Reinfection and Mixed Infection Cause Changing *Mycobacterium tuberculosis* Drug-Resistance Patterns

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***Rationale:* Multiple infections with different strains of *Mycobacterium tuberculosis* may occur in settings where the infection pressure is high. The relevance of mixed infections for the patient, clinician, and control program remains unclear. *Objectives:* This study aimed to describe reinfection and mixed infection as underlying mechanisms of changing drug-susceptibility patterns in serial sputum cultures. *Methods:* Serial *M. tuberculosis* sputum cultures from patients diagnosed with multi-drug-resistant (MDR) tuberculosis were evaluated by phenotypic drug-susceptibility testing and mutation detection methods. Genotypic analysis was done by IS*6110* DNA fingerprinting and a novel strain-specific polymerase chain reaction amplification method. *Measurements and Main Results:* DNA fingerprinting analysis of serial sputum cultures from 48 patients with MDR tuberculosis attributed 10 cases to reinfection and 1 case to mixed infection. In contrast, strain-specific polymerase chain reaction amplification analysis in 9 of the 11 cases demonstrated mixed infection in 5 cases, reinfection in 3 cases, and laboratory contamination in 1 case. Analysis of clinical data suggests that first-line therapy can select for a resistant subpopulation, whereas poor adherence or second-line therapy resulted in the reemergence of the drug-susceptible subpopulations. *Conclusions:* We have shown that, in some patients with MDR tuberculosis, mixed infection may be responsible for observations attributed to reinfection by DNA fingerprinting. We conclude that treatment and adherence determines which strain is dominant. We hypothesize that treatment with second-line drugs may lead to reemergence of the drug-susceptible strain in patients with mixed infection.**

Keywords: drug resistance; mixed infections; *Mycobacterium tuberculosis*; reinfection

Traditionally, infection by *Mycobacterium tuberculosis* was assumed to be caused by a single strain, and recurrences were believed to be due to reactivation of the strain that caused the first episode (1). Infection with multiple *M. tuberculosis* strains within one patient before, during, or after successful treatment was rarely considered. The development of DNA fingerprinting methods to differentiate *M. tuberculosis* strains (2) has made it possible to document reinfection with a genetically different *M. tuberculosis* strain in patients with recurrent tuberculosis who were resident in high or low tuberculosis incidence settings, and who were either immunocompetent or immunosuppressed (3–11).

Evidence of mixed infection in a single host at a single point in time, suggesting reinfection before or during disease, was first observed using the phage typing method (12, 13) and was later confirmed using DNA fingerprint analysis (8, 14–19). The infrequency of observing mixed infections probably reflects the insensitivity of the DNA fingerprinting method (16). It has recently been shown, using a highly specific polymerase chain reaction (PCR)–based genotyping method, that patients with tuberculosis in a high-incidence setting often have different *M. tuberculosis* strains in the same sputum specimen (20).

It has been suggested that mixed infections may be of concern for tuberculosis control because such infections could influence the diagnosis of drug resistance if a patient is infected with both a sensitive and a resistant strain (21). Furthermore, it is possible that undetected drug-resistant strains may emerge under the pressure of antibiotic treatment (21).

This study aimed to describe whether reinfections and mixed infections can be underlying mechanisms of drug-susceptibility variation in serial *M. tuberculosis* sputum cultures collected from patients diagnosed with multi-drug-resistant (MDR) tuberculosis (defined as bacillary resistance to at least isoniazid and rifampin). We show that the type of information available (clinical, microbiological, DNA fingerprinting, or PCR results) influences the interpretation of the observed variation in drug-susceptibility patterns. We demonstrate how the selective pressure of the antibiotic therapy determines which strain is dominant in the sputum culture. This work was presented at the South African Society for Biochemistry and Molecular Biology conference held in Stellenbosch, South Africa, in 2005 (31).

