ANTIMICROBIAL RESISTANCE

Genome and MIC stability in *Mycobacterium tuberculosis* and indications for continuation of use of isoniazid in multidrug-resistant tuberculosis

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Mycobacterium tuberculosis strains resistant to two or more of the first line antituberculosis drugs (MDR) are a serious threat to successful tuberculosis control programmes. For this retrospective study, 85 follow-up drug resistant isolates from 23 patients residing in a community with a high incidence of tuberculosis were collected and the level of in-vitro resistance to antibiotics determined quantitatively. PCR-SSCP and sequencing techniques were used to screen for gene mutations associated with resistance in 31 follow-up samples from a smaller group of eight patients. DNA fingerprint analysis was done on sequential isolates to confirm identity. Although treatment had a profound effect on changes in drug resistance patterns, the MIC for a particular agent remained constant in follow-up isolates. DNA fingerprinting and mutational analysis (14 different loci) showed that the genome of MDR strains of *M. tuberculosis* is relatively stable during the course of therapy. The *rpo*B gene was the most frequently mutated structural gene involved in drug resistance and a novel C to T mutation upstream of open reading frame (ORF)1 of the *inh*A operon was detected. No evidence was found of the presence of strain W (New York) in this group of MDR strains. The results stress the importance of confirming individuality of strains for the accurate calculation of frequencies of particular mutations associated with drug resistance, particularly in a high incidence area. Approximately one-half (47.8%) of the patients had isolates resistant to concentrations just above the critical concentration for isoniazid (MICs of 0.2–5 mg/L). Therefore, these patients and their contacts who develop primary drug-resistant tuberculosis may respond to higher dosages of treatment which could have a considerable impact on the cost and the ease of management of resistant tuberculosis.

Introduction

A recent resurgence in the incidence of tuberculosis in developed and developing countries, linked partly to the human immunodeficiency virus (HIV) pandemic, has re-awakened public concern. Strains resistant to one or more of the major antituberculosis drugs threaten tuberculosis control programmes in developing and developed countries. Multidrug-resistant (MDR) strains have been associated with rapid progression to death of HIV-infected individuals [1, 2] and transmission of MDR strains to health care personnel is an added cause for concern.

Streptomycin (SM) was the first drug shown to be effective in the treatment of tuberculosis [3]. However, monotherapy led to the selection of resistant strains of *Mycobacterium tuberculosis* [4] and subsequently combination chemotherapy was shown to prevent the development of drug resistance. A combination of isoniazid (INH), rifampicin (RMP) and pyrazinamide (PZA) (the first-line drugs) is currently used with success in short course chemotherapy of tuberculosis. Various other drugs, such as SM or other aminoglycosides, ethionamide (ETH), ethambutol (EMB), thiacetazone (TH) and cycloserine (CS) are considered as alternatives for the management of MDR tuberculosis. Resistance to INH arises at a frequency of $10^{-5} - 10^{-7}$ comparable to that reported for RMP [5]. RMP is essential in short course chemotherapy regimens as, without it, treatment must be prolonged to 9 or 12

months to ensure sterilisation of the lesions. In developing countries the management of tuberculosis becomes extremely difficult in the presence of significant RMP resistance. A better understanding of the mechanisms leading to the development of MDR is essential to minimise this problem.

Mechanisms responsible for resistance to the first-line drugs have been the focus of research by many investigators and have been reviewed recently [6]. Resistance to INH develops as a result of mutations in the catalase-peroxidase gene (*kat*G) or the *inh*A gene, which encodes an enzyme involved in mycolic acid synthesis. Resistance to ETH may also be mediated by mutations in the *inhA* gene. RMP resistance is mediated by mutations in the *rpoB* gene encoding the β-subunit of RNA polymerase. SM resistance can occur as a result of mutations in either the *rrs* gene or the S12 ribosomal protein gene (*rps*L). It has been proposed that resistance in MDR strains is the result of an accumulation of mutations in the gene targets of a specific antibiotic. This may suggest a degree of inherent genome instability in these strains and implies that second-line drugs may also be of limited use, as their use may rapidly lead to resistance to these agents. Given this situation, it is essential that the use of first-line drugs such as INH and RMP be optimised.

To test the genome-instability hypothesis, the genome of serial *M. tuberculosis* isolates from patients in a high incidence community was studied by DNA fingerprinting and by mutational analysis of genes previously shown to be involved in resistance. The level of in-vitro resistance amongst MDR strains to antituberculosis drugs was also quantified.

Materials and methods

Clinical isolates and patient data

Drug-resistant isolates of *M. tuberculosis* (n = 85; group A samples, Table 1) were obtained during the follow-up of 23 patients residing in the Western Cape Province, South Africa, between Oct. 1993 and Aug. 1994 and the degree of in-vitro resistance to anti-tuberculosis drugs was determined quantitatively for INH, RMP, SM and ETH. Follow-up isolates (n = 31; group B samples, Table 2) from a smaller group of eight patients were subjected to further molecular analysis. DNA fingerprint analysis with the *IS*6110 probe was performed on all sequential isolates to confirm identity. All patients had evidence of tuberculosis on chest radiography and diagnosis was confirmed by culture of *M. tuberculosis* from sputum. The clinical isolates were resistant to at least one antituberculosis drug as determined by the proportional methods [7]. The susceptibility tests were performed on Löwenstein Jensen (LJ) medium containing critical concentrations (mg/L) of: INH, 0.2 and 1; RMP, 30; rifabutin (RBU),

10 and 20; SM, 5; ETH, 20; EMB, 2; ofloxacin (OF), 2; while kanamycin (KM) at, 5; CS, 50; and TH, 2 mg/L respectively were incorporated into Middle-brook 7H10 agar. Although all patients had active tuberculosis for periods longer than indicated in Tables 1 and 2, only the follow-up samples listed here were available and used for this study.

