Original Report

Genetic Diversity and Evidence for Acquired Antimicrobial Resistance in *Mycobacterium tuberculosis* at a Large Hospital in South India

Kenneth A. Harris Jr., PhD;* Umadevi Mukundan, MSc;† James M. Musser, PhD;‡ Barry N. Kreiswirth, PhD;§ and M. K. Lalitha, PhD†

ABSTRACT

Objectives: To assess genetic diversity and drug resistance of *Mycobacterium tuberculosis* isolates collected at Christian Medical College Hospital (CMCH), Vellore, India, between July 1995 and May 1996.

Materials and Methods: Isolates were subjected to IS*6110*-based restriction fragment length polymorphism (RFLP) analysis and tested for resistance to isoniazid, rifampin, ethambutol, streptomycin, and pyrazinamide, and DNA from selected strains was sequenced in regions associated with drug resistance.

Results: One hundred and one *M. tuberculosis* isolates were collected from 87 patients with pulmonary tuberculosis. Charts of 69 patients were reviewed for history of tuberculosis illness and treatment. DNA from 29 strains was sequenced in *kat*G, *rpo*B, and *gyr*A, and sometimes *pnc*A regions. Analysis by RFLP revealed a high degree of genetic diversity, with no identifiable clusters of infection. Of the strains tested, 51% were resistant to at least one antibiotic, and 43% were resistant to more than one drug. There was a high rate of resistance observed in patients whose charts indicated a history of improperly administered tuberculosis treatment, whereas little drug resistance was observed in patients never previously treated for tuberculosis. Sequencing of genes associated with drug resistance revealed several previously unreported mutations in resistant strains.

Conclusions: This analysis suggests that the cases of tuberculosis in the sample are largely reactivation of long-standing infections and that the drug resistance among patients in CMCH is largely acquired or secondary rather than attributable to the spread of drug-resistant strains.

Key Words: *India, multidrug resistance,* Mycobacterium tuberculosis, *restriction fragment length polymorphism, tuberculosis*

Int J Infect Dis 2000 4:140–147.

It is estimated that one-third of the world's population is infected with *Mycobacterium tuberculosis*.[1] Tuberculosis (TB) is the leading cause of death from a single infectious agent worldwide, and if present trends continue unchecked, TB deaths are predicted to increase from 3 million in 1995 to 3.5 million in 2000.[2] From the vast number of people with latent TB infection, approximately 8 million develop active disease per year, maintaining a pool of about 16 million cases of TB worldwide.[3]

Many factors contribute to the rise in TB in developing countries such as India. There has been a recent increase in TB incidence worldwide, particularly in the developing world. Poor socioeconomic conditions, such as poverty, crowded living conditions, and malnutrition parallel high tuberculosis infection rates.[3-5] Fifty percent of adults in India are estimated to be infected with *M. tuberculosis*.[6] India presently harbors approximately 30% of the world's cases of tuberculosis, or about 3.8 million active cases of disease, and the prevalence of active TB in the general Indian population in 1995 was estimated to be 422 per 100,000.[6] The most recent survey of the prevalence of TB in an area served by Christian Medical College Hospital (CMCH), a rural section of the North Arcot Ambedkar District in Tamil Nadu, South India, found a lower prevalence of 241 per 100,000 in the early 1980s.[7]

An alarming aspect of the tuberculosis pandemic is the development and spread of drug-resistant strains of *M. tuberculosis*. Multidrug resistance (MDR) is defined by the World Health Organization as resistance to both rifampin and isoniazid.[8] Multidrug resistant strains of TB have gravely compounded the problem posed by this

*Yale University School of Medicine, New Haven, Connecticut; †Department of Clinical Microbiology, Christian Medical College and Hospital, Vellore, India; ‡Institute for the Study of Bacterial Pathogenesis, Baylor College of Medicine, Houston, Texas; §TB Center, Public Health Research Institute, New York, New York.

This work was made possible by a Yale University Wilbur G. Downs International Health Travel Fellowship and the generous gift of reagents from the Amersham Corporation, Arlington Heights, Illinois.

Supported by Public Health Services grant AI-37004 to J.M.M.

Address correspondence to Dr. Barry N. Kreiswirth, Director, PHRI TB Center, Public Health Research Institute, 455 First Ave., Room 953, New York, NY 10016. E-mail: barry@phri.org.

disease in India; therefore, it is important to determine the modes of development and extent of spread of MDR strains in India. This study is an example of a preliminary effort to do so, using IS*6110* restriction fragment length polymorphism (RFLP) analysis and sequencing of drug resistance mutations in TB strains isolated at a large hospital in South India. Christian Medical College Hospital in Vellore, Tamil Nadu, is a major tertiary care facility that serves the North Arcot Ambedkar District and also attracts patients from all over India and South Asia. Together these data demonstrate the diversity of genotypes and patterns of drug resistance present in a cross-section of TB strains found in patients from across India.

