensitive when tested on 122 cultures of mycobacteria belonging to the MTBC, including *M. tuberculosis* devoid of IS*6110* elements or mpt40 (192). Apart from the MIRUs, other types of tandem repeats have also been described (193, 194). There are no reports of VNTR application in clinical specimens.

Spoligotyping

Spacer oligotyping (spoligotyping) depends on the polymorphism of the chromosomal Direct Repeat (DR) locus of MTBC strains containing a variable number of short repetitive DNA sequences (DRs) of 36-bp separated by nonrepetitive genomic sequences of 35–41-bp, the spacers (195). After PCR using biotinylated primers, the amplicons are hybridised to 43 spacer-specific oligonucleotides immobilised on a membrane. Detection is achieved via a streptavidin-peroxidase conjugate and substrate and detected by chemiluminescence (196). Spoligotyping can be used to differentiate between *M. tuberculosis* and *M. bovis,* and *M. microti.* It was successfully used to detect and type 34/35 *M. bovis* in lymph node specimens using sequence capture PCR (197). Goyal et al. (198) obtained successful spoligotyping results in 159 of 175 patients tested using DNA extracted from sputum samples. The technique has also been used to characterise MTBC DNA from historic tissue samples (199, 200). It can be used to test as many as 43 samples in one run and can be completed within one day after PCR amplification. However, the method needs further optimisation if it is to be routinely used for clinical samples.

FINAL REMARKS

Tremendous advancements in the development and performance of new technological ap-

proaches for the rapid diagnosis of mycobacterioses, and especially tuberculosis, and prediction of drug resistance have been made in the last few years. These methods use various targets such as genes coding for the 16S rRNA, 23S rRNA, *oxyR*, *recA*, mpt40, p27, MPB64, heat shock proteins, a number of repetitive elements and genes involved in drug resistance. So far, a number of commercial and 'in-house' techniques are available for direct application in respiratory specimens with a few being adapted for nonrespiratory specimens as well. The obvious advantages of molecular techniques are their ability to provide results within hours, with increased sensitivities and specificities compared to microscopy, increased sensitivity compared to culture in some cases of extrapulmonary infections (66), and the possibility of being performed on heat-inactivated materials thereby minimising biosafety concerns. However, these techniques are still fairly complicated and require expensive equipment, e.g. thermocyclers, electrophoresis equipment, sequencing and other automated systems, as well as experienced personnel and specific PCR facilities, such as pre- and post-PCR rooms, to prevent cross-contamination.

Molecular techniques are affected by a high risk of cross-contamination. As became clear from recent proficiency testing studies, a considerable reduction in false positives (111) was obtained compared to previous results (108–110), probably by using better-trained staff and improved PCR facilities. Although there have been improvements in the performance of molecular techniques for detecting MTBC in clinical specimens in many laboratories, concern regarding lack of sufficient sensitivity for application to smear-negative and nonrespiratory samples still remains (201). Presence of DNA-polymerase inhibitors and uneven distribution of bacilli due to the paucibacillary nature of some specimens are the main causes of the low sensitivities observed. In order to detect false-negative results due to inhibition, use of tests with internal amplification controls is encouraged. More extended DNA-extraction procedures, as applied in most noncommercial assays, could improve sensitivities (86). On the other hand, improved sensitivity would imply loss of rapidity. However, the question of how quickly results of chronic diseases should be available to the physician still remains. Should we aim at a few hours (with a lower sensitivity) or are we better off with a rapid assay with a turnaround time of 2 to 3 days (possibly with a higher sensitivity)? Furthermore, the theoretical turnaround time of most currently existing commercial tests (a few hours) approaches that of microscopy, but in practice this may take up to 2 days owing to the common practice of examining samples daily, twice weekly, or even weekly in many clinical laboratories. Also, these tests not only require well-trained personnel but also increase the workload of already overburdened laboratories. This may have an effect on the performance of NAATs.

In terms of performance, there appears to be no significant difference between commercial tests and in-house tests (111). Further, as nearly all commercial assays do not include pretreatment procedures for decontamination and liquefaction of samples, performance of these assays will largely depend on local practices of these procedures (202, 203). In contrast to direct microscopy, currently used NAATs can give specific information on the presence of bacilli belonging to the MTBC, differentiating them from NTM but not between the different species belonging to the complex, with an exception in the case of the 65 kDa-based PCR that can distinguish between *M. bovis* and *M. tuberculosis*, and the mpt40 for differentiating *M. bovis* from other mycobacteria. Interspecies differentiation of MTBC does not affect the choice of treatment, whereas MTBC-NTM differentiation is important for proper treatment.

Therefore, molecular identification of MTBC and NTM directly from clinical specimens saves considerable time, and allow detection and identification of difficult-to-grow mycobacteria such as *M. ulcerans, M. genavense* (204), *M. paratuberculosis* and noncultivable species, notably *M. leprae*. The MTBC-NTM differentiation appears to be mainly relevant in settings with a low prevalence of TB, where disease due to NTM is more frequent. In high prevalence countries where nearly all ZN smear-positive cases are due to MTBC, the added value of simultaneous screening for MTBC and NTM would be unjustified.

Rapid information on drug resistance can be of great consequence, especially in high-risk areas, in order to detect MDRTB and either

adapt the treatment and/or isolate the patient to prevent transmission. So far, most molecular techniques have focused on the rapid detection of RMP resistance, the most potent drug, and a good marker for MDRTB in high prevalence settings (6, 68). INNO-LiPA-Rif.TB has been evaluated on a large scale, providing rapid and reliable information. Direct sequencing of the *rpoB* gene or microarrays could be future applications, but require further evaluation. The INNO-LiPA-Rif.TB test is easy to perform, and apart from PCR-facilities there are no additional costs for equipment though costly materials are required, whereas the two latter techniques require both expensive equipment and materials. Theoretically, all techniques could be applied to genes related to resistance to other drugs as well but the nature of drug resistance in *M. tuberculosis* does not allow this. Therefore, culture remains indispensable for DST, and rapid culture-based techniques such as metabolic tests or microcolony detection could serve as cheap and easier to implement alternatives in some settings (205). In most cases the patient has no urgent need for the rapid information on the type of MTBC isolate, but PCR-based typing methods could be helpful in large molecular epidemiological studies.

Current opinion on the use of molecular techniques in clinical management has been to use them in combination with smear results and clinical suspicion (206, 207). In this regard, patients who are positive by both smears and molecular techniques can be taken to have TB. However, as treatment is mostly initiated in smear-positive patients based on clinical suspicion and sometimes on chest X-ray results in high incidence settings, molecular techniques may have little impact in these patients. Nonetheless, a combination of techniques can be very useful in confirming a diagnosis of TB in smear-negative patients and in extrapulmonary cases with a high clinical suspicion, though not for ruling out these cases owing to their overall low sensitivity. Thus, molecular techniques are thought to be more cost effective in cases with doubtful clinical suspicion but are not suited for following the effect of treatment.

Finally, molecular methods hold considerable promise in the early diagnosis and detection of drug resistance of MTBC directly from clinical specimens. However, rapid diagnosis of TB in nonrespiratory disease and smear-negative patients with high clinical suspicion remains a major challenge. Thus, molecular techniques will only play a complementary role to classical and culture-based techniques, which will remain indispensable for definitive diagnosis in some cases and determination of drug resistance in the foreseeable future.

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