Quantitative drug resistance determination

The MICs of INH, RMP, SM and ETH were determined by the agar dilution method [8]. Serial dilutions of INH, RMP, SM and ETH were made and incorporated into Middlebrook 7H10 agar medium. Test organisms, maintained on LJ medium, were inoculated into Dubos broth and incubated for 10 days at 37°C. The cultures were then adjusted to an optical density of a McFarland no. 1 standard, equivalent to *c.* 2×10^7 organisms/ml, with a Klett-Summerson colorimeter. Ten μl of each culture were then inoculated (in duplicate) on to drug-containing and drug-free control plates, sealed in plastic bags and incubated at 37°C for 21 days. The lowest drug concentration that inhibited the growth of > 99% of the bacterial population was considered to be the MIC. *M. tuberculosis* H37Rv (ATCC 27294) was included as a control. Critical concentrations of 0.2 mg/L for INH, 1 mg/L for RMP, 2 mg/L for SM and 5 mg/L for ETH were used to discriminate between susceptible and resistant isolates.

Semi-quantitative catalase assays

Test organisms were grown to an optical density of a McFarland no. 1, standard in 20×150-mm screw-capped tubes containing Dubos medium. One ml of a mixture (1:1) of hydrogen peroxide 30% and Tween 80 10% solution, was applied to the surface of growth. After 5 min, the highest level (in mm) of the column of O_2 bubbles generated was recorded [9]. The H37Rv strain of *M. tuberculosis* and uninoculated tube of medium were included as controls. Additional controls consisted of *M. avium* (ATCC 25291, low catalase) and *M. gordonae* (ATCC 14470, high catalase).

DNA extraction and fingerprinting

Sputum samples were grown in a BACTEC TB-460 System (Becton Dickinson, USA) and subcultured on LJ slants. Organisms were heat-killed at 80°C for 1 h before DNA extraction [10] and fingerprinted by restriction fragment-length polymorphism with the *IS*6110 element as described elsewhere [11].

Mutational analysis

Primers were synthesised to amplify small regions (*c.* 200 bp each) of the *kat*G, *inh*A, *rpo*B, *rrs* and *rps*L genes (Table 3). A 50-ng sample of DNA from each isolate was amplified in a 100-μl reaction mixture and 5 μl of the amplified products were subjected to SSCP

Table 1. Data for MDR isolates (group A)