MATERIALS AND METHODS

Mycobacterial Strains and Epidemiologic Features

A retrospective study was conducted of 101 *M. tuberculosis* isolates from sputum samples collected from 87 patients with TB, at CMCH between July 1995 and May 1996. Mycobacteria were grown on Lowenstein-Jensen slants. These isolates represent all of the sputum cultures positive for *M. tuberculosis* accumulated at the CMCH Department of Clinical Microbiology during this period. Of the 101 isolates in the sample, 63 had been tested for drug susceptibility. Charts for 69 of the 87 patients were available for review, and any history of treatment for tuberculosis was noted. Inadequate treatment was defined as any form of improper drug therapy either before or during treatment at CMCH, including taking medications irregularly; taking fewer medications than prescribed; or being treated with an insufficient regimen, such as a one- or two-drug protocol for any length of time. Lack of previous treatment was confirmed only if specifically mentioned as such in the chart. If there was no mention of prior treatment history in the chart, the patient was included in the same "unknown" category as those whose charts were unavailable for review.

DNA Analysis and Drug Susceptibility

DNA was extracted from mature cultures by standard methods.[9] Restriction fragment length polymorphism patterns were compared with each other and with the database at the Public Health Research Institute (PHRI) TB Center in New York City, using BioImage Whole Band Analyzer (BioImage, Ann Arbor, MI). The PHRI database contains approximately 6500 RFLP patterns, collected primarily in New York City over the past 5 years. These methods were successfully applied to isolates from 77 of the 87 patients; insufficient or degraded DNA from isolates from 10 patients made RFLP analysis impossible.

Drug susceptibility assays were performed at CMCH, using standard methods.[10,11] Because of technical difficulties inherent to pyrazinamide-susceptibility testing, susceptibility but not resistance can be reliably determined. Extracted DNA was sequenced by standard polymerase chain reaction (PCR) methods, using probes specific to genes associated with drug resistance.[12] DNA isolates from the study sample were randomly selected for sequencing. Unfortunately mycobacterial strains could not be transported from India, therefore repeat drug susceptibility studies could not be conducted when sequence data conflicted with susceptibility data.

RESULTS

Clinical Characteristics

In the 10 cases where multiple isolates were obtained from a patient, the same RFLP pattern was observed in all isolates from that patient. Most TB patients at CMCH are diagnosed by acid-fast smear alone, and therefore, the 87 patients in the study from whom the 101 *M. tuberculosis* isolates were obtained are a small subset of the thousands of TB patients treated at CMCH each year. Consistent with the previously observed higher TB incidence in males in India,[13] 71% of the study patients were male. The age range of patients was 12 to 72 years (mean, 37 y; median, 35 y). Patients came from 10 Indian states, Nepal, and Bhutan (Table 1), and of the patients whose origins were known, about half (49%) came from the North Arcot Ambedkar (NAA) District in northern Tamil Nadu state, of which Vellore is the administrative headquarters. Most of the patients (76%) were from south India (Tamil Nadu or the adjacent states of Andhra Pradesh and Karnataka).

Table 1. Geographic Origins of the 87 Study Patients

Region	*Number of Patients (%)*	*Total Patients in Area*
Number of patients of known origin (n = 75)		
Vellore (town and adjacent area)	17 (23)	
NAA District (other than Vellore)	20 (27)	
Total NAA District		37 (49)
Tamil Nadu (outside of NAA District)	11 (15)	
Total Tamil Nadu		48 (64)
Andhra Pradesh	7	
Karnataka	2	
Total South India		57 (76)
Madhya Pradesh	1	
Uttar Pradesh	1	
Bihar	1	
West Bengal	9	
Assam	1	
Manipur	2	
Mizoram	1	
Nepal	1	
Bhutan	1	
Unknown/chart not available (n = 12)		

NAA = North Arcot Ambedkar.