METHODS

Study Population

Clinical data and sputum specimens were collected as part of an ongoing, prospective study in Cape Town, South Africa (22). The incidence of new smear and/or culture-positive tuberculosis in the study communities (population, ∼ 35,000) was, on average, 313 per 100,000 per year (1993–1998) (22). A database search was performed to identify patients with MDR tuberculosis and who had at least two serial sputum cultures of *M. tuberculosis*. Only data from patients fulfilling these criteria were included in the analysis. This study was approved by the Ethics Review Board at Stellenbosch University.

Drug-Susceptibility Testing

Drug-susceptibility testing was done by the National Health Laboratory Service using the indirect proportion method on Löwenstein-Jensen

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TABLE 1. PRIMER SEQUENCES

Primer Name	Sequence	IS*6110* Insertion Point
Universal forward	TTC AAC CAT CGC CGC CTC TAC	
Strain a reverse	GAG CGC GCC GAA GGC GGC CAT GAA C	2602188
Strain b reverse	GGC CAA ATC CAG CAC CAC GGT GAA C	3096762
Strain c reverse	GCG CCA ATG AAG CCA GCA ACG CCG T	3379767
Strain d reverse	CAC CCT CTA CTC TGC GCT TTG	3127922
Strain e reverse	TTG CTT TGA GGC GAC TTC C	3125705
Strain f reverse	GCG CGT GTC CCGA TGT GAG GTG GT	1989058
Strain g reverse	TCA GCC CGC CGC GAC TGT ATG AAC C	2627510
Strain h reverse	CAG GAC AAA GGT CGG CAA CCT GAA CC	1996100
Internal control forward	GAG CAG CAG TGG AAT TTC GC	
Internal control reverse	TCC CAG TGA CGT TGC CTT C	

medium containing critical concentrations of 0.2 μg/ml isoniazid and 30 μg/ml rifampin. Drug-susceptibility testing for other drugs was done for sputum cultures resistant to isoniazid and/or rifampin.

Mutations conferring resistance to isoniazid, rifampin, streptomycin, and ethambutol were determined by DNA sequencing or PCR dot-blot hybridization (23).

Genotyping by Molecular Techniques

M. tuberculosis strains present in serial sputum cultures were genotyped by the IS*6110* DNA fingerprinting method (2). In addition, each sputum culture was assessed by strain-specific PCR amplification (20), using a universal forward primer (complementary to an internal sequence of the IS*6110* element) in combination with the respective reverse primer (complementary to the strain-specific IS*6110* insertion junction; Table 1). Strain-specific primers were designed after sequencing of the IS*6110* insertion junctions from different strains identified in serial sputum cultures by DNA fingerprinting (*see* the online supplement). PCR reactions were performed in a total volume of 25 μl, according to previously described reaction conditions (20). PCR-amplified products were electrophoretically fractionated in 2.0% agarose and visualized by staining with ethidium bromide. Sensitivity and specificity of each primer set were determined by amplification of pure DNA from a panel of genetically unrelated and related strains (20), and were shown to be 100% (95% confidence interval, 85–100%) when compared with the gold standard of IS*6110* DNA fingerprinting. Using the described amplification conditions (20), underlying strains could be detected at a molar ratio of 1:125.

To minimize laboratory cross-contamination, sputum cultures from each patient were PCR-amplified on separate days, and each procedure (preparation of the PCR reaction mixes, the addition of the DNA, the PCR amplification, and the electrophoretic fractionation) was conducted in physically separated rooms. Primers complementary to the *M. tuberculosis* gene Rv3875 were included in each amplification reaction as a positive amplification control. Negative controls (water) were included to detect reagent contamination.

Reagent contamination could not be detected because all negative controls were negative on amplification. The inability to detect specific strains was not due to the presence of PCR inhibitors because positive controls produced amplification products in all sputum cultures. This PCR method was highly consistent as amplification of each sputum culture gave identical products on repeated amplification.

Definitions of Reinfection and Mixed Infection

Reinfection was confirmed, according to the gold standard (5), when analysis of serial sputum cultures from a single patient showed the appearance of a genetically different strain (in two or more cultures) during the course of disease. If the genetically different strain was only identified in a single sputum culture, reinfection was considered as probable. Mixed infection was confirmed when two genetically distinct strains were present in the initial sputum culture as well as in at least one subsequent sputum culture.