Patient	Isolate no.	Date	Initial resistance patterns*	Catalase (mm)	MIC (mg/L) INH	SM	RIF	ETH
A	1	2/94	INH, RMP	4	20	< 2.5	100	< 5
	2	4/94	INH, RMP	1	20	< 2.5	100	< 5
	3	4/94	INH, RMP, TH	16	20	< 2.5	100	> 5
B	12	5/94	INH, RMP, RBU, KM	0	> 40	< 2.5	> 100	< 5
	11	5/94	INH, RMP, RBU, KM	0	> 40	< 2.5	> 100	< 5
	10	6/94	INH, RMP	0	> 40	< 2.5	> 100	< 5
C	14	2/94	INH, RMP	5	20	5	> 100	< 5
	17	6/94	INH, RMP	5	10	5	> 100	< 5
D	24	10/93	INH, RMP, SM, TH	5	10	5	> 100	< 5
	28	3/94	INH, RMP, SM,	1	10	5	> 100	< 5
	25	4/94	INH, RMP, SM,	5	10	5	> 100	< 5
	26	5/94	INH, RMP, SM,	5	10	5	> 100	< 5
	27	7/94	INH, RMP, SM,	4	10	5	> 100	< 5
E	29	12/93	INH, RMP, KM, TH	20	5	10	> 100	20
	30	12/93	INH, RMP, KM, TH	6	5	10	> 100	20
	31	4/94	INH, RMP, SM	10	5	20	> 100	20
	32	5/94	INH, RMP, SM, ETH	20	5	10	> 100	20
	33	5/94	INH, RMP, SM, ETH	17	5	10	> 100	20
	34	6/94	INH, RMP, SM, ETH	40	5	10	> 100	20
F	35	3/94	INH, RMP, EMB, RBU, KM TH	13	10	< 2.5	> 100	< 5
	36	4/94	INH, RMP, EMB, RBU, KM TH	9	10	< 2.5	NA	< 5
G	40	2/94	INH, RMP	7	10	> 1000	> 100	< 5
	42	3/94	INH, RMP, SM	21	10	> 1000	100	< 5
	41	4/94	INH, RMP, SM	10	10	> 1000	> 100	< 5
	45	6/94	INH, RMP, SM	10	10	> 1000	> 100	< 5
H	46	1/94	INH, RMP, SM, EMB	45	5	> 1000	> 100	< 5
	50	2/94	INH, RMP, CS, SM, EMB	19	5	> 1000	> 100	< 5
	47	3/94	INH, RMP, SM, EMB, CS, TH	16	5	> 1000	> 100	< 5
	48	4/94	INH, RMP, SM, EMB, CS, TH	3	5	> 1000	> 100	< 5
	49	6/95	INH, RMP, SM, EMB, CS, TH, KM	2	5	> 1000	> 100	< 5
I	81	2/94	INH, RMP	1	2.5	< 2.5	> 100	< 5
	78	3/94	INH, RMP, KM	1	2.5	< 2.5	> 100	< 5
	79	3/94	INH, RMP, EMB, KM	0	2.5	< 2.5	100	< 5
	83	4/94	INH, RMP, EMB, KM	2	5	< 2.5	> 100	< 5
	82	6/94	INH, RMP, EMB, RBU, KM	1	2.5	< 2.5	> 100	< 5
	85	7/94	INH, RMP, RBU, CS, KM	3	5	< 2.5	> 100	< 5
J	86	1/94	INH, RMP, SM, KM	> 40	2.5	20	> 100	< 5
	87	2/94	INH, RMP, SM, KM	2	2.5	20	> 100	< 5
K	94	3/94	INH, SM, KM, TH	9	10	10	NA	< 5
	95	3/94	INH, KM	13	10	10	NA	< 5
	96	5/94	INH, RMP, SM, KM	5	10	5	NA	< 5
L	97	1/94	INH, RMP	5	10	< 2.5	> 100	< 5
	99	4/94	INH, RMP, RBU, KM	3	10	< 2.5	> 100	< 5
M	110	1/94	INH, RMP, CS	10	5	< 2.5	> 100	< 5
	103	1/94	INH, RMP	6	5	< 2.5	100	< 5
	104	2/94	INH, RMP, SM, CS	4	5	< 2.5	NA	< 5
	111	2/94	INH, RMP	4	5	< 2.5	> 100	< 5
	105	4/94	INH, RMP, CS	6	5	< 2.5	NA	< 5
	106	4/94	INH, RMP, SM, CS, TH	4	5	< 2.5	> 100	< 5
	107	4/94	INH, RMP, SM	> 40	5	NA	> 100	< 5
	108	5/94	INH, RMP, SM, KM, TH	20	5	< 2.5	NA	< 5
N	113	5/94	INH, RMP	17	2.5	< 2.5	> 100	< 5
	114	5/94	INH, RMP	4	2.5	< 2.5	NA	< 5
O	117	3/94	INH, RMP, KM	9	5	< 2.5	> 100	< 5
	119	4/94	INH, RMP, RBU, KM, TH	> 40	5	< 2.5	> 100	< 5
P	121	2/94	INH, RMP, SM, EMB	20	10	> 1000	> 100	< 5
	123	3/94	INH, RMP, SM, KM, TH	5	10	> 1000	> 100	< 5
	122	4/94	INH, RMP, SM, KM, TH	12	10	> 1000	> 100	< 5
Q	129	5/94	INH, RMP, SM, RBU	9	5	> 1000	> 100	< 5
	130	5/94	INH, RMP, SM	6	5	> 1000	> 100	< 5
R	134	1/94	INH, RMP, RBU, CS, ETH	> 40	40	< 2.5	> 100	> 20
	135	2/94	INH, RMP, ETH	> 40	40	< 2.5	> 100	> 20
	136	2/94	INH, RMP, SM, RBU, CS, ETH	> 40	40	< 2.5	> 100	> 20
	138	3/94	INH, RMP, SM, RBU, CS, ETH	17	40	< 2.5	> 100	20
	139	3/94	INH, RMP, SM, ETH	5	40	< 2.5	> 100	20
	137	4/94	INH, RMP, SM, RBU, CS, KM, ETH	> 40	40	< 2.5	> 100	> 20
S	144	3/94	INH, RMP	5	5	> 1000	> 100	< 5
	142	3/94	INH, SM, RBU, CS, TH	1	5	> 1000	> 100	< 5
	145	4/94	INH, RMP, SM, TH	5	5	> 1000	> 100	< 5
	143	5/94	INH, RMP, SM	6	5	> 1000	> 100	< 5
T	146	1/94	INH, RMP, SM, EMB, CS	13	5	> 1000	NA	< 5
	147	2/94	INH, RMP, SM, EMB, KM	25	5	> 1000	NA	< 5
	148	3/94	INH, RMP, SM, EMB, CS, KM	9	5	> 1000	NA	< 5

Table 1. (*continued*)

Patient	Isolate no.	Date	Initial resistance patterns*	Catalase (mm)	MIC (mg/L)			
					INH	SM	RIF	ETH
U	149	1/94	INH, RMP, SM, EMB, CS, KM	35	10	100	> 100	< 5
	151	3/94	INH, RMP, SM	12	10	100	> 100	< 5
	152	3/94	INH, RMP, SM, EMB, CS, KM	11	10	100	> 100	< 5
V	155	1/94	INH, RMP, KM	1	2.5	< 2.5	100	< 5
	158	3/94	INH, RMP, KM	1	2.5	< 2.5	> 100	< 5
W	160	12/93	INH, RMP, SM, KM	37	10	5	> 100	< 5
	162	1/94	INH, RMP, SM	> 40	10	NA	> 100	< 5
	169	3/94	INH, RMP, SM	1	10	5	> 100	< 5
	167	6/94	INH, RMP, SM	4	10	5	> 100	< 5
	166	7/94	INH, RMP, SM	24	10	5	> 100	< 5
	168	7/94	INH, RMP, SM	27	10	5	> 100	< 5
	170	8/94	INH, RMP, SM, KM	13	10	NA	100	< 5

*Initial resistance patterns were results from the routine screening of INH, Isoniazid; SM, streptomycin; EMB, ethambutol; RBU, rifabutin; ETH, ethionamide; RMP, rifampicin; KM, kanamycin; CS, cycloserine; TH, thiacetazone.
NA, not available.

analysis as described previously [12]. A mobility shift with regard to the *M. tuberculosis* H37Rv reference strain was scored as a mutation which was confirmed by restriction endonuclease digestion in cases where a restriction enzyme site was affected (Table 3) or by direct sequencing of the polymerase chain reaction (PCR) product. Direct sequencing of PCR products was performed with the Sequenase PCR Product sequencing kit (United States Biochemical, Cleveland, OH, USA) according to the manufacturer's instructions.