Table 2. Drug Resistance and History of Inadequate Anti-tuberculosis Chemotherapy

Inadequate ATT?	*Number in Study**	*Number Tested†*	*Number Resistant to*				
			Isoniazid‡	*Rifampin‡*	*Ethambutol‡*	*Streptomycin‡*	*Other†*
Yes	27 (31)	22 (35)	19 (86)	16 (73)	6 (27)	8 (36)	2 (9)
No	18 (21)	11 (17)	0	0	0	0	1 (9)
Unknown	42 (48)	30 (48)	12 (40)	6 (20)	3 (10)	8 (27)	1 (3)
Total	87 (100)	63 (72)	31 (49)	22 (35)	9 (14)	16 (25)	4 (6)
Delhi state§		2240	(28.8)	(14.0)	(7.0)	(18.1)	

Figures in parentheses denote percentages *of total subjects; †of total tested; ‡of number tested in the category. ATT = anti-tuberculosis chemotherapy; other = strain found resistant to another drug (capreomycin, ciprofloxacin, ofloxacin, ethionamide, or cycloserine; most strains tested for resistance only to the four first-line drugs); §data from WHO/IUATLD study for comparison.[28]

RFLP Strain Typing by IS*6110*

From the 87 patients, 77 isolates were successfully typed. There were no identifiable clusters of patients infected with the same strain; 49 strains (64%) had patterns unique both to the group and to the PHRI database. Similarity among strains was not significantly correlated with factors such as geographic origin of patients.

When few copies of the insertion sequence are present, IS*6110*-based typing alone is not sufficient to differentiate these strains and determine if any clusters of patients infected with the same strain are present. In this study, 22 isolates (29%) had only a single copy of the IS*6110* insertion sequence; in 19 of these isolates, the fragment containing the insertion sequence was approximately 1.5 kilobases (kb), a size seen in the strains designated BE in the PHRI database.[14] Strains with a single band in this position are common; 123 BE strains are present in the database, dating from 1992. In two "single-band" isolates the fragment size was approximately 5.0 kb (designated BE3) and in one the fragment was 1.1 kb, a pattern new to the PHRI database (designated BE6). A large proportion of single-band isolates has been observed in other RFLP studies in India and southeast Asia.[15,16]

Only three strains in the sample with a large number of copies of IS*6110* matched patterns in the PHRI database. Strain GD1, isolated from a man from the NAA District, had an RFLP pattern that differed by one band from a strain isolated in Kenya in 1995. Strain EZ2, also from a NAA District man, matched exactly with a strain isolated from a New Jersey man in 1996. Strain E, from a woman from Thiruchchirapalli, Tamil Nadu, matched a large New York City cluster, with 60 examples in the PHRI database, collected from 1991 to 1996. None of the matches are known to be from patients of Indian origin. The matches indicate that there could be epidemiologic connections between the CMCH patients and those in the database, but more information is required to trace these connections.

Drug Resistance

Drug susceptibility testing was performed at CMCH for 63 of the isolates in the sample; 32 of 63 patients (51%) were infected with a strain resistant to at least one drug. The most common resistance was to isoniazid (49% resistant), followed by rifampin (35%), streptomycin (25%), and ethambutol (14%) (Table 2). It was determined that 74% of the isolates were susceptible to pyrazinamide. The mean age of the patients with resistant strains (39 y) was not significantly different from that of patients with susceptible strains (36 y) or the mean of all patient ages (37 y).

Patient charts were reviewed to correlate drug resistance with previous TB and treatment. As indicated in Table 2, 31% of the patients in the sample were reported to have a history of inadequate chemotherapy, as previously defined (see Materials and Methods); 21% had never been treated with antibiotics for TB; and drug therapy history for the rest of the patients either was not recorded in the chart, was unknown, or the charts of the patients

Table 3. Pattern of Drug Resistance with History of Inadequate Anti-tuberculosis Chemotherapy

Inadequate ATT?	*Number Tested*	*Number Resistant to*									
		1 Drug		*2 Drugs*			*3 Drugs*			*4 Drugs*	
		I	*S*	*IR*	*IE*	*IS*	*IRE*	*IRS*	*IES*	*IRES*	*Total (%)*
Yes	22	1	0	7*	0	1	3	4†	1	2	19 (86)
No	11	0	0	0	0	0	0	0	0	0	0§
Unknown	30	3	1	0	1	2	1	4‡	0	1	13 (43)
Total (%)	63	5 (8)			11 (17)			13 (21)		3 (5)	32 (51)
Delhi state (%)#	2240	(10.9)			(10.9)			(7.1)		(3.5)	(32.4)

ATT = anti-tuberculosis chemotherapy; I = isoniazid; S = streptomycin; R = rifampin; E = ethambutol.
*One strain also resistant to ciprofloxacin and ethionamide; †one strain also resistant to capreomycin, ofloxacin, and cycloserine; ‡one strain also resistant to ciprofloxacin, capreomycin, and cycloserine; §one otherwise pan-susceptible strain resistant to cycloserine; #data from WHO/IUATLD study for comparison.[28]