Statistical Methods

Fisher's exact test was used to identify significant differences between patient groups.

RESULTS

Study Population

During the period from January 1993 to December 1998, 1,023 patients resident in the epidemiologic field site in Cape Town, South Africa, were culture-positive for *M. tuberculosis* (22). Sputum cultures from 768 of these patients were available for further analysis. Cultures from the remaining 255 patients were contaminated, were lost, or failed to produce usable DNA fingerprints. Phenotypic drug-susceptibility testing classified 48 of the 768 patients as having an episode of MDR tuberculosis.

Genotyping by DNA Fingerprinting

DNA fingerprinting showed a single strain in serial sputum cultures from 37 of the 48 patients with MDR tuberculosis. Serial sputum cultures from the remaining 11 patients showed the presence of genetically distinct strains (Figure 1). Among these, two different strains were isolated from one patient (Patient 2) on the day of diagnosis, as well as from subsequent sputum cultures, demonstrating mixed infection (Figure 2). In the remaining 10 patients, DNA fingerprinting data suggested reinfection. Reinfection could be confirmed by using fingerprint methods in two patients (HIV-uninfected Patient 1 [Figure 2] and HIV-infected Patient 7 [Table 2]), and was suggested (probable reinfection) in the remaining eight patients, for whom confirmatory sputum cultures for both genetically distinct *M. tuberculosis* strains were not available.

Genotyping by PCR Amplification

According to the strain-specific PCR amplification method results, only 3 of the 11 patients with genetically distinct strains present on DNA fingerprint analysis were classified as confirmed or probable reinfection (Figure 1). Reinfection with an MDR strain during treatment with first-line antituberculous drugs was confirmed in Patient 7, an HIV-infected patient (Table 2). Reinfection could not be distinguished from mixed infection in the remaining two patients (Table 2, Patients 3 and 4) because their initial sputum culture was not available for genotypic analysis.

Five cases were classified as mixed infection based on analysis of the strain-specific PCR amplification results. The diagnosis of mixed infection on the basis of the DNA fingerprinting results in Patient 2 was confirmed by the PCR method (Figures 1 and 2). In addition, four patients classified as probable reinfection by DNA fingerprinting were reclassified as cases of mixed infection, because two genetically distinct strains could be identified by PCR in both the initial and subsequent sputum cultures (Figure 2 and Table 2; Patients 1, 5, 6, and 8).

In one patient classified as probable reinfection by the DNA fingerprinting method, the presence of a second strain could not