Dot-blot hybridisation for strain W

Previously described primers RTB59 and RTB38 [12] were used to amplify a region around codon 315 in the *katG* gene. The amplified products were subjected to separate dot-blot hybridisation assays [13] with radiolabelled oligonucleotides RTB 39 (normal for codon 315, [12]) and RTBmu: dGCGATCACC*ACA*-GGCATCGA (dinucleotide mutant for codon 315), respectively.

Results

Treatment and drug resistance patterns

Patient M (group A patients, Table 1) had the most isolates available for the time period under investigation (n = 8) and results from the hospital records for this patient were used as an example to demonstrate the effect of treatment on susceptibility patterns (Table 4). Not unexpectedly, a trend of initial resistance to INH and development of resistance to RMP (and other drugs) was frequently noticed in isolates from patients in this study. Following diagnosis in Sept. 1991, patient M presented with an INH resistant strain in July 1992, which became resistant to RMP after prolonged treatment with the fixed combination of INH, RMP and PZA (Rifater). From Dec. 1993, the treatment was changed to include INH, SM, ETH, EMB, TH and OF and the isolates became resistant to SM, CS, TH and

KM within the following 5 months, in addition to INH and RMP. It is interesting that although this patient at no time received KM or CS (according to the hospital and clinic records), the organism became resistant to these two antibiotics. Once resistant to INH and RMP, the isolates retained this resistance profile.

MICs

MICs for INH, RMP, SM and ETH, four of the agents most commonly used in the treatment of tuberculosis and for which the principal molecular mechanisms of resistance have been reported [6], were determined in this study. The results for group A samples show that despite changes that may occur in resistance patterns over time, the MIC for a particular agent stayed constant in follow-up isolates from a particular patient (Table 1). In most cases, the MIC corresponded to the drug resistance patterns obtained during the initial routine screening. The isolates could be grouped into low-level and high-level resistance, based on a low or a high MIC. Isolates from only 2 (9%) of the patients were resistant to high levels of INH (MIC 40 mg/L), and 48% of the patients had isolates that were resistant to levels just above the normal critical concentration for INH (MICs of 0.2–5 mg/L). Intermediate resistance (MICs of 10–20 mg/L for INH) was observed for isolates from 10 (44%) of the patients. This is in contrast to the results for RMP, where isolates from 21 (91%) of the patients had MICs of 100 mg/L, which is far above the normal critical concentration for RMP. Isolates from 7 (30%) of the 23 patients had MICs for SM of 100–> 1000 mg/L, isolates from 5 (22%) of the patients had MICs between 5 and 20 mg/L, while 10 (44%) of the patients were infected with strains susceptible to SM. It was noted that 12 isolates from 2 (9%) of the patients were resistant to ETH with MICs of 20 mg/L (Table 5). The low frequency of ETH resistance is probably a reflection of the restriction of its use to the management of drug-resistant tuberculosis in this region.

Table 2. Mutational data, group B isolates

Patient	Isolate no.	Date	Position of mutations					Catalase (mm)	MIC (mg/L)			
			katG	inhA	rpoB	rrs	rpsL		INH	SM	RMP	ETH
B1	320	06.10.93	463, 315	–	+	513	–	1	NA*	NA	NA	NA
	463	02.02.94	463, 315	–	+	513	–	0	10	20	>100	>20
B2	45	12.08.92	–	–34	516	–	–	9	2.5	<2.5	100	<5
	161	11.05.93	–	–34	516	–	–	10	2.5	<2.5	100	20
	241	16.07.93	–	–34	516	–	–	11	2.5	5	100	>20
	391	11.01.94	–	–34	516	–	–	5	2.5	5	>100	20
	457	27.01.94	–	–34	516	–	–	NA	NA	NA	NA	NA
B3	310	27.09.93	–	–	+	–	43	5	5	>1000	>100	<5
	341	23.11.93	–	–	+	–	43	6	10	>1000	>100	<5
B4	115	07.01.93	–	–	531	–	43	7	10	>1000	>100	<5
	259	03.08.93	–	–	531	–	43	8	5	>1000	>100	<5
	323	06.10.93	–	–	531	–	43	4	10	>1000	>100	<5
	413	12.01.94	–	–	531	–	43	4	10	>1000	>100	<5
B5	81	12.10.92	315	–	+	–	–	25	10	5	>100	<5
	213	25.06.93	315	–	+	–	–	8	10	5	>100	5
	331	06.10.93	315	–	+	–	–	4	10	5	>100	<5
	347	05.11.93	315	–	+	–	–	8	10	5	>100	<5
	353	14.09.93	315	–	+	–	–	5	10	5	>100	<5
	409	10.02.94	315	–	+	–	–	5	10	5	>100	<5
	461	09.03.94	315	–	+	–	–	5	10	5	>100	<5
	580	06.06.94	315	–	+	–	–	7	10	5	>100	<5
B6	42	03.09.92	+*	–	531	–	–	0	>40	5	<2.5	<5
	194	22.05.93	+*	–	531	–	–	0	>40	5	>100	<5
	188	10.06.93	+*	–	531	–	–	NA	NA	NA	NA	NA
	257	16.08.93	+*	–	531	–	–	0	>40	10	>100	<5
	421	01.02.94	+*	–	531	–	–	0	>40	5	100	<5
B7	328	23.08.93	315	–	+	–	–	1	10	<2.5	<2.5	<5
	429	11.02.94	315	–	+	–	–	7	10	<2.5	<2.5	<5
	519	14.04.94	315	–	+	–	–	10	10	5	<2.5	<5
B8	513	14.04.94	463; 315	–	+	513	–	0	10	10	>100	>20
	508	19.05.94	463; 315	–	+	513	–	0	10	20	>100	>20

+ or – refers to SSCP results; NA, not available.
*Mobility shift detected by PCR-SSCP with primer-set RTB51/RTB31, but mutation not confirmed.

Table 3. Mutational analysis of samples

Primer	Sequence	Restriction endonuclease	Reference
*kat*G			
RTB51	GTGCCCGAGCAACACCCACC		12
RTB31	TCAAGTCGACTGGTCGCGAC		
RTB59	TGGCCGCGGCGGTCGACATT		12
RTB39	GCTGGTGATCGCGTCCTTAC		
RTB57	CCGACGATGCTGGCCACTGA		12
RTB37	AGGTCGTGCTGACCGCAGGG		
RTB56	CCCTGCGGTCAGCACGACCT	*Msp* I	12
RTB36	TCGGGGTCGTTGACCTCCCA		
RTB54	CTACAAGCGCTTACCGCTGG	*Hae* III	12
RTB34	GACCTCGACAAGCGCCCGCA		
RTB59	GCTGGTGATCGCGTCCTTAC	*Hpa* II	12
RTB38	GGTCAGTGGCCAGCATCGTC		
katGup51	GACTACGCCGAACAGCTCC		17
katGup32	GCGATAACCCCGCAAGACC		
katGup52	GCGGGGTTATCGCCGATG		17
katGup32	GCCCTCGACGGGGTATTTC		
*inh*A			
inhA51	CGGGCAACAAGCTCGACGGG		Current
inhA31	GGGTTCATGATCGGCAGGAG		
inhp5	CGCAGCCAGGGCCTCGCTG		25
inhp3	CTCCGGTAACCAGGACTGA		
*rpo*B			
TR8	TGCACGTCGCGGACCTCCA		30
TR9	TCGCCGCGATCAAGGAGT		
rrs			
STR51	TTCCTTGGGATCCGTGCCGT		Current
STR31	CTAGACGCGTCCTGTGCATG		
STR53	TCACCATCGACGAAGCTCCG	*Rsa* I, *Bsr* I, *Pvu* II	Current
STR33	CGCCCGCACGCTCACAGTTA		
*rps*L			
STR52	GTGAAGACCGCGGCTCTGAA	*Mbo* II, *Hph* I	Current
STR34	TTCTTGACACCCTGCGTATC		

Table 4. Effect of treatment on drug resistance patterns for patient M*

Treatment	Date of isolation	Drug resistance pattern
RIFA	9/91	Unknown
(Sept. 1991 intermittent until Aug. 1993)	7/92	INH
	2/93	INH
	4/93	INH
	8/93	INH, RMP
	11/93	INH, RMP
INH, SM, ETH, EMB, TH, OF	12/93	INH, RMP
(Dec. 1993–)	1/94	INH, RMP, CS
	1/94	INH, RMP
	2/94	INH, RMP, CS, SM
	2/94	INH, RMP
	3/94	INH, RMP, SM
	4/94	INH, RMP, CS
	4/94	INH, RMP, CS, SM, TH
	4/94	INH, RMP, SM
INH, ETH, EMB, TH, OF	5/94	INH, RMP, SM, TH, KM
(May 1994–)		
INH, ETH, EMB, KM, TC, OF, CLO	6/94	INH, RMP, SM
(June 1994–)	8/94	INH, RMP, SM
INH, ETH, EMB, TH, KM, Z, OF	9/94	INH, RMP, SM
(Oct. 1994–July 1995)	4/95	INH, RMP, SM, TH, KM

INH, isoniazid; KM, kanamycin; SM, streptomycin; CS, cycloserine; EMB, ethambutol; TH, thiacetazone; RBU, rifabutin; OF, ofloxin; ETH, ethionamide; TC, thiacetozone; RMP, rifampicin; Z, pyrazynamide; RIFA, rifater; CLO, clothamatazine.
*Date of first tuberculosis diagnosis: Sept. 1991.

Table 5. Grouping of MIC results

Antituberculosis agent	MIC (mg/L)	Number of isolates	Number (%) of patients (n = 23)
INH	⩾ 40	9	2 (8.7)
	10–20	34	10 (43.5)
	> 0.2–⩽ 5	42	11 (47.8)
SM	100–> 1000	24	7 (30.4)
	5–20	23	6 (26.1)
	Susceptible	35	10 (43.5)
	NA	3	...
RMP	⩾ 100	74	21 (91.3)
	NA	11	...
ETH	⩾ 20	12	2 (8.7)
	Susceptible	73	21 (91.3)

INH, isoniazid; SM, streptomycin; RMP, rifampicin; ETH, ethionamide; NA, not available.

Isoniazid resistance and catalase activity

It has been recognised previously that development of resistance to isoniazid is often associated with loss of catalase and peroxidase activities [14] and recent genetic evidence has clearly demonstrated a link between isoniazid resistance and changes in the catalase-peroxidase enzyme [15]. All the isolates in this study were resistant to INH and the catalase activity was reduced in most isolates (compared to H37Rv that had a catalase activity of 40 mm). Some isolates (10 of 85) had normal or even elevated catalase activities, although resistant to INH (Table 1). Particularly interesting to note are isolates from patient R, which were highly resistant to INH (MIC = 40 mg/L) and yet had normal levels of catalase activity. This suggests a resistance mechanism not involving catalase, confirming previous reports [16]. However, this procedure is semi-quantitative and despite determining catalase activities in duplicate, not all the follow-up isolates from particular patients had the same activity. These discrepancies are currently under further investigation.

DNA fingerprint analysis

Serial isolates collected from 29 patients with MDR tuberculosis were characterised by DNA fingerprinting with *IS*6110 [11], Fig. 1 shows a sample result. No fingerprint data are available for isolates from patients Q and W in Table 1. Twenty-one strains were identified containing between two (e.g., all isolates from patient I, Table 1) and 23 (e.g., isolates from patient O, Table 1) insertion elements. Three strains were shown to have infected more than one patient: one strain had 19 *IS*6110 elements (patients B1 and B8, Table 2), one had four *IS*6110 elements (patient B5, Table 2; patient C, Table 1) and one had five *IS*6110 elements (patients B3 and B4, Table 2 and patients G, H, N, P, T, Table 1). No epidemiological data were available for these patients and it is not known whether these infections were due to recent transmission. The banding patterns of 15 of the serial isolates remained constant, demonstrating that the respective infections were due
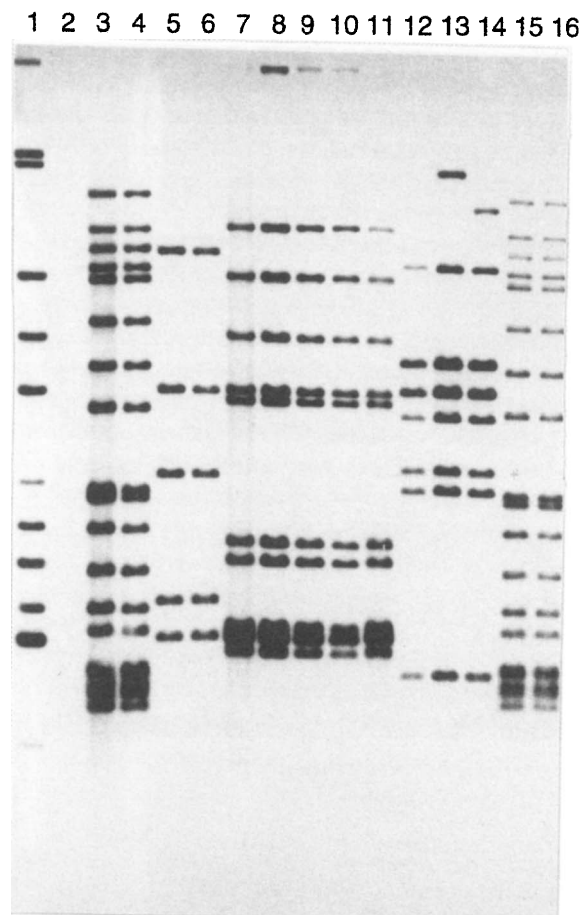

Fig. 1. *IS*6110 DNA fingerprints. Serial isolates from patients B1 (lanes **3, 4**), B3 (**5, 6**), B6 (**7–11**), B7 (**12–14**) and B8 (**15, 16**) were fingerprinted with the *IS*6110 element. Lane 1, control strain MT14323.

to a single strain rather than to multiple infections selected by therapy or re-infection with different strains. Six of the serial isolates (isolates from patients B2 and B7, Table 2 and isolates from patients A, E, O and U, Table 1) showed slight DNA fingerprint perturbations as a function of time. These variations could be either the result of replicative transposition (additional bands) or anomalies in *Pvu*II restriction as

a result of DNA methylation (band shifts) [16]. These small DNA fingerprint changes do not imply re-infection but rather suggest strain evolution and thus these results suggest that all patients were infected with only a single strain.

Identification of mutations by PCR-SSCP

Primers were selected to amplify 14 different genetic loci in the group B isolates (Table 2) where mutations that are associated with resistance to INH, ETH, RMP and SM were previously reported. Ten of these loci are in structural genes, while three sets of primers were directed towards putative promoter regions of the *kat*G gene and the *inh*A operon. The results are summarised in Table 2.

Structural genes. The *rpo*B gene was the most frequently mutated gene in this set of MDR clinical isolates (Table 2). Although the precise nature of the *rpo*B gene mutations was not determined for most isolates in this study, the SSCP analysis suggests that the same mutation is present in sequential isolates from a particular individual, as the nature of the mobility shift remained constant. Sequence analysis indicated that the mobility shifts for all the isolates of patient B2 were due to codon 516 mutations, changing from GAC to GTC, and all the isolates of patients B4 and B6 were due to codon 531 mutations, changing from TCG to TTG.

Mutations were detected in the *kat*G gene in isolates from five of eight patients (Table 2). An Arg463Leu mutation has been reported previously [12, 16, 18] and the presence of this mutation in isolates 320, 463, 513 and 508 (Table 2) as initially determined by SSCP analysis, was confirmed by restriction endonuclease analysis with *Msp*I. No other isolate showed this mutation by SSCP or *Msp*I analysis. A Ser315Thr mutation was also detected by SSCP and confirmed by *Hpa*II analysis with primer set RTB59/38 in all isolates from four patients.

Sequential isolates from two patients had both the Arg463Leu and the Ser315Thr mutation. PCR-SSCP analysis of amplified products or RTB51/31 showed the same change in all five sequential isolates of patient B6, but the specific nature of the mutation is as yet unconfirmed. No mutations were detected in the other regions of the *kat*G gene investigated in this study. Complete deletion of the *kat*G gene has been proposed as a mechanism of resistance to INH [15], but as several different regions of the gene could be amplified in this study, it can be concluded that none of the samples tested had significant *kat*G gene deletions, thus confirming previous reports [6, 12, 17]. No differences in mutational spectrum were detected in serial isolates. The *inh*A gene may also be associated with resistance to INH, but no mutations were detected in a 169-bp region of the structural gene that has been shown previously to be involved in resistance to INH and ETH [19, 20].