Table 4. Extent of Drug Resistance by Geographic Origin of Study Patients

Region	Number of Patients	Number Tested (%)	Resistant to at Least One Drug n (%)	MDR n (%)
North Arcot Ambedkar District	37	23 (62)	10 (43)	6 (26)
South India	57	39 (68)	17 (44)	10 (26)
North-northeast India and subcontinent	18	15 (83)	11 (73)	9 (60)

MDR = multidrug resistant.

were not available for review. Drug resistance in the previously untreated group may be interpreted as a marker for primary resistance (i.e., new infection with a drug-resistant strain). Of 11 patients in the sample never treated for TB, only one was infected with a drug-resistant strain. Acquired resistance likely was present among the patients with a history of inadequate treatment, as the rates of resistance to first-line drugs among this group were high: 86% were resistant to isoniazid, 73% were resistant to rifampin, and 73% were resistant to both and, therefore, qualify as MDR strains.

The pattern of drug resistance in the sample is shown in Table 3. Multidrug resistance was common: the largest number of the resistant strains were resistant to three first-line drugs. Of 22 patients with a history of inadequate chemotherapy, 19 (86%) had drug-resistant TB and only one of these was resistant to only one drug. Resistance to both isoniazid and rifampin (MDR) was seen in 22 (69%) of the 32 resistant strains.

Of the patients from South India who were tested, 44% had drug-resistant TB, and 26% had MDR TB (Table 4). A much larger proportion of the patients travelling to CMCH from North and Northeast India, Nepal, and Bhutan had drug susceptibilities performed (83%) and had drug-resistant TB or MDR TB (73% and 60%, respectively).

Sequencing of Genes Associated with Drug Resistance and Evolution

Antibiotic resistance in *M. tuberculosis* is mediated by chromosomal mutations rather than by plasmids carrying drug resistance genes. *Mycobacterium tuberculosis* genes in which mutations are associated with drug resistance have been identified for several antibiotics: *rpo*B (rifampin), *rrs* and *rps*L (streptomycin), *kat*G and *inh*A (isoniazid), *gyr*A (fluoroquinolones), and *pnc*A (pyrazinamide).[12] DNA from 29 of the strains in the study sample was sequenced in the resistance-associated regions of *kat*G, *rpo*B, and *gyr*A, and 13 of these were also sequenced in the *pnc*A region.

Mutations in *kat*G were detected in 18 of the 29 strains sequenced (Table 5). Two of these mutations had not been previously reported: strain 35, with a single point mutation (428 Gly to Arg); and strain 44, with one novel point mutation (328 Asp to Ala) as well as a polymorphism seen in a large division of *M. tuberculosis* strains (463 Arg to Leu).[12] Ten of the mutated strains occurred in patients with a known history of inadequate chemotherapy, whereas only one was from a patient with no prior drug therapy (Table 6). This observation is consistent with the hypothesis that these are examples of acquired resistance (i.e., these mutations arose in these patients).

Mutations in *rpo*B that result in amino acid changes were identified in 12 of the 29 strains sequenced (see Table 5). Two mutations had not been previously reported: strain 82, with two amino acid changes (516 Asp to Glu; 522 Ser to Leu); and strain 58, with three point mutations changing two amino acids (525 Thr to Thr; 526 His to Pro; 527 Lys to Gln).[12] Eight of the mutated strains occurred in patients with a known history of inadequate chemotherapy compared to one from a patient without prior drug therapy (see Table 6).

Two of 13 strains sequenced in the *pnc*A region possessed single point mutations that change the amino acid sequence of pyrazinamidase: strain 35 (132 Gly to Asp) and strain 78 (His to Arg) (Table 7). Both of these mutations, which previously have been reported, were from patients with known histories of inadequate chemotherapy (see Table 6).[12]

None of the 29 strains sequenced had mutations in *gyr*A that are expected to result in drug resistance. However, two of these strains (5 and 82) were tested for ciprofloxacin susceptibility and strain 5 was found to be resistant. In addition, strain 1 was reported resistant to ofloxacin. The resistance seen in these two strains could be attributable to mutations in the B subunit of gyrase (the *gyr*B region).

