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A Molecular Approach to Identification and Profiling of First-Line-Drug-Resistant Mycobacteria from Sputum of Pulmonary Tuberculosis Patients

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Conventional and molecular techniques were applied to detect and characterize drug resistance of mycobacteria in the sputum samples of clinically confirmed tuberculosis. The sensitivities of mycobacterium detection by ZN staining, culture, multiplex PCR, and restriction fragment length polymorphism (RFLP) were 27.7%, 19.9%, 92.9%, and 95.7%, respectively, but all were 100% specific. The conventional and multiple-allele-specific PCR (MAS-PCR) methods enabled establishment of the drug resistance in 19.3% and 86.9% cases, respectively. We demonstrated that molecular techniques have potential in the accurate diagnosis of tuberculosis.

In addition to *Mycobacterium tuberculosis*, nontuberculous mycobacteria (NTM) also contribute to life-threatening pulmonary tuberculosis (TB) (6, 22). Because of the differential pathogenic potentials and responses to antitubercular drugs (ATDs), it is necessary to accurately identify mycobacteria at the early stage of disease diagnosis (17, 18).

Clinically important mycobacteria rapidly develop resistance to first-line ATDs (5). Information on the comparative characteristics of these strains, their mutation patterns, and occurrence of resistance phenotypes among patients is currently lacking. Conventional methods like microscopy, biochemical tests, and drug sensitivity on culture medium are labor-intensive and time-consuming for identification of the organisms and detection of their resistance patterns. These tests may also yield ambiguous results (2). Hence, accurate methodology enabling rapid identification up to the subspecies level and assessment of drug susceptibility are necessary.

DNA-based techniques allow specific identification and characterization of drug resistance patterns with speed and high sensitivity (8, 11, 14). It is envisaged that a thorough understanding of the genetic basis of strain differentiation and their drug susceptibility will facilitate better management of the disease. The objectives of the present study were to (i) detect species and (ii) analyze resistance patterns of mycobacteria in sputum samples of clinically suspected first-line drug (rifampin, isoniazid, and ethambutol) failure in TB patients by using the molecular approach.

This study was approved by the institutional ethics committee, and written informed consents were obtained. Morning sputum samples from TB patients ($n = 145$) were collected on three consecutive days. Clinical diagnosis was supported by persistent symptoms, radiological abnormalities, and Mantoux test.

Digestion and decontamination of sputum samples were carried out using the *N*-acetyl-L-cysteine (NALC)/NaOH method (10). Briefly, NALC-NaOH solution (2% NaOH, 1.45% Na-citrate, and 0.5% NALC) and sputum samples were mixed at a 1:1 dilution, vortexed, and incubated for 15 min at room temperature. After digestion and decontamination, samples were concentrated by adjusting the volume to 50 ml with phosphate buffer (0.067 M, pH 6.8), followed by centrifugation of the mixture at 3,500 × *g* for 20 min. The sediments were resuspended in 2 ml of distilled water. Around 500 μl of each resuspended sediment was removed and kept at 2 to 8°C for DNA extraction. The remaining was used for Ziehl-Neelsen (ZN) staining and inoculation on Lowenstein-Jensen (LJ) solid and Middlebrook 7H9 liquid medium (10). For each sample, inoculations on both types of media were done in triplicate and incubated at 25°C, 37°C, and 42°C for 6 weeks (15). LJ slopes were examined on every alternate day for the first week and twice every week thereafter. Middlebrook 7H9 bottles were read every other day for up to 6 weeks for positive culture. Drug-resistant *M. tuberculosis* and other slow-growing mycobacteria grew more slowly than drug-susceptible ones, as found by earlier workers (12). In order to not miss these organisms, all culture tubes found negative at 6 weeks were further incubated up to 12 weeks for final identification. Antimicrobial drug susceptibility testing was performed by the resistance ratio method according to the manufacturer's instructions (Tulip Diagnostics Pvt. Ltd., Goa, India). Culture-positive samples were identified to the species level by biochemical tests, namely niacin, catalase, nitrate reduction, heat stable catalase, pigment production, and aryl sulfatase tests.