Mutations in both the *rrs* and *rps*L genes are associated with resistance to SM and in both cases the mutations are located in two regions of these genes: codons 43 or 88 for *rpsL* and mutations located near or in codons 530 or 915 of *rrs* [21]. PCR-SSCP analysis of the *rpsL* region showed mobility shifts for all the isolates from patients B3 and B4. Restriction endonuclease analysis with *Mbo*II confirmed that the shifts were due to an A to G mutation at codon 43. No mutations were detected at codon 88 by SSCP or *Hph*I (codon 88) restriction endonuclease analysis. All the sequential isolates from patients B1 and B8 showed an A to C mutation at position 513 (confirmed by sequence analysis) of the *rrs* gene. Restriction endonuclease analysis with *Rsa*I (position 491) and *Bsr*I (position 512) did not identify any further mutations in the 530 region and no mutations were identified in the 950 region of the *rrs* gene in this group of MDR isolates.

The mutation at codon 43 in the *rps*L gene is associated with a high level of resistance (MIC > 1000 mg/L) for SM in isolates from both patients B3 and B4 (Table 2). DNA fingerprint analysis indicated that these two patients were infected with the same strain of *M. tuberculosis* (see DNA fingerprint analysis). It was subsequently found that isolates from patients G, H, P, T, Q and S (Table 1) which had similar MICs for SM, also had the codon 43 mutation. Although isolates from patient N had fingerprints similar to those of isolates from patients G, H, P and T, they (from patient N) not only lacked this mutation, but also had considerably lower MICs. The precise mutation is not yet known.

Isolates from patients Q and S also had high MICs for SM. Unfortunately, no fingerprint data were available for isolates from patient Q (see DNA fingerprint analysis), but it was noted that the fingerprint patterns of isolates from patient S (six *IS*6110 bands) differed by only one additional *IS*6110 element compared to isolates from patients G, H, N, P and T (these had five *IS*6110 elements). Futhermore, isolates from patients B1 and B8 (Table 2) also had the same mutations in drug resistance genes and showed the same DNA fingerprint pattern. These data suggest that certain mutations are associated with given levels of resistance and, furthermore, that although DNA fingerprint analysis is a useful technique to monitor the spread of disease, it has no direct diagnostic application, as strains with the same or similar DNA fingerprint patterns may or may not be drug resistant.

Mutations in the putative control elements. To determine whether mutations in control elements of relevant known genes may play a role in drug resistance, a domain spanning *c.* 400 bp upstream of the *kat*G gene

and *c*. 300 bp upstream of the ORF1 of the *inhA* gene locus was analysed by PCR-SSCP. No mutations were detected in the putative *katG* gene control element. However, four of five sequential isolates from patient B2 showed a mobility shift in the putative control region of the *inh*A gene (Table 2). Sequence analysis (Fig. 2) confirmed that the mobility shift was due to a novel C to T mutation at nucleotide −34 upstream of ORF1 of the *inh*A operon. It is important to note that despite the identical fingerprints of all the isolates from this patient, no mutation was detected in isolate 45, the first isolate collected from this patient, which was susceptible to ETH according to the criteria used in this study. Further analysis of 15 other ETH-resistant isolates from this community failed to show this particular mutation.

Has strain W (New York) spread to our community?

Some of the MDR strains originating from this community in South Africa have similar drug resistance patterns and a high *IS*6110 copy number which is similar to the highly drug-resistant *M. tuberculosis* strain W which has spread from New York to other cities in America and to France [22]. One of the striking features of strain W is a rare dinucleotide missense mutation at codon 315 (AGC to ACA) of the *katG* gene [22]. PCR amplification of this region and dot-blot hybridisation assays with radiolabelled oligonucleotides RTB39 (normal for codon 315 [12]) and RTBmu (dinucleotide mutant for codon 315) were used to analyse the codon 315 region in one isolate from each of the patients B1–B8 in Table 2. Another 14 MDR isolates, which were not part of isolates in Tables 1 and 2, but which had a high *IS*6110 copy number, were also examined. All the samples hybridised

strongly to oligonucleotide RTB39 and no hybridisation was observed with primer RTBmu under stringent conditions (results not shown), suggesting that none of these isolates belonged to the strain W family. However, this does not exclude the possibility that other members of this family which have undergone clonal expansion are present in our community [22].

Discussion

It has been shown previously in limited studies that the DNA fingerprints of *M. tuberculosis* isolates from patients undergoing chemotherapy do not change during the development of resistance [23]. The results of DNA fingerprinting and mutational analysis (14 different loci) in the present study show that the genome of MDR *M. tuberculosis* strains is relatively stable, even while under pressure from different drugs during treatment. Furthermore, the acquisition of resistance to antituberculosis drugs in these patients is not due to secondary infection with a new resistant strain.

RpoB was the most frequently mutated gene in drug resistant *M. tuberculosis* and this gene is a useful target for rapid molecular screening for MDR tuberculosis. There have been many reports on the incidence of mutations in structural genes associated with drug resistance in *M. tuberculosis* [6, 12, 21]. Only the presence and stability of specific mutations in these genes in serial isolates have been investigated in the present study and the results are not an indication of the frequency of the overall mutational pattern in drug-resistance genes in *M. tuberculosis*. However, this study enphasises the importance of confirming strain identity with fingerprint analysis at
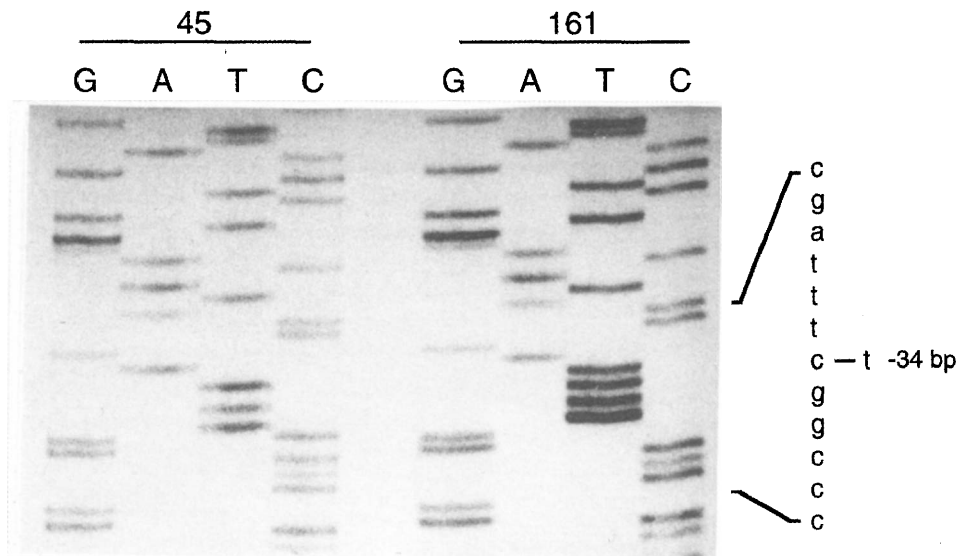

Fig. 2. Mutation in promoter of *inh*A gene. Isolates 45 and 161 (Table 2) were amplified with primer set inhp5/inhp3 (Table 3) and the PCR products were sequenced directly. A C to T mutation at position −34 bp upstream of ORF1 of the *inhA* operon was detected in samples 161 and samples 241, 391 and 457 compared to sample 45.

all times when reporting on the overall incidence of given mutations. This is particularly important when samples originate from a high incidence community for tuberculosis (700/100 000 cases/year), such as in South Africa [24] or when samples are analysed retrospectively from a databank.

Mutations in promotor regions of genes can potentially affect expression and, therefore, also contribute towards drug resistance. This possibility was investigated in the *kat*G and *inhA* genes. However, none of the samples showed any mutations in the promoter sequences immediately upstream of the *kat*G gene. This result is consistent with an earlier report where the isolates originated from a different geographical area [17]. In contrast, a novel C to T mutation at nucleotide −34 upstream of ORF1 of the *inh*A operon was detected. This mutation, which has not been described previously, falls within the promotor region of this operon and could affect transcription and drug response, as the first isolate from patient B2 did not have the mutation at position −34 bp and only this isolate in the batch from one patient was susceptible to ETH. Mutations 1 bp on either side of a putative ribosome binding site of ORF1 in *inh*A have been reported [6, 25], and although we have found the same mutations in other South African isolates (results not shown), these mutations were not detected in these particular isolates. Results from this study support accumulated evidence [6, 19, 25] that mutations in the structural region of the *inh*A gene are rare. Although mutations in the promotor region have been described in this paper and by others, few ETH-resistant samples have these promotor mutations. However, recent biochemical and genetic evidence suggests that *inh*A may not be the major target for activated INH in *M. tuberculosis* [26] and may explain why only some of the isolates have *inh*A mutations.

Accurate drug susceptibility testing of *M. tuberculosis* by conventional techniques is difficult and may perhaps explain the intermittent resistance patterns in follow-up isolates observed in this study. The development of antituberculosis drug resistance is best prevented by combination chemotherapy administered under direct observation. Failure to comply with this requirement on the part of the health services or the patient may lead to monotherapy. The use of combinations of drugs that do not offer optimal protection against the selection of drug-resistant mutants may also elicit resistance in some instances. This study illustrates the manner in which the sequential addition of drugs to the therapeutic regimen can be followed by the accumulative acquisition of resistance.

It is significant that despite changes in drug resistance patterns that occurred during treatment, the level of resistance (based on MICs) for a particular antibiotic remained constant in our patients. This has been observed previously [27] and is an important consideration for treatment, as the results show that although the MICs are above the normal in-vitro critical concentrations for *M. tuberculosis*, a significant number of these organisms have MICs at achievable in-vivo concentrations and patients may still respond to treatment with these antibiotics, particularly if higher, but still clinically acceptable dosages of agents are used. The low MICs found in *c.* 50% of the INH-resistant isolates indicate that this agent, given in a higher dose, may still be active in many MDR tuberculosis patients [28]. Evidence from early clinical trials in which INH was used as the sole agent in patients with INH-resistant organisms suggests that the use of higher dosages of INH in patients with lower levels of resistance may be clinically beneficial [27]. However, it is known that the rate of elimination of INH is under genetic control [29, 30] and varies greatly from one individual to another. The rate of acetylation should therefore be taken into account in evaluating the activity of INH in MDR tuberculosis.

It is noteworthy that almost half of the isolates evaluated in this study were susceptible *in vitro* to concentrations of INH which are easily achievable, even in rapid acetylators of INH. As INH is relatively inexpensive compared to other drugs available for the management of MDR tuberculosis, we suggest that MIC (INH) testing of drug resistant strains of *M. tuberculosis* should be considered and that, where appropriate, INH should be included in any multidrug regimen for the treatment of MDR tuberculosis [28]. Given the limited number of drugs available for the management of MDR tuberculosis and the increased costs and toxicity associated with their use, it is important to take note of existing drugs that could possibly be used in a mutidrug regimen to treat a signficant proportion of the cases detected.

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