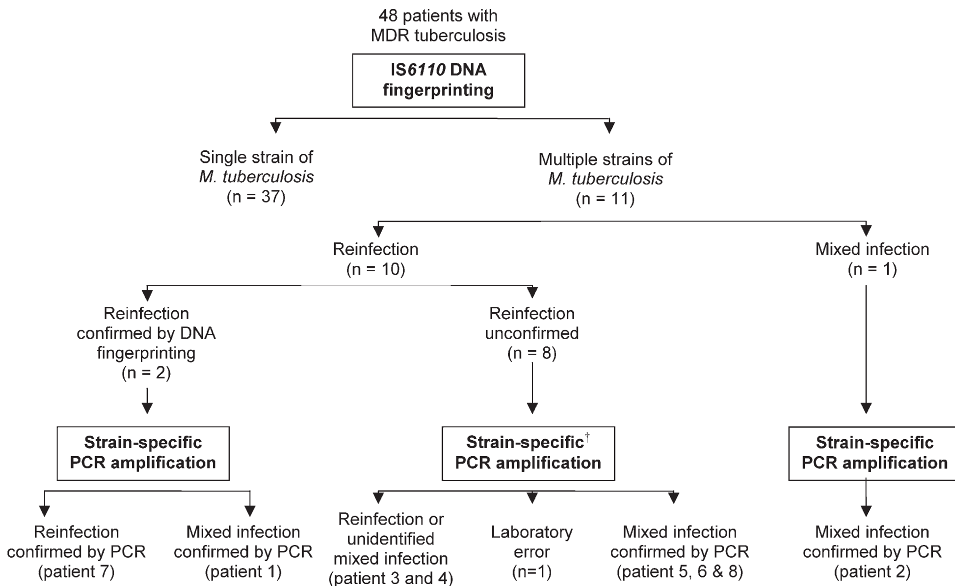

Figure 1. Schematic diagram showing the grouping of patients according to DNA fingerprinting and strain-specific polymerase chain reaction (PCR) amplification methods. †Sputum culture from two patients could not be tested. MDR = multi-drug-resistant.

be confirmed by PCR amplification in more than one sputum culture (data not shown), suggesting laboratory error (Figure 1).

Strain-specific primers were not available for the analysis of serial sputum cultures from the remaining two patients. Similarly, strain-specific primer sets were also not available for the 37 patients with MDR tuberculosis with genetically identical strains in serial sputum cultures, because the development of primers was dependent on the identification of different *M. tuberculosis* strains in serial sputum cultures.

Clinical and Demographic Characteristics of Patients with Mixed Infection and Reinfection

No difference could be found between patients infected with either a single or two genetically distinct strains, when comparing sex, age, type of tuberculosis, and treatment outcome (Table 3).

Mechanisms for Emergence and Reemergence of Strains in *M. tuberculosis* Sputum Cultures of Mixed Infection Cases

To explore for an association between treatment regimen, adherence, and changing strain populations during therapy in patients with MDR tuberculosis, the genotype data were compared with the drug-susceptibility patterns, the treatment regimen, and the adherence records (Figure 2 and Table 2).

Emergence of a drug-resistant strain on treatment with first-line antituberculosis drugs. Analysis of the drug-susceptibility results of the sputum cultures of Patient 1 (Figure 2, Patient 1, *lane A* through *D*) shows fully susceptible initial sputum cultures followed by the appearance of MDR tuberculosis. Without additional information of genotypic data, this would have been interpreted as acquisition of drug resistance in the same strain. Conversely, according to the analysis of the DNA fingerprinting data, this patient would have been classified as confirmed reinfection with a genetically distinct MDR strain during therapy (Figure 2, Patient 1; compare *lanes A* and *B* to *lanes C* and *D*). In contrast to this, the PCR-based method showed that both the drug-susceptible and the MDR strains were present at diagnosis (Figure 2, Patient 1, *lane A*), classifying the patient as a case of mixed infection. These findings suggest that antibiotic pressure with a standard first-line regimen may have led to reduced growth of the drug-susceptible strain population, and selection and subsequent culture dominance of the previously undetected genetically distinct MDR strain population. Similar results were observed for Patient 2 (Figure 2), as well as Patients 5 and 6 (Table 2).

Treatment interruption and reemergence of a drug-susceptible strain in the patient with MDR tuberculosis. Analysis of the drug-susceptibility results of the subsequent sputum cultures from Patient 1 shows the reemergence of drug-susceptible sputum cultures (Figure 2, Patient 1, *lanes E* and *F*) followed again by MDR tuberculosis (Figure 2, Patient 1, *lanes G* to *I*). Without additional information, the presence of the drug-susceptible strain would have been interpreted as inaccuracy of the phenotypic drug-susceptibility tests. Analysis of DNA fingerprinting data, however, confirms the presence of the drug-susceptible strain (the strain the patient was initially dually infected with), thereby refuting the hypothesis of inaccuracy of the phenotypic drug-susceptibility tests. The PCR-based method showed that both the drug-susceptible and the MDR strain were present throughout the 17-month treatment period. These results have demonstrated that in this patient with MDR tuberculosis, the underlying drug-susceptible strain reemerged and became the dominant strain population in the sputum cultures after partial or complete removal of the antibiotic pressure through poor adherence or default (Figure 2, Patient 1, *lane D*). The data for Patients 3 and 8 showed a similar pattern, with the underlying drug-susceptible strain reemerging after a period of poor treatment adherence or default (Table 2, Patient 3, *lane J* and *K*, and Patient 8, *lane C* and *D*).

Treatment with second-line drugs and reemergence of a drug-susceptible strain in a patient with MDR tuberculosis. Analysis of the drug-susceptibility results of the sputum cultures from Patient 2 shows the presence of drug-susceptible and drug-resistant strains at time of diagnosis (Figure 2, Patient 2, *lanes A* and *B*). Without additional information, this would have been interpreted as inaccuracy of the phenotypic drug-susceptibility tests. Analysis of DNA fingerprinting data and PCR-based data confirmed the presence of both a drug-susceptible and a drug-resistant strain at diagnosis, indicating mixed infection. Three months after starting second-line treatment, the drug-susceptibility tests demonstrate the presence of a fully susceptible sputum culture (Figure 2, Patient 2, *lane F*), which was confirmed by DNA fingerprinting and PCR-based genotyping methods. Together, these results suggest that, in this patient, the lowered antibiotic pressure was not due to poor adherence (adherence

	Patient 1 (HIV neg)									Patient 2 (HIV neg)					
	A	B	C	D	E	F	G	H	I	A	B	C	D	E	F
Date - Day	23	23	23	22	14	29	10	19	09	10	10	01	06	20	20
Date - Month	09	09	12	03	04	04	11	01	02	04	04	09	10	10	01
Date - Year	93	93	93	94	94	94	94	95	95	94	94	94	94	94	95
Susceptibility - isoniazid	S	nd	R	R	S	nd	R	R	R	nd	nd	R	R	R	S
- rifampin	S	nd	R	R	S	nd	R	R	R	nd	nd	R	R	R	S
- streptomycin	S	nd	R	R	S	nd	R	R	R	nd	nd	S	S	S	nd
- ethambutol	S	nd	S	S	S	nd	S	S	S	nd	nd	S	S	S	nd
Treatment - Rifafour (Inh, Rif, Pza, Emb)	X	X	X	X	X	X									
- Inh, Rif, Pza, Emb							X	X	X						
- Inh, Rif, Pza										X	X	X	X		
- Emb, Eth, Sm, Teri, Pza														X	X
Adherence (%)		62	63	D	D	U	62	U	U		100	92	95	95	100
Point mutations - *katG*315	-	-	+	+	-	-	+	+	+	+	-	+	+	+	-
- *rpoB*531	-	-	+	+	-	-	+	+	+	+	-	+	+	+	-
- *rpsL*43	-	-	+	+	-	-	+	+	+	nd	nd	-	-	-	-
- *rrs*513	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	-	nd	-
- *emb*306	-	-	nd	nd	nd	nd	-	nd	-	nd	-	-	-	-	-
Strain designation	a	a	b	b	a	a	b	b	b	c	d	c	c	c	d

IS*6110* DNA fingerprinting

Strain-specific PCR

PCR (strain a) *

PCR (strain b) *

PCR (strain c) *

PCR (strain d) *

Figure 2. Phenotypic and genotypic characterization of sputum cultures from Patients 1 and 2. Serial *M. tuberculosis* sputum cultures were obtained from patients diagnosed with MDR tuberculosis. Phenotypic culture-based drug-susceptibility testing was performed by the direct proportion method. Treatment regimen implemented at each visit is indicated, whereas adherence was measured for the period between each visit. Mutations conferring resistance were detected by DNA sequencing or PCR dot blot (23). All sputum cultures were genotyped by IS*6110* DNA fingerprinting (2), and the strain(s) present was randomly assigned an alphabetic designation according to its strain family classification. Presence of multiple strains in each sputum culture was determined using strain-specific PCR amplification (20). D = default (stopped therapy for a period of > 2 months); Emb = ethambutol; Eth = ethionamide; Inat = isoniazid and thiacetazone; Inh = isoniazid; Kana = kanamycin; nd = not determined; neg = negative; Oflox = ofloxacin; pos = positive; Pza = pyrazinamide; R = drug resistant; Rif = rifampin; S = drug sensitive; Sm = streptomycin; Teri = terizidone; thia = thiacetazone; U = unknown; + = mutation present; − = mutation absent; *internal positive PCR control (Rv3875).

was 92 to 100% throughout treatment) but rather due to treatment with less effective second-line drugs. A similar result was observed for Patient 4 (Table 2).

The analysis of the combined data (clinical, phenotypic drug susceptibility test, mutation detection analysis, IS*6110* DNA fingerprinting analysis, and strain-specific PCR amplification) allowed most observations to be resolved; however, some results remained discordant. In all of these instances, the phenotypic drug-susceptibility test data were in conflict with the results of mutation analysis. In three cases, the culture-based phenotyping method failed to detect the presence of the drug-resistant strain, despite this strain being overrepresented in these cultures (Table 2, Patients 3, *lanes B* and *H*, 4, *lane F*, and 7, *lane C*). This result demonstrates poor-quality drug-susceptibility testing. In the remaining two cases, the mutation analysis failed to detect the presence of the drug-resistant strain, probably as a result of the preferential amplification of the overrepresented genomic locus of the drug-susceptible strain in comparison to the underrepresented genomic locus in the underlying drug-resistant strain (Table 2, Patients 6, *lane A*, and 8, *lane D*).

DISCUSSION

The use of the standard IS*6110* DNA fingerprinting method has provided insight into the relative importance of recent infection and reactivation (24, 25) and has established reinfection as a mechanism leading to the recurrence of tuberculosis (3, 5, 9). DNA fingerprinting has also been used to gain insight into the mechanisms resulting in changing drug-susceptibility patterns during the course of disease (17, 26, 27). These studies have demonstrated that reinfection, before or during therapy, can be a mechanism leading to the development of drug resistance. Using DNA fingerprinting as the gold standard, our study supports reinfection as a mechanism leading to changing drug-susceptibility patterns.

Our PCR-based strain-typing method, challenged, in certain instances, the validity of the interpretation based on the DNA fingerprinting data. The only patient with confirmed reinfection by both methods was coinfected with HIV and therefore was probably unable to resist reinfection with a drug-resistant strain despite receiving therapy for the drug-susceptible tuberculosis (4). The PCR-based strain-typing method contested the DNA

TABLE 2. SUMMARY OF THE PHENOTYPIC AND GENOTYPIC CHARACTERISTICS OF SPUTUM CULTURES FROM PATIENTS 3 TO 8

	Patient 3 (HIV neg)													Patient 4 (HIV neg)							
	A	B	C	D	E	F	G	H	I	J	K	L	M	A	B	C	D	E	F	G	H
Day	U	25	20	16	29	05	09	31	29	04	15	05	14	U	14	09	20	21	28	05	08
Month	11	05	07	08	09	10	11	01	02	04	10	02	08	11	04	06	06	07	07	01	06
Year	94	95	95	95	95	95	95	96	96	96	96	98	98	93	94	94	94	94	94	95	95
Susceptibility																					
Isoniazid	nd	R	R	R	R	R	R	S	R	R	S	R	R	R	R	S	R	R	S	R	R
Rifampin	nd	S	R	R	R	R	R	S	R	R	S	R	R	R	R	S	R	R	S	R	R
Treatment																					
Rifafour (Inh, Rif, Pza, Emb)	U	X	X	X	X	X															
Rifater (Inh, Rif, Pza)														X							
Emb, Eth, Inat, Kana							X	X	X	X	X	X	X								
Emb, Eth, Inat, Kana, Teri															X						
Eth, Inat, Kana, Teri,																X	X	X	X		
Emb, Eth Inat,																				X	
Emb, Eth, Inat, Teri, Pza																					X
Adherence, %	U	87	79	90	90	95	99	71	79	53	49	D	U		83	80	82	100	100	68	57
Point mutations																					
*katG*315	nd	+	+	+	+	+	+	+	+	+	−	+	+	nd	+	−	+	+	+	+	+
*rpoB*531														nd	+	−	+	+	+	+	+
*rpoB*516	nd	+	+	+	+	+	+	+	+	+	−	+	+								
Strain designation																					
(DNA fingerprinting)	nd	b	b	b	b	b	b	b	b	b	e	b	b	nd	d	f	d	d	d	d	d
Strain population																					
present (PCR)	nd	b	b	b	b	b	b + e	b	b	b + e	b + e	b	b	nd	d + f	f	d	d + f	d	d + f	d

	Patient 5 (HIV nd)				Patient 6 (HIV nd)			Patient 7 (HIV pos)						Patient 8 (HIV nd)			
	A	B	C	D	A	B	C	A	B	C	D	E	F	A	B	C	D
Day	03	26	05	05	17	23	10	01	02	07	15	15	21	19	24	24	05
Month	06	11	01	01	06	09	11	07	07	12	12	12	12	02	02	02	03
Year	93	93	95	95	93	93	93	98	98	98	98	98	98	98	98	98	99
Susceptibility																	
Isoniazid	S	nd	R	R	R	R	R	nd	nd	S	R	R	R	nd	R	R	R
Rifampin	S	nd	R	R	R	R	R	nd	nd	S	R	R	R	nd	R	R	R
Treatment																	
Rifater (Inh, Rif, Pza)	X				X	X	X										
Emb, Eth, Kana, Inat, Oflox, Sm, Teri, Pza		X	X	X													
Rifinah (Inh, Rif)									X	X	X	X	X				
Emb, Pyrifin (Inh, Rif, Pza)															X	X	
Emb, Eth, Sm, Inat, Pza																	X
Adherence, %		U	78	78		97	U			95	85	85	100			38	D
Point mutations																	
*KatG*315	−	+	+	+	−	+	+	−	−	+	+	+	+	+	+	+	−
*rpoB*531	−	+	+	+	−	+	+	−	−	+	+	+	+	+	+	+	−
Strain designation																	
(DNA fingerprinting)	g	d	d	d	h	b	b	e	e	d	d	d	d	c	c	c	e
Strain population																	
present (PCR)	g + d	g + d	g + d	g + d	b + h	b	b + h	e	e	d + e	d + e	d + e	d + e	c	c + e	c	c + e

Definition of abbreviations: D = default (stopped therapy for a period of > 2 months); Emb = ethambutol; Eth = ethionamide; Inat = isoniazid and thiacetazone; Inh = isoniazid; Kana = kanamycin; nd = not determined; neg = negative; Oflox = ofloxacin; PCR = polymerase chain reaction; pos = positive; Pza = pyrazinamide; R = drug resistant; Rif = rifampin; S = drug sensitive; Sm = streptomycin; Teri = terizidone; U = unknown; + = mutation present; − = mutation absent.

For complete dataset, *see* online supplement (Figure E1).

fingerprinting classification of reinfection in certain cases by demonstrating the presence of undetected drug-resistant strains (not detected by drug-susceptibility testing or DNA fingerprinting) in the initial sputum culture. It is unlikely that these results reflect cross-contamination because the strains causing mixed infection were found to be present in multiple sputum cultures taken from these patients on different occasions. Only one isolate from one patient was classified as cross-contamination. This level of cross-contamination is similar to the 3.8% previously described for this laboratory (19).

We present new data to support three mechanisms whereby mixed infections can lead to changing drug-susceptibility patterns during therapy. We suggest first that, during the initial treatment period, the first-line antibiotics reduced the drug-susceptible strain population, while allowing the drug-resistant strain population to grow, thereby converting the patient from an apparently drug-susceptible tuberculosis case to an MDR tuberculosis case. We propose that this represents a mechanism of selection through antibiotic pressure.

Second, when the antibiotic pressure was removed by poor

TABLE 3. CHARACTERