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Identification of *Mycobacterium* Species and *Mycobacterium tuberculosis* Complex Resistance Determinants by Use of PCR-Electrospray Ionization Mass Spectrometry

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PCR coupled with electrospray ionization mass spectrometry (PCR–ESI-MS) is a novel technology that has recently been used to identify pathogens from clinical specimens or after culture within about 6 h. We evaluated the MDR-TB (multidrug-resistant tuberculosis) assay, which uses PCR–ESI-MS for detection and identification of *Mycobacterium* spp. and *Mycobacterium tuberculosis* complex (MTBC) resistance determinants from solid and broth Middlebrook culture media. The performance of the MDR-TB assay was compared to identification using nucleic acid hybridization probes and 16S rRNA gene sequencing for 68 MTBC and 97 nontuberculous mycobacterial (NTM) isolates grown on agar and 107 cultures grown in Bactec MGIT broth. MTBC resistance profiles from the MDR-TB assay were compared to results with the agar proportion method. The PCR–ESI-MS system correctly identified all MTBC isolates and 97.9% and 95.8% of the NTM isolates from characterized agar cultures and MGIT broth cultures to the species level, respectively. In comparison to the agar proportion method, the sensitivity and specificity for the detection of drug resistance using the MDR-TB assay were 100% and 92.3% for rifampin, 100% and 93.8% for isoniazid, 91.6% and 94.4% for ethambutol, and 100% and 100% for fluoroquinolones, respectively. The MDR-TB assay appears to be a rapid and accurate method for the simultaneous detection and identification of mycobacterial species and resistance determinants of MTBC from culture.

Tuberculosis (TB) persists as a global health concern, with 8.7 million new cases and 1.4 million deaths in 2011 (1). Further, drug-resistant and multidrug-resistant TB is established throughout the world (2). Since pulmonary TB is highly transmissible, rapid diagnosis and infection control make up essential elements of control. The poor sensitivity of smear microscopy and the untimely nature of culture (requiring up to 6 weeks) have hindered diagnoses (3). Thus, the development of rapid and accurate diagnostic tests is critical to help establish appropriate clinical management and infection control measures to further prevent transmission and the amplification of resistance (2).

Over the past decade, there have been increasing efforts, funding, and advances in diagnostic technologies for detecting *Mycobacterium tuberculosis* complex (MTBC) and its determinants of drug resistance. These new technologies include liquid media for culture and drug susceptibility testing (DST), line probe assays, and real-time PCR technologies (4). Currently, the detection of MTBC and characterization of drug resistance markers using molecular methods is largely limited to the use of separate assays for individual markers (e.g., 16S rRNA, *rpoB*, *katG*, etc). Exceptions include the real-time PCR GeneXpert MTB/RIF assay (MTBC and rifampin) and the InnoLiPA Rif.TB (MTBC and rifampin), GenoType MTBDRPlus (MTBC, rifampin, and isoniazid) and GenoType MTBDRsl (MTBC, fluoroquinolone, amikacin-capreomycin, and ethambutol) line probe assays. Recently, whole-gene sequencing for determining TB drug resistance mutations in a research setting has been reported, but it remains to be seen if this can be reasonably adapted to clinical laboratories (5). Another novel technology couples PCR with electrospray ionization mass spectrometry (PCR–ESI-MS) to permit the identification of microorganisms from culture or directly from clinical specimens within 6 h (6, 7).

In this study, we evaluated the ability of the MDR-TB (multidrug-resistant tuberculosis) assay (Ibis Biosciences, Carlsbad, CA), which utilizes PCR–ESI-MS, to simultaneously detect *M. tuberculosis* complex and nontuberculous mycobacteria from positive cultures (11). In addition, the MDR-TB assay was evaluated to determine its ability to detect resistance determinants in MTBC for isoniazid (INH), rifampin (RIF), ethambutol (EMB), and the fluoroquinolones (FQ).

MATERIALS AND METHODS

Mycobacteria. The ability of the MDR-TB assay to accurately identify previously characterized mycobacterial isolates was evaluated by testing 68 previously characterized MTBC and 97 previously characterized nontuberculous mycobacterial (NTM) isolates. Isolates included ATCC strains and culture isolates identified using either the AccuProbe nucleic acid hybridization probes (GenProbe, San Diego, CA) or 16S rRNA gene sequencing as previously described (8). In addition, 57 positive and 50 negative Bactec MGIT broth cultures from patients suspected of having a mycobacterial infection and for which routine mycobacterial testing was requested were also tested with the MDR-TB assay using the AccuProbes or 16S sequencing as the reference methods. The ability of the MDR-TB assay to determine genetic markers of drug resistance of MTBC was evaluated using a subset of 48 well-characterized MTBC isolates described above for which agar proportion results were available for use as the reference method. The genetic markers detected by the MDR-TB assay include *katG* and the promoter regions of *inhA* and *ahpC* for INH, *rpoB* for RIF, *embB* for EMB, and *gyrA* for the FQ.

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(i) Mycobacterial culture isolates. The isolates were freshly subcultured onto Middlebrook 7H10 (BBL, Sparks, MD) and were lysed by placing a loopful of the organism into a 2.0-ml tube containing 500 μl of sterilized water, 50 μl of 0.1-mm silica glass beads, and 100 μl of 2.4-mm zirconia beads (BioSpec Products, Inc., Bartlesville, OK). The tubes were heated at 95°C for 5 min and then placed on a Disruptor Genie instrument (Scientific Industries, Bohemia, NY) for 2 min to mechanically lyse the organisms and release the nucleic acid. From the stock lysates, a 1:20 dilution was performed by adding 20 μl of lysate into 180 μl of sterile water. The diluted lysates were used as the template for the PCRs (10).

(ii) MGIT broth cultures. After routine culture identification and susceptibility testing was completed, 1 ml of the MGIT culture was added to 2.0-ml tube containing 50 μl of 0.1-mm silica glass beads and 100 μl of 2.4-mm zirconia beads (BioSpec Products, Inc., Bartlesville, OK) and centrifuged at 17,900 × *g* for 10 min. The supernatant was removed, and the pellet was resuspended in 300 μl of sterile water. The tubes were then heated at 95°C for 5 min and then placed on a Disruptor Genie instrument (Scientific Industries, Bohemia, NY) for 2 min to mechanically lyse the organisms and release the nucleic acid. The lysates were used as the template for the PCRs.

PCR–ESI-MS analysis. PCR plates (Broad Fungal Assay; Abbott Laboratories) were thawed and centrifuged at 1,800 × *g* (IEC Centra MP4R; Thermo Scientific) for 1 min. PCR plates were loaded with extracted DNA using a CAS-1200 precision liquid-handling system (Corbett Research [Qiagen], Valencia, CA). Eight wells containing two primer sets per well were filled with 10 μl of extracted sample, allowing testing of 12 samples per 96-well plate. Primer pairs were previously described by Massire et al. (11). Following sample loading, the plates were sealed with Easy Pierce 20-μm heat-sealing tape (catalog no. AB-1720; Thermo Scientific) at 175°C for 1.5 s (ThermoSci ALPS 50V; Thermo Scientific), centrifuged at 1,800 × *g* for 1 min, and loaded on to the Eppendorf Mastercycler proS thermocycler (Eppendorf, Hamburg, Germany. The PCR was carried out using the manufacturer's recommended thermocycler protocol as previously reported (10).

Sample analysis. Sample analysis was performed following PCR amplification by loading the plate onto the Plex-ID system (Abbott Molecular, Des Plaines, IL), where the PCR product was desalted and analyzed using electrospray ionization mass spectrometry (ESI-MS). The base count of each amplicon was determined for each primer pair and compared to database version NFDU.415.455.349. The MDR-TB assay utilizes signal thresholds (cutoffs) designed to limit reporting of irreproducible detections. Cutoffs are applied to two measurements, the level and the Q score, as previously described (9, 10). The level is an indication of the amount of the amplicon present in the sample in comparison to a calibrant, and the Q score is a rating between 0 (low) and 1 (high) which represents the strength of the data supporting identification. For the MDR-TB assay, a Q score of ≥0.85 is considered a reportable result. Results included the *Mycobacterium* species identification and resistance determinants for INH, RIF, EMB, and FQ if MTBC was identified.

Work flow. The overall work flow to process 12 samples (one MDR-TB assay plate) on the PCR–ESI-MS system from start to finish is approximately 6 h, with approximately 1 h of hands-on time. Following a strict unidirectional workflow, the overall turnaround time (TAT) to reporting of results was 24 h, since the plates were placed on the PCR–ESI-MS system at the end of the work day and the data were analyzed the following morning, as previously described (10).

RESULTS

Mycobacterial culture isolates. PCR–ESI-MS identified all (100%; 68/68) *M. tuberculosis* complex culture isolates as MTBC (Table 1). To challenge the specificity of the MDR-TB assay to identify NTMs, we evaluated 97 previously characterized NTM isolates (Table 2). Of the 97 NTM isolates, PCR–ESI-MS was able to identify 99.0% (96/97) and 96.9% (94/97) of the isolates to the genus and species levels, respectively. Only one misidentification occurred, where *M. rhodesiae* was identified as *M. aichiense*. *M. alvei* was not detected by the MDR-TB assay, and an *M. xenopi* isolate was identified as *Mycobacterium* species JDM601. Three isolates were correctly identified but also had a second NTM identified (*M. austroafricanum* was identified as *M. austroafricanum*/*M. vanbaalenii*, while *M. murale* and *M. tokaiense* were each identified as *M. murale*/*M. tokaiense*). The three results with multiple NTM matches reported were counted as correct identifications, but it should be recognized that the MDR-TB assay cannot separate these species. *M. murale* and *M. tokaiense* are also indistinguishable by 16S rRNA gene sequencing.

TABLE 1 Identification of previously characterized *M. tuberculosis* complex isolates grown on agar medium and resistance profiles determined by the agar proportion method compared to those determined by the MDR-TB assay[a]

No. of isolates[b]	Agar proportion result				MDR-TB assay result			
	INH	RIF	EMB	OFL	INH	RIF	EMB	FQ
26	S	S	S	S	S	S	S	S
4	R	S	S	S	R	S	S	S
3	R	R	R	S	R	R	R	S
2	S	S	R	S	S	S	R	S
2	R	R	R	R	R	R	R	R
1	R	S	R	S	R	S	R	S
1	S	R	S	S	S	R	S	S
1	R	**S**	R	S	R	**R**	R	S
1	**S**	S	S	S	**R**	S	S	S
1	S	**S**	S	S	S	**R**	S	S
1	R	R	**R**	S	R	R	**S**	S
1	**S**	S	**S**	S	**R**	S	**R**	S
1	R	S	**R**	S	R	S	**S**	S
1	S	R	R	S	S	R	R	S
1	R	**S**	S	S	R	**R**	S	S
1	R	R	**S**	S	R	R	**R**	S

[a] S, susceptible; R, resistant; INH, isoniazid; RIF, rifampin; EMB, ethambutol; OFL, ofloxacin. Discordant results are in bold.

[b] All isolates were appropriately identified as *M. tuberculosis* complex by the MDR-TB assay. An additional 20 isolates were appropriately identified by PCR–ESI-MS but are not included in this table because agar proportion method susceptibility results were not available for comparison.

MGIT broth cultures. The accuracy of the MDR-TB assay to identify MTBC and NTM directly from positive MGIT broth cultures was compared to identification using the AccuProbes for MTBC, *M. avium* complex, and *M. gordonae*, while 16S rRNA gene sequencing was used for identification of all other species. Fifty-seven positive and 50 negative MGIT tube broth cultures were tested. Of the positive MGIT broth cultures, 9 of 9 (100%) MTBC isolates were identified correctly (Table 3). In addition, all 9 isolates were found to be susceptible to the first-line agents EMB, INH, and RIF by broth susceptibility on the VersaTREK system (Trek Diagnostics, Cleveland, OH) and by the MDR-TB genotypic assay. For the NTM-positive MGIT broth cultures, the MDR-TB assay identified 100% (48) and 95.8% (46/48) of the NTM isolates correctly to the genus and species levels, respectively. Two *M. gordonae* isolates were identified by the MDR-TB assay as *Mycobacterium* species. One *M. avium* complex isolate identified by AccuProbe had a multiple detection of *M. avium* (Q score, 1; level, 666) and *M. gordonae* (Q score, 0.92; level, 37) by the MDR-TB assay. Sequencing of the MGIT tube lysate confirmed the presence of *M. avium* complex. Of the 50 MGIT tube-negative cultures, all were negative except 1, which was identified as *M. gordonae* (Q

TABLE 2 Previously characterized nontuberculous mycobacteria identified by AccuProbes and/or 16S sequencing and comparison with results of MDR-TB assay[a]

Mycobacterium species (AccuProbe or 16S sequencing ID)	No. of isolates	MDR-TB assay result(s)
M. acapulcensis	1	*M. acapulcensis*
M. agri	1	*M. agri*
M. aichiense	1	*M. aichiense*
M. alvei	1	Not detected
M. aurum	1	*M. aurum*
M. austroafricanum	1	*M. austroafricanum; M. vanbaalenii*
M. avium	5	*M. avium*
M. avium subsp. *silvaticum*	1	*M. avium*
M. boenickei	1	*M. boenickei*
M. bohemicum	1	*M. bohemicum*
M. bolletii	1	*M. bolletii*
M. branderi	1	*M. branderi*
M. brumae	1	*M. brumae*
M. canariasense	1	*M. canariasense*
M. chitae	1	*M. chitae*
M. chlorophenolicum	1	*M. chlorophenolicum*
M. conceptionense	1	*M. conceptionense*
M. confluentis	1	*M. confluentis*
M. cookii	1	*M. cookii*
M. cosmeticum	1	*M. cosmeticum*
M. diernhoferi	1	*M. diernhoferi*
M. doricum	1	*M. doricum*
M. duvalii	1	*M. duvalii*
M. elephantis	1	*M. elephantis*
M. flavescens	1	*M. flavescens*
M. fortuitum	1	*M. fortuitum*
M. fortuitum subsp. *acetamidolyticum*	1	*M. fortuitum*
M. frederiksbergense/gilvum	1	*M. gilvum*
M. gadium	1	*M. gadium*
M. gallinarum	1	*M. gallinarum*
M. gastri	1	*M. gastri*
M. gilvum	1	*M. gilvum*
M. gordonae	1	*M. gordonae*
M. haemophilum	1	*M. haemophilum*
M. hassiacum	1	*M. hassiacum*
M. heckeshornense	1	*M. heckeshornense*
M. heidelbergense	1	*M. heidelbergense*
M. hiberniae	1	*M. hiberniae*
M. hodleri	1	*M. hodleri*
M. holsaticum	1	*M. holsaticum*
M. houstonense	1	*M. houstonense*
M. immunogenum	1	*M. immunogenum*
M. interjectum	1	*M. interjectum*
M. intermedium	1	*M. intermedium*
M. intracellulare	4	*M. intracellulare*
M. kansasii	1	*M. kansasii*
M. kubicae	1	*M. kubicae*
M. lacus	1	*M. lacus*
M. lentiflavum	1	*M. lentiflavum*
M. mageritense	1	*M. mageritense*
M. malmoense	1	*M. malmoense*
M. marinum	1	*M. marinum*
M. massiliense	1	*M. massiliense*
M. moriokaense	3	*M. moriokaense*
M. murale	1	*M. murale; M. tokaiense*
M. neoaurum	1	*M. neoaurum*
M. neworleansense	1	*M. neworleansense*
M. nonchromogenicum	1	*M. nonchromogenicum*
M. novocastrense	1	*M. novocastrense*
M. obuense	1	*M. obuense*
M. palustre	1	*M. palustre*
M. parafortuitum	1	*M. parafortuitum*
M. parascrofulaceum	1	*M. parascrofulaceum*
M. peregrinum	1	*M. peregrinum*
M. phlei	1	*M. phlei*
M. phocaicum	1	*M. phocaicum*
M. porcinum	1	*M. porcinum*
M. poriferae	1	*M. poriferae*
M. psychrotolerans	1	*M. psychrotolerans*
M. pulveris	1	*M. pulveris*
M. rhodesiae	1	*M. aichiense*
M. saskatchewanense	1	*M. saskatchewanense*
M. scrofulaceum	1	*M. scrofulaceum*
M. senegalense	1	*M. senegalense*
M. seoulense	1	*M. seoulense*
M. septicum	1	*M. septicum*
M. setense	1	*M. setense*
M. shimoidei	1	*M. shimoidei*
M. simiae	1	*M. simiae*
M. smegmatis	1	*M. smegmatis*
M. sphagni	1	*M. sphagni*
M. szulgai	1	*M. szulgai*
M. terrae	1	*M. terrae*
M. thermoresistibile	1	*M. thermoresistibile*
M. tokaiense	1	*M. murale*; *M. tokaiense*
M. triplex	1	*M. triplex*
M. wolinskyi	1	*M. wolinskyi*
M. xenopi	1	*Mycobacterium* sp. JDM601
Total	97	
No. (%) with agreement to the species level	94 (96.9)	

[a] Shaded areas are discordant results.

score, 1; level, 379). Sequencing of the lysate was negative and confirmed the negative culture results.

Genetic markers of drug resistance. The second aim of the study was to evaluate the ability of the MDR-TB assay to determine genetic markers of drug resistance of MTBC using a subset of 48 well-characterized isolates (Table 1). The sensitivities and specificities for each antimicrobial agent, comparing the MDR-TB assay results to the reference standard method of agar proportion, are summarized in Table 4. The MDR-TB assay had a sensitivity and specificity of 100% and 93.8% for the detection of INH resistance. Two major errors were detected in which a *katG* S315T nucleotide substitution which would suggest INH resistance was detected, but the isolates were susceptible by the agar proportion method. The MDR-TB assay had a sensitivity and specificity of 100% and 92.3% for detection of RIF resistance. Three discordant results were observed. All three were determined to be susceptible by agar proportion, but previously described resistance substitutions were detected to be encoded in *rpoB*, L511P, D516G, and D516F, by PCR–ESI-MS. The MDR-TB assay also demonstrated a sensitivity and specificity of 91.6% and 94.4% for the detection of EMB resistance. Two major errors occurred (*embB*, M306V and M306I), and one very major error occurred (no resistance muta-

TABLE 3 Identification of *Mycobacterium* species from Bactec MGIT broth positive bottles by AccuProbes and/or 16S sequencing compared with identification by MDR-TB assay

Mycobacterium species or group	No. of isolates	MDR-TB result
M. avium complex	17	*M. intracellulare*
M. tuberculosis complex	9	*M. tuberculosis*
M. avium complex	8	*M. avium*
M. gordonae	5	*M. gordonae*
M. abscessus group	3	*M. abscessus*
M. kansasii	2	*M. kansasii*
M. chelonae	2	*M. chelonae*
M. gordonae	2	*Mycobacterium* sp.
M. fortuitum	1	*M. fortuitum*
M. avium complex	1	*M. avium*/*M. gordonae*
M. xenopi	1	*M. xenopi*
M. scrofulaceum	1	*M. scrofulaceum*
M. mucogenicum/*phocaicum*	1	*M. phocaicum*
M. abscessus group	1	*M. massiliense*
M. abscessus group	1	*M. bolletii*
M. obuense	1	*M. obuense*
M. simiae	1	*M. simiae*
Total	57	
No. (%) with agreement to the species level	55 (96.5)	

TABLE 4 Comparison of *M. tuberculosis* complex resistance profiles determined by agar proportion and MDR-TB assay

Drug and MDR-TB assay result	No. of isolates with result by agar proportion method		Performance of MDR-TB assay	
	Resistant	Susceptible	% sensitivity	% specificity
Isoniazid			100	93.8
Resistant	16	2		
Susceptible	0	30		
Rifampin			100	92.3
Resistant	9	3		
Susceptible	0	36		
Ethambutol			91.6	94.4
Resistant	11	2		
Susceptible	1	34		
Fluoroquinolones			100	100
Resistant	2	0		
Susceptible	0	46		

tions were detected in an isolate identified as resistant by the agar proportion method). The MDR-TB assay had a sensitivity and specificity of 100% for the detection of FQ resistance. Notably, the negative predictive value (NPV) for INH, RIF, and FQ resistance was 100%, and that for EMB resistance was 97.1%.

DISCUSSION

There are only two published studies of the MDR-TB assay using the PCR–ESI-MS system. The first report, by Massire et al., described the development of the assay by Ibis Biosciences (11). The second article, by Wang et al., described the molecular characterization of drug resistance profiles in 96 *Mycobacterium tuberculosis* isolates circulating in China (7). This is the first study evaluating the clinical utility of the MDR-TB assay for simultaneously detecting MTBC, NTM, and MTBC resistance determinants in comparison to conventional laboratory methods.

The first aim of this study was to determine the accuracy of the MDR-TB assay in correctly identifying previously characterized MTBC and NTM isolates. The MDR-TB assay correctly identified 100% of the MTBC and 96.9% of the NTM culture isolates to the species level. One *M. alvei* isolate was not detected, one *M. xenopi* isolate was identified as *Mycobacterium* species, and one misidentification of *M. rhodesiae* as *M. aichiense* was observed. The accuracy of the MDR-TB assay was further challenged by comparing results from AFB-positive MGIT tube broth cultures to those of standard methods used in the clinical microbiology laboratory. As seen with the isolates cultured on solid Middlebrook agar, 100% of MTBC and 95.8% of NTM isolates were identified correctly to the species level from positive MGIT broths. Two *M. gordonae* isolates were identified to the genus level only as *Mycobacterium* species. These results are similar to those described by Massire et al., where all MTBC and 96.3% of NTM isolates from positive MGIT broth cultures were appropriately identified by the MDR-TB assay (11). Most important, none of the MTBC isolates from our study were incorrectly identified as NTM or vice versa. The MDR-TB assay detected *M. gordonae* isolates in one positive *M. avium* complex MGIT tube and one negative MGIT tube that were not detected by culture. Sequencing was performed on both lysates, and *M. gordonae* was not detected. This suggests the possibility that a reagent may have been contaminated with *M. gordonae* DNA. The MDR-TB assay can reliably detect MTBC and does not appear to "cross-react" with NTM species, which is important in a setting with a high NTM prevalence.

The second aim of the study was to compare the ability of the MDR-TB assay to determine MTBC genetic markers of drug resistance compared to the gold standard agar proportion method. The study by Wang et al. characterized the resistance determinants of 96 MTBC isolates from China using the MDR-TB assay; however, no comparison with phenotypic DST or other comparator method was performed (7). In comparison to phenotypic DST, the sensitivity and specificity values for the detection of first-line and second-line drug resistance using the MDR-TB assay were 100% and 92.3% for RIF, 100% and 93.8% for INH, 91.6% and 94.4% for EMB, and 100% and 100% for FQ, respectively. These results are comparable to those with other rapid molecular methods, such as Sanger dideoxy sequencing, pyrosequencing, the Cepheid GeneXpert MTB/RIF assay, and the InnoLiPA Rif.TB, GenoType MTBDRPlus, and GenoType MTBDRsl line probe assays (12–15).

Fortunately, genotypic and phenotypic correlation for the most frequently used first-line drugs, INH and RIF, is quite reliable (15). Rifampin resistance was detected by the MDR-TB assay with a sensitivity and specificity of 100% and 92.3%, respectively. Mutations within the *rpoB* gene, which encodes the DNA-dependent RNA polymerase, generally correlate with phenotypic results and predict resistance to RIF in 90 to 95% of the isolates (16). Likewise, a study comparing sequencing of the *rpoB* gene to the agar proportion method revealed a sensitivity of 97% and specificity of 93.6% (17). Discordant results by the MDR-TB assay resulting in a decreased specificity of the assay included L511P,

TABLE 5 Comparison of methodologies for simultaneous detection and identification of MTBC, NTM, and MTBC resistance determinants[a]

Methodology	Detection of MTBC	Detection of NTMs	Detection of 1st–line agents	Detection of 2nd–line agents	Advantages	Limitations
GeneXpert MTB/RIF (2, 15)	Yes	No	*rpoB* only	No	Direct from sputum	Cost of cartridge and instrumentation (2)
					Automated closed system	Only one marker for resistance included
					Random access	
					Highly trained staff not required	
					WHO endorsed (25)	
					Rapid [~2 h]	
Line probe assays[b] (2, 15)	Yes	Yes	Yes	Yes	Validated from specimens and culture isolates	Multiple different line probes required for simultaneous detection of mycobacterial species and resistance determinants
					WHO endorsed (26)	Cost/strip (26)
					Rapid [~4–6 h]	Cost of instrumentation [thermocycler and automated washer]
						Open system
						Labor-intensive
						Sensitivity decreases if performed direct from smear-negative specimens (15)
Pyrosequencing (12, 27)	Yes (27)	Yes (27)	*rpoB*, *katG*, *embB*, *inhA* (12)	*rrs*, *gyrA*, *eis* (12)	Moderately rapid [~1 day]	Short sequence reads
					High throughput [12 samples/96 well plate]	Not yet performed on direct specimens
						Multiple targets needed
						Cost/isolate
						Cost of instrumentation
						Experienced personnel required
Sanger sequencing for speciation and for all known resistance-determining regions (15)	Yes	Yes	*rpoB*, *inhA*, *katG*, *embB*, *pncA*	*gyrA*, *rrs*, *eis*, *tlyA*	Referenced against a large database of isolates	Labor-intensive
					Ability to identify novel mutations associated with resistance	Cost/gene
					Performed from smear positive specimens and culture isolates	Cost of instrumentation
					Provides a resistance marker for PZA	Most suitable for reference laboratories
					Performed at CDC	Experienced personnel required
					Moderately rapid [~1 day]	
Next-generation sequencing (5)	Not at this time; only use of known positive MTBC isolates (5)	Not at this time	*katG*, *rpoB*, *pncA*	*rrs*, *gyrA*	Full-length characterization of genes	Not yet performed on direct specimens
					Ability to identify novel mutations associated with resistance	Cost/sample
					Moderately rapid [~2 days]	Cost of instrumentation
					Provides a resistance marker for PZA	Labor-intensive
						Experienced personnel required
						Most suitable for reference laboratories
PCR–ESI-MS; current study (7, 11)	Yes	Yes	*katG*, *inhA*, *ahpC*, *rpoB*, *embB*	*gyrA*	Simultaneous detection of mycobacterial species [MTBC or NTMs] and MTBC drug resistance markers from culture isolates	Short sequence reads
					High throughput [12 samples/MDR-TB panel]	Not yet performed on direct specimens
					Moderately rapid [~1 day]	Open system
						Cost/sample
						Cost of instrumentation (10)
						Labor intensive
						Experienced personnel required
						Most suitable for reference laboratories

[a] References are given in parentheses.

[b] Commercial tests include INNO-LiPA Rif.TB (MTBC and rifampin), GenoType MTBDRPlus (MTBC, rifampin, and isoniazid) and GenoType MTBDRsl (MTBC, fluoroquinolone, amikacin-capreomycin and ethambutol), INNO-LiPA Mycobacteria v2 assay (mycobacterial species; MTBC and NTM), GenoType Mycobacterium CM/AS (mycobacterial species; NTMs).

D516G, and D516F substitutions. These substitutions have been associated with discordant susceptibility tests in previous studies (17, 18). It has been proposed that they could confer low-level but clinically relevant resistance (18).

Resistance to INH has a high correlation with mutations present within two genes: *katG* (encoding a catalase-peroxidase enzyme required for isoniazid activation) and *inhA* (promoter region). Mutations within the *katG* gene provide high-level resistance and account for the majority of phenotypic resistance (~85%) (15). Isoniazid resistance was detected by the MDR-TB assay with a sensitivity and specificity of 100% and 93.8%, respectively. The MDR-TB assay includes a third target for the detection of INH resistance, the promoter region of *ahpC*. The *ahpC* gene encodes an alkyl hydroperoxide reductase, which is involved in the cellular response to oxidative stress (19). Studies have demonstrated an association of mutations in the *ahpC* promoter region with an INH resistance phenotype (20). However, the inclusion of the *ahpC* gene in molecular studies has been questioned because there are limited data on the role in INH resistance (12). None of the isolates that were resistant to INH in this study had mutations detected in the promoter region of the *ahpC* gene. Based on our results, the *ahpC* target does not appear to play a substantial role in increasing the sensitivity of the assay to detect INH resistance. However, the number of INH-resistant isolates tested in this study was small ($n = 16$), so further testing with a larger cohort of INH-resistant isolates is required to substantiate the role of *ahpC*. The decreased specificity of the MDR-TB assay was owing to two isolates with *katG* S315T nucleotide substitutions that were found to be susceptible by the agar proportion method. Generally, mutations at codon 315 within *katG* are the most common providing resistance to INH.

Although the resistance-determining region of *embB* (arabinosyl transferase) has been described and used as the primary molecular target for EMB resistance, it represents only ~60% of phenotypic resistance (21). On the other hand, conventional DST for EMB is notoriously problematic due to a narrow range between the MICs of susceptible and resistant isolates (12, 22). The MDR-TB assay exploits the resistance-determining region of *embB* to predict EMB susceptibility with a sensitivity and specificity of 91.6% and 94.4%, respectively. These results are in line with those of other studies demonstrating both false-positivity and false-negativity issues with *embB* as the sole genetic marker for EMB resistance in comparison to phenotypic DST (15, 17, 22).

Interestingly, the resistance-determining region of *gyrA* covered by the MDR-TB assay provided 100% sensitivity and 100% specificity for determining phenotypic susceptibilities to the FQ. Our study supports the high specificity of the *gyrA* marker but contrasts with other studies that found that the resistance-determining region of *gyrA* accounts for only 80% of phenotypic resistance (17). These differences could be due to the region of the target gene used in each assay, differences in strains tested, and the small number of FQ-resistant strains in our cohort.

In recent years, several techniques that simultaneously detect MTBC and drug resistance markers for MTBC have been described. These methods are generally nucleic acid amplification (NAAT) tests, including line probe assays, real-time PCR assays, and sequencing methods. A comparison of these methodologies with the PCR–ESI-MS method described in this article is presented in Table 5. PCR–ESI-MS has the advantage over the current NAAT methods of being able to identify both MTBC and NTM species, and if MTCB is detected, it will simultaneously provide markers for drug resistance in a single assay. The line probe assays are the NAAT method which comes closest to providing this information, but at least two distinct strips/assays are needed at this time. Another advantage of the PCR–ESI-MS method is the ability to test for a wide variety of organisms on the same platform, including bacteria, viruses, fungi, and parasites (6, 10, 23, 24). This can be accomplished by some of the current NAAT tests (PCR and sequencing) but generally requires the performance of a variety of PCR assays and the use of multiple sequencing targets. However, the PCR–ESI-MS technology does not come without limitations, including the open system format, the cost of supplies and labor, and the requirement for experienced personnel, as previously described (10). Most important, the high instrument costs of the PCR–ESI-MS system (~$500,000) may hamper the implementation of this technology in small clinical microbiology laboratories, and therefore this technology may be limited to use in reference laboratories or state health laboratories.

Overall, the MDR-TB assay appears to be a rapid and accurate method for the simultaneous detection and identification of mycobacterial species and resistance determinants to MTBC directly from culture (solid or broth) compared to standard laboratory methods (nucleic acid hybridization probes or 16S rRNA gene sequencing). Implementation of this assay in a clinical microbiology laboratory could provide a turnaround time for identification of MTBC and NTM isolates that rivals that of Sanger dideoxy sequencing while simultaneously providing genotypic drug resistance patterns to physicians, allowing them to tailor the treatment regimens for *M. tuberculosis* prior to receiving phenotypic susceptibility results. Future studies evaluating the use of the MDR-TB assay directly with specimens will be important to determine whether the assay performs as well without waiting for growth in culture.

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