Mycobacterial DNA was extracted directly from the concentrated sputum samples followed by identification and differentiation of species using multiplex PCR and restriction fragment length polymorphism (RFLP) analysis (1, 3, 19). A single-step multiple-allele-specific PCR (MAS-PCR) assay was also performed for analysis of the most frequently occurring gene muta-

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TABLE 1 Comparison of molecular techniques with conventional methods for diagnosis of pulmonary tuberculosis[a]

	Conventional method				Molecular technique				MAS-PCR analysis			
	Biochemical test		Genotype		Multiplex PCR		PCR-RFLP analysis					
Mycobacterium species	Organism	No.[b]	Resistance pattern	No.[b]	Organism	No.[b]	Organism	No.[b]	Organism	No.[b]	Resistance pattern	Mutation site
M. tuberculosis	*M. tuberculosis*	25	$I^R + R^R + ER$	1	*M. tuberculosis*	99	*M. tuberculosis*	102	*M. tuberculosis*	1	$I^R + R^R + E^R$	315, −15, 306, 516
			M I^R	2					*M. tuberculosis*	4	M I^R	315
			$E^R + R^R$	1					*M. tuberculosis*	4	M I^R	−15
									M. tuberculosis	2	M R^R	526
									M. tuberculosis	1	M R^R	531
									M. tuberculosis	1	M R^R	516, 531
									M. tuberculosis	2	M R^R	516
									M. tuberculosis	2	M E^R	306
									M. tuberculosis	1	$I^R + R^R$	315, −15, 531
									M. tuberculosis	1	$I^R + R^R$	−15, 516
									M. tuberculosis	1	$I^R + R^R$	−15, 526
									M. tuberculosis	1	$I^R + R^R$	315, 516, 526
									M. tuberculosis	1	$I^R + R^R$	315, 531
									M. tuberculosis	1	$I^R + R^R$	−15, 531
									M. tuberculosis	2	$E^R + I^R$	−15, 306
									M. tuberculosis	1	$E^R + R^R$	306, 526
									M. tuberculosis	1	$E^R + R^R$	306, 516, 531
M. bovis	*M. bovis*	1	–	–	*M. bovis*	7	*M. bovis* subsp. *bovis*	8	*M. bovis* subsp. *bovis*	1	M I^R	−15
									M. bovis subsp. *bovis*	1	M I^R	315, −15
									M. bovis subsp. *bovis*	1	R^R	516, 531
M. avium					*M. avium* subsp. *avium*	5	*M. avium* subsp. *avium*	5	*M. avium* subsp. *avium*	1	M I^R	−15
							M. avium subsp. *intracellulare*	3	*M. avium* subsp. *intracellulare*	1	M I^R	−15
M. kansasi	*M. kansasi*	1	$I^R + R^R$	1	NTM	20	*M. kansasii*	9	*M. kansasi*	1	M I^R	−15
									M. kansasi	1	M I^R	315
									M. kansasi	1	R^R	526
									M. kansasi	1	$I^R + R^R$	−15, 516, 531
M. fortuitum	*M. fortuitum*	1	–	–			*M. fortuitum*	2	*M. fortuitum*	1	M I^R	315
									M. fortuitum	1	M I^R	−15
M. chelonae							*M. chelonae* subsp. *abscessus*	3	*M. chelonae* subsp. *abscessus*	1	$I^R + R^R + E^R$	315, 306, 531
									M. chelonae subsp. *abscessus*	1	$I^R + R^R$	315, −15, 526
									M. chelonae subsp. *abscessus*	1	$E^R + I^R$	315, −15, 306
M. malmoense							*M. malmoense*	1	*M. malmoense*	1	M E^R	306
M. szulgai							*M. szulgai*	1	*M. szulgai*	1	M E^R	306
M. terrae							*M. terrae*	1		–	–	–

[a] $I^R + R^R + E^R$, resistance to isoniazid, rifampin, and ethambutol; M I^R, monoresistance to isoniazid; $I^R + R^R$, resistance to both isoniazid and rifampin; $I^R + E^R$, resistance to both isoniazid and ethambutol; M R^R, monoresistance to rifampin; $R^R + E^R$, resistance to both rifampin and ethambutol; M E^R, monoresistance to ethambutol; NTM, nontuberculous mycobacteria.

[b] No., number of isolates showing positive results.

tions of *katG*, *rpoB*, *embB*, and the promoter site of the *mab-inhA* gene (23).

One positive control (DNA of *M. tuberculosis* $H_{37}Rv$) and one negative control (no-template DNA) were included in every set of PCR. Stringency and reproducibility of PCR were established using reference strains obtained from National Jalma Institute of Leprosy and Other Mycobacterial Diseases, Agra, and Tuberculosis Research Centre, Chennai, India. Amplicons were analyzed on an agarose gel using UVP Doc.It LS image analysis software, version 6.3.3.

We evaluated the effectiveness of multiplex PCR and RFLP in comparison to conventional diagnostic tests. Of the total 145 sputum samples from clinically suspected first-line-drug-failure TB patients, four samples were later found to be due to pneumonia (2 cases) and histoplasmosis (2 cases) by clinicians. ZN staining for acid-fast bacilli (AFB) was positive in 39 samples (26.9%), and culture was positive in 28 samples (19.3%). In contrast, multiplex PCR and RFLP confirmed a total of 131 (90.3%) and 135 (93.1%) positive samples, respectively. ZN staining, culture, multiplex PCR, and RFLP with respect to clinical assessment were 100% specific, but sensitivities were 27.7%, 19.9%, 92.9%, and 95.7%, respectively. All four diagnostic methods had a 100% positive predictive value. Negative predictive values with ZN staining and culture methods were 3.8% and 3.4%, while multiplex PCR

and RFLP methods had values of 28.6% and 40%, respectively. In our case, multiplex PCR amplified three DNA targets: heat shock protein (encoded by *hsp65*), cold shock gene (*dnaJ*), and insertion sequence (IS*6110*) (1). However, sequences homologous to IS*6110* have now also been found in mycobacteria that is not part of the *M. tuberculosis* complex (9, 13). Several *M. tuberculosis* strains that lack this insertion sequence have been isolated, and this may account for the false identification (16, 21, 24). The RFLP method involving *hsp65* offers the possibility of exploring the diversity within and between members of the *Mycobacterium* genus. This method distinguishes individual subspecies of the *M. fortuitum* complex. It also separates *M. avium* complex (MAC) into subspecies *avium*, *paratuberculosis*, and *intracellulare*. Furthermore, the *hsp65*-RFLP also distinguishes *M. kansasii* and *M. gastri*. We applied RFLP to achieve more precise identification of NTM up to the subspecies level, which is not possible by multiplex PCR.

Resistance to first-line drugs has been linked to mutations in at least 10 genes (7). Rapid turnaround time is highly desirable in assessing mycobacterial drug sensitivity (4, 20). In this study, 28 (19.3%) culture-positive samples were tested for susceptibility to the first-line ATDs through conventional sensitivity testing. In contrast, drug resistance pattern was established in 126 cases (86.9%) using MAS-PCR (Table 1). The main advantage of MAS-PCR is simultaneous detection of resistance to three first-line drugs, which is otherwise difficult to diagnose by other molecular methods (23). It offers additional advantages of simplicity, speed, and economy.

In conclusion, our results suggest that combined application of multiplex PCR, RFLP, and MAS-PCR is a powerful strategy, enabling accurate diagnosis of mycobacterial infection from sputum sample and profiling of susceptibility to first-line drugs. This can greatly facilitate the management of pulmonary and allied mycobacterial infections.

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REFERENCES

1. **Bhattacharya B, et al.** 2003. Development of a new sensitive and efficient multiplex polymerase chain reaction (PCR) for identification and differentiation of different mycobacterial species. Trop. Med. Int. Health **8**:150–157.
2. **Boy JC, Marr J.** 1975. Increased threats of TB in new form of MDR-TB. Ann. Intern. Med. **82**:489.
3. **Brunello F, et al.** 2001. Identification of 54 mycobacterial species by PCR-restriction fragment length polymorphism analysis of the *hsp65* gene. J. Clin. Microbiol. **39**:2799–2806.
4. **Centers for Disease Control and Prevention.** 2002. Reported tuberculosis in the United States. 2003. Centers for Disease Control and Prevention, U.S. Department of Health and Human Services, Atlanta, GA.
5. **Davies PD.** 1994. Infection with *Mycobacterium kansasii*. Thorax **49**:435–436.
6. **Falkinham JO, III.** 2011. Nontuberculous mycobacteria from household plumbing of patients with nontuberculous mycobacteria disease. Emerg. Infect. Dis. **17**:419–424.
7. **Johnson R, et al.** 2006. Drug resistance in *Mycobacterium tuberculosis*. Curr. Issues Mol. Biol. **8**:97–111.
8. **Jonas V, Longiaru M.** 1997. Detection of *Mycobacterium tuberculosis* by molecular methods. Clin. Lab. Med. **17**:119–128.
9. **Kent L, McHugh TD, Billington O, Dale JW, and Gillespie SH.** 1995. Demonstration of homology between IS*6110* of *Mycobacterium tuberculosis* and DNAs of other *Mycobacterium* spp. J. Clin. Microbiol. **33**:2290–2293.
10. **Kent PT, Kubica GP.** 1985. Public health mycobacteriology: a guide for the level III laboratory. U.S. Department of Health and Human Services, Centers for Disease Control, Atlanta, GA.
11. **Kocagöz T, et al.** 1993. Detection of *Mycobacterium tuberculosis* in sputum samples by polymerase chain reaction using a simplified procedure. J. Clin. Microbiol. **31**:1435–1438.
12. **Levin BR, et al.** 1997. The population genetics of antibiotic resistance. Clin. Infect. Dis. **24**:S9–S16.
13. **Lie′bana E, Aranaz A, Francis B, Cousins D.** 1996. Assessment of genetic markers for species differentiation within the *Mycobacterium tuberculosis* complex. J. Clin. Microbiol. **34**:933–938.
14. **Mokrousov I, et al.** 2003. PCR-based methodology for detecting multidrug-resistant strains of *Mycobacterium tuberculosis* Beijing family circulating in Russia. Eur. J. Clin. Microbiol. Infect. Dis. **22**:342–348.
15. **Rajalahti I, Vuorinen P, Nieminen MM, Miettinen A.** 1998. Detection of *Mycobacterium tuberculosis* complex in sputum specimens by the automated Roche Cobas Amplicor *Mycobacterium Tuberculosis* test. J. Clin. Microbiol. **36**:975–978.
16. **Sahadevan R, Narayanan S, Paramasivan CN, Prabhakar R, Narayanan PR.** 1995. Restriction fragment length polymorphism typing of clinical isolates of *Mycobacterium tuberculosis* from patients with pulmonary tuberculosis in Madras, India, by use of direct-repeat probe. J. Clin. Microbiol. **33**:3037–3039.
17. **Smith NH, Gordon SV, de la Rua-Domenech R, Clifton-Hadley RS, Hewinson RG.** 2006. Bottlenecks and broomsticks: the molecular evolution of *Mycobacterium bovis*. Nat. Rev. Microbiol. **4**:670–681.
18. **Somoskovi A, et al.** 2007. Sequencing of the *pncA* gene in members of the *Mycobacterium tuberculosis* complex has important diagnostic applications: identification of a species-specific *pncA* mutation in "*Mycobacterium canettii*" and the reliable and rapid predictor of pyrazinamide resistance. J. Clin. Microbiol. **45**:595–599.
19. **Sritharan V, Barker RH, Jr.** 1991. A simple method for diagnosing *M. tuberculosis* infection in clinical samples using PCR. Mol. Cell. Probes **5**:385–395.
20. **Tenover FC, et al.** 1993. The resurgence of tuberculosis: is your laboratory ready? J. Clin. Microbiol. **31**:767–770.
21. **van Soolingen D, de Haas PE, Hermans PW, Groenen PM, van Embden JD.** 1993. Comparison of various repetitive DNA elements as genetic markers for strain differentiation and epidemiology of *Mycobacterium tuberculosis*. J. Clin. Microbiol. **31**:1987–1995.
22. **Wolinsky E.** 1979. Nontuberculous mycobacteria and associated diseases. Am. Rev. Respir. Dis. **119**:107–159.
23. **Yang Z, et al.** 2005. Simultaneous detection of isoniazid, rifampin, and ethambutol resistance of *Mycobacterium tuberculosis* by a single multiplex allele-specific polymerase chain reaction (PCR) assay. Diagn. Microbiol. Infect. Dis. **53**:201–218.
24. **Yuen LK, Ross BC, Jackson KM, Dwyer B.** 1993. Characterization of *Mycobacterium tuberculosis* strains from Vietnamese patients by Southern blot hybridization. J. Clin. Microbiol. **31**:1615–1618.