Recently, groups of TB strains from around the world have been subdivided into evolutionarily related groups based on sequences of the *kat*G and *gyr*A genes.[17] It is thought that the ancestral group (Group 1) is described by *kat*G 463 Leu and *gyr*A 95 Thr; these sequences are shared by the other *M. tuberculosis* complex species, *M. bovis*, *M. microti*, and *M. africanum*. Group 2 is characterized by a change to *kat*G 463 Arg and in Group 3 *gyr*A is Ser; these polymorphisms are found only in *M. tuberculosis*. Twenty of the strains sequenced (69%) were Group 1 strains and only two were Group 3. Two strains had deletions in *kat*G, and it could not be determined whether they were Group 1 or Group 2. Sreevatsan and colleagues present the hypothesis that Group 3 strains, found largely in Latin America and Africa, arose and spread approximately 450 years ago.[17] The predominance of Group 1 strains in India is consistent with the notion that these strains have been endemic there for a much longer time.

Table 5. Drug Resistance Predicted from Sensitivity Testing at CMCH and from *katG* and *rpoB* Gene Sequencing Data

Reference Number	FP (Number of Bands)	Isoniazid Resistance: Interpretation Phenotypic	Isoniazid Resistance: Interpretation Genotypic	katG Sequence	Rifampin Resistance: Interpretation Phenotypic	Rifampin Resistance: Interpretation Genotypic	rpoB Sequence
1	001 (17)	R	R	315S>R; 463R>L	R	R	526H>Y
3	BE (1)	S			S		
5	001 (15)	R	R	315S>T; 463R>L	R	R	531S>L
8	BE (1)	S			S		
10	BE (1)	R	R	315S>T; 463R>L	R	S	wt
13	BE (1)		S	463R>L		S	wt
15	001 (5)	R	R	315S>T; 463R>L	S	S	wt
19	BE (1)		S	463R>L		S	wt
20	001 (12)		R	315S>T		S	wt
21	001 (10)	R			S		
22	E (9)	S			S		
23	BE (1)	S	S	463R>L	S	S	wt
24	EZ2 (12)		S	wt		S	wt
26	001 (15)	S			S		
27	001 (12)	S			S		
28	001 (3)	R	S	463R>L	S	S	wt
32	001 (12)	S			S		
34	001 (15)	R	R	315S>N; 463R>L	S	S	wt
35	BE (1)	R	R	428G>R *	R	S	wt
36	BE (1)	S			S		
37	001 (9)	S	S	wt	S	S	wt
39	BE3 (1)	S	S	463R>L	S	S	wt
40	001 (12)	S			S		
42	001 (9)	S			S		
44	001 (10)	R	R	329D>A *; 463R>L	R	R	531S>L
45	BE6 (1)	R	R	315S>T; 463R>L	S	S	wt
47	AH (4)	S			S		
49		R			R		
50	001 (9)	R			R		
51	001 (13)	R			R		
53	BE (1)		S	463R>L		S	wt
55	BE (1)	S			S		
56	L1 (3)	R	R	315S>T; 463R>L	R	R	513 Q>K
57		S			S		
58	001 (15)	R	S	463R>L	R	R	525T>T; 526H>P; 527K>E *
59	BE (1)	S			S		
60	BE (1)	S			S		
61	001 (16)	S			S		
62	001 (10)	R	S	wt	R	R	531S>L
63	001 (10)	R	S	463R>L	R	S	wt
64	001 (10)	S			S		
65		S			S		
66	BE (1)	S			S		
68	BE (1)	R	R	315S>T; 463R>L	R	S	wt
70	001 (5)	R			S		
71	001 (16)	S			S		
72	001 (14)	R	R	315S>N; 463R>L	R	R	533L>P
73		S			S		
74	BE (1)	S			S		
75	001 (20)	R	R	deletion	R	R	531S>W
76	001 (5)	S			S		
78	001 (13)	R	R	deletion	R	R	526H>C
80	001 (14)	R			S		
81	GD1 (13)	R			R		
82	BE3 (1)	S	R	315S>T	S	R	516D>E *; 522S>L
83	001 (17)	R	R	315S>T; 463R>L	R	R	531S>L
86		R			S		
87	001 (15)	R	R	315S>T; 463R>L	R	R	526H>R
88	001 (6)	R			R		
89	001 (17)	S			S		
90	001 (5)	S			S		
92	001 (4)	R			R		
96		S			S		
98	001 (13)	S			S		
99	BE (1)	S	R	315S>T; 463R>L	S	S	wt
100	001 (9)	R			S		
101	001 (10)	S			S		
102	BE (1)	R			R		

FP = fingerprint; *novel mutation; wt = wild type.

Table 6. Presence of Mutations in Four Drug Resistance-Associated *M. tuberculosis* Genes Correlated with History of Inadequate Anti-tuberculosis Chemotherapy

Inadequate ATT?	katG *R*	katG *S*	rpoB *R*	rpoB *S*	gyrA *S*	pncA *R*	pncA *S*
Yes	10	2	8	4	12	2	6
No	1	1	1	1	2	0	0
Unknown	7	8	3	12	15	0	5
Total	18	11	12	17	29	2	11

ATT = anti-tuberculosis chemotherapy; R = mutation consistent with resistance to drug; S = wild-type sequence or mutation not associated with drug resistance; drugs associated with the genes shown are: *kat*G, isoniazid; *rpo*B, rifampicin; *gyr*A, fluoroquinolones; *pnc*A, pyrazinamide.

DISCUSSION

Restriction Fragment Length Polymorphism Strain Typing

Typing by RFLP has shown that diversity of strains varies widely in different regions of the world. In east Asia, a single family of RFLP patterns dominates the population of TB strains.[18] In Madras, RFLP typing studies found a high proportion (40%) of strains with zero or one copy of IS*6110*.[15] This situation reduces the usefulness of IS*6110*-based RFLP typing for epidemiologic analysis, but it does not render the method useless. The other 60% of strains in that study displayed a wide diversity of RFLP patterns, in contrast to observations in east Asia.[19,20] Other probes also may be used for RFLP typing in populations where IS*6110* is less useful; the direct-repeat (DR) probe was used in Madras to demonstrate the presence of reactivation of incompletely cured infections after treatment.[19]

Strain type clustering is not necessarily expected in the subset of patients from regions distant from CMCH, as the sample is small and these patients are not likely to have been in contact when they acquired TB infections. However, there is a lack of clustering even among patients from Vellore. Clustering of patients with the same strain would suggest that patients are ill with infections acquired around the same time and in the same place. Whereas this small sample may not detect the full extent of any clustering that may be present among TB strains in Vellore, the lack of clustering in the sample is consistent with the explanation that these TB cases are attributable to reactivation of previously acquired infections. Given the high prevalence of TB infection in India, the result is not surprising, because these patients had opportunities to be exposed to TB throughout their lives.

Drug Resistance

Various studies reveal that the patterns of drug resistance in India vary from region to region, but that the problem is great.[8,13,21-24] The studies differ in their methodologies and populations studied. In Jaipur in 1993, resistance to at least one drug was seen in 19.9% of cases.[25] Primary resistance (acquisition of strains that are already drug-resistant) was observed in TB infections of kidney transplant patients at CMCH from 1991 to 1993.[10] One study, conducted from 1985 to 1989, determined that 25% of patients with TB in North Arcot, Tamil Nadu, had a strain resistant to one of three first-line drugs, isoniazid (I), rifampin (R), or streptomycin (S).[26] Another study reported the prevalence of primary resistance in South India to be 4.8% for I, 5.7% for S, and 0.12% for R.[27] The resistance rates calculated by the World Health Organization (WHO)/International Union against Tuberculosis and Lung Disease (IUATLD) for Delhi state from 1994 to 1997 were lower than observed in this study, but the trend in drug resistance is similar to that observed here (see Table 2).[28] The WHO study observed significantly less resistance to multiple drugs than is present in the sample studied here (see Table 3).

Owing to cost constraints, drug susceptibility studies and even mycobacterial cultures are not routinely ordered for TB patients at CMCH. The studies are performed either if cost is not a factor or if drug resistance is suspected, either clinically or from a known history of inadequate treatment. Therefore, the sample studied here undoubtedly is enriched for drug resistant strains. The subset from North India enriches the sample further for serious drug resistance, because patients with unsuccessfully treated

Table 7. Pyrazinamide Resistance Predicted from Sensitivity Testing at CMCH and from *pncA* Gene Sequencing Data

Reference Number	*Pyrazinamide Resistance Interpretation* *Phenotypic*	*Genotypic*	pncA *Sequence*
1	S	S	65 S>S
5	NA	S	wt
28	S	S	wt
34	S	S	65 S>S
35	NA	R	132 G>D
58	S	S	65 S>S
62	S	S	wt
63	S	S	wt
68	NA	S	wt
75	S	S	wt
78	S	R	51 H>R; 65 S>S
83	S	S	wt
87	NA	S	wt

S = wild-type sequence or mutation not associated with drug resistance; R = mutation consistent with resistance to drug; NA = not assessed; wt = wild type.[12]

TB may seek treatment at national referral centers such as CMCH. The correlation in this sample between prior inadequate treatment and drug resistance as well as the lack of drug resistance in the patients with no prior treatment does suggest that drug resistance in the sample was largely acquired rather than primary. Unfortunately, the exact details of each history of inadequate treatment were generally not known or not documented in the chart. Therefore the effects of various forms of inadequate therapy (e.g., too short a course, too few drugs, irregular dosing) on the development of drug resistance could not be assessed in this study. Although the present study suggests that only a small proportion of drug resistance may be attributable to primary resistance, the growing rates of drug resistance in India set the stage for the spread of drug-resistant TB in the future.

In general the DNA sequencing predictions correlated well with the drug susceptibility test results from CMCH. Four strains were found to be isoniazid-resistant but lacked mutations in the region of *kat*G sequenced, and may have had mutations elsewhere in *kat*G or in *inh*A, that also confer resistance. It is more difficult to explain the two strains that were reported susceptible to isoniazid but had *kat*G mutations known to confer resistance (82 and 99; see Table 5). Strain 82 also had a novel mutation in *rpo*B at position 516 as well as a mutation at position 522 known to confer resistance, yet it was reported susceptible to rifampin. Four additional strains were reported resistant to rifampin but no *rpo*B mutations were found. Since over 95% of rifampin-resistant strains have mutations in this region of *rpo*B,[12] these cases are likely attributable to random error in susceptibility testing. Strain 78 was reported susceptible to pyrazinamide but had a mutation in the *pnc*A gene. It is possible that this mutation (51 His to Arg) causes no disruption in pyrazinamidase activity. This case may also be attributable to testing error, because pyrazinamide susceptibility is particularly difficult to test accurately.

The data presented here characterize a diverse sample of Indian *M. tuberculosis* strains with a high rate of drug resistance. In resource-poor developing countries, MDR TB poses a grave threat to public health, especially with the concurrent spread of HIV infection. The long duration of treatment required to cure TB combined with the cost of drugs makes compliance with anti-tuberculosis chemotherapy difficult to achieve in India.[6] Most people in India seek health care from private providers who often treat TB improperly.[6,29,30] Directly Observed Therapy (DOT) programs are being piloted in India at a cost of $100 million to cover 30% of the country.[31] The most conservative estimate states that successful implementation of DOT will provide a 4% increase per year ($8.3 billion in 1993-1994) in India's Gross Domestic Product owing to recovered productivity.[31] Proper implementation of DOT will require the extent of drug resistance to be assessed. Christian Medical College Hospital has one of the most comprehensive clinical microbiology laboratories in India, and even there, culturing and drug susceptibility testing are not performed on all cases of TB, owing to the prohibitive cost. Sufficient funds should be allotted to TB control programs to permit routine culturing and drug susceptibility testing.

ACKNOWLEDGMENTS

For assistance and advice on this project the authors thank Dr. G. Sridharan, Department of Clinical Virology (CMCH), the staff of the Department of Clinical Microbiology (CMCH), Drs. Benjamin Samuel, Elisabetta Ullu, and Michael O. Rigsby of the Infectious Diseases Group (Yale), and Bill Eisner, Soraya Moghazeh, and Barun Mathema, TB Center (PHRI).

REFERENCES

1. Snider J, Dixie E, Raviglione M, Kochi A. Global burden of tuberculosis. In: Bloom BR, ed. Tuberculosis: pathogenesis, protection, and control. Washington, DC: American Society for Microbiology Press, 1994:3-11.
2. Dolin PJ, Raviglione MC, Kochi A. Global tuberculosis incidence and mortality during 1990-2000. Bull World Health Organ 1994; 72:213-220.
3. Enarson DA, Murray JF. Global epidemiology of tuberculosis. In: Rom WN, Garay SM, eds. Tuberculosis. New York: Little, Brown, 1996:57-75.
4. World Health Organization. Tuberculosis control and research strategies for the 1990s: memorandum from a WHO meeting. Bull World Health Organ 1992; 70:17-21.
5. Sudre P, ten Dam G, Kochi A. Tuberculosis: a global overview of the situation today. Bull World Health Organ 1992; 70:149-159.
6. Pathania V, Almeida J, Kochi A. TB patients and private for-profit health care providers in India. Geneva: World Health Organization, 1997:39.
7. Ray D, Abel R. Incidence of smear-positive pulmonary tuberculosis from 1981 to 1983 in a rural area under an active health care programme in South India. Tuber Lung Dis 1995; 76:190-195.
8. Dey AB, Nagarkar K. Multidrug resistant tuberculosis. Trop Gastroenterol 1995; 16:92-100.
9. van Embden JDA, Cave MD, Crawford JT, et al. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. J Clin Microbiol 1993; 31:406-409.
10. John GT, Mukundan U, Vincent L, Jacob CK, Shastry JCM. Primary drug resistance to *Mycobacterium tuberculosis* in renal transplant recipients. Natl Med J India 1995; 8:211-212.
11. Canetti G, Fox W, Khomenko A, et al. Advances in techniques of testing mycobacterial drug sensitivity, and the use of sensitivity tests in tuberculosis control programmes. Bull World Health Organ 1969; 41:21-43.
12. Ramaswamy S, Musser JM. Molecular genetic basis of antimicrobial resistance in *Mycobacterium tuberculosis*: 1998 update. Tuber Lung Dis 1998; 79:3-29.
13. Prabhakar R. Tuberculosis: the continuing scourge of India. Indian J Med Res 1996; 103:19-25.
14. Casper C, Singh SP, Rane S, et al. The transcontinental transmission of tuberculosis: a molecular epidemiological assessment. Am J Public Health 1996; 86:551-553.

15. Das S, Paramasivan CN, Lowrie DB, Prabhakar R, Narayanan PR. IS*6110* restriction fragment length polymorphism typing of clinical isolates of *Mycobacterium tuberculosis* from patients with pulmonary tuberculosis in Madras, South India. Tuber Lung Dis 1995; 76:550-554.
16. Yuen LKW, Ross BC, Jackson KM, Dwyer B. Characterization of *Mycobacterium tuberculosis* strains from Vietnamese patients by Southern blot hybridization. J Clin Microbiol 1993; 31:1615-1618.
17. Sreevatsan S, Pan X, Stockbauer KE, et al. Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination. Proc Natl Acad Sci U S A 1997; 94:9869-9874.
18. van Soolingen D, Qian L, de Haas PEW, et al. Predominance of a single genotype of *Mycobacterium tuberculosis* in countries of East Asia. J Clin Microbiol 1995; 33:3234-3238.
19. Sahadevan R, Narayanan S, Paramasivan CN, Prabhakar R, Narayanan PR. 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 1995; 33:3037-3039.
20. Das S, Chan SL, Allen BW, Mitchison DA, Lowrie DB. Application of DNA fingerprinting with IS*986* to sequential mycobacterial isolates obtained from pulmonary tuberculosis patients in Hong Kong before, during, and after short-course chemotherapy. Tuber Lung Dis 1993; 74:47-51.
21. Cohn DL, Bustreo F, Raviglione MC. Drug-resistant tuberculosis: review of the worldwide situation and the WHO/IUATLD global surveillance project. Clin Infect Dis 1997; 24(Suppl 1):S121-S130.
22. Khilnani GC, Sharma SK, Pande JN. Multidrug resistant tuberculosis. Indian J Chest Dis Allied Sci 1994; 36:137-145.
23. Mahajan M, Agarwal DS, Gadre DJ, Singh NP, Gupta HC, Talwar V. Initial and acquired drug resistance of *Mycobacterium tuberculosis* in East Delhi. J Commun Dis 1996; 28:15-19.
24. Siddiqi N, Shamim M, Jain NK, et al. Molecular genetic analysis of multidrug resistance in Indian isolates of *Mycobacterium tuberculosis*. Mem Inst Oswaldo Cruz 1998; 93:589-594.
25. Gupta PR, Singhal B, Sharma TN, Gupta RB. Prevalence of initial drug resistance in tuberculosis patients attending a chest hospital. Indian J Med Res 1993; 97:102-103.
26. Paramasivan CN, Chandrasikaran V, Santha T, Sudarsanam NM, Prabhakar R. Bacteriological investigations for short-course chemotherapy under the tuberculosis programme in two districts of India. Tuber Lung Dis 1993; 74:23-27.
27. Balasubramanian R, Sivasubramanian S, Vijayan VK, et al. Five-year results of a 3-month and two 5-month regimens for the treatment of sputum-positive pulmonary tuberculosis in South India. Tubercle 1990; 71:253-258.
28. WHO Global Tuberculosis Programme G. Anti-tuberculosis drug resistance in the world. Geneva: WHO, 1997.
29. Uplekar MW, Shepard DS. Treatment of tuberculosis by private general practitioners in India. Tubercle 1991; 72:284-290.
30. Uplekar MW, Rangan S. Private doctors and tuberculosis control in India. Tuber Lung Dis 1993; 74:332-337.
31. Dholakia R, Almeida J. The potential economic benefits of the DOTS strategy against TB in India. Geneva: World Health Organization, 1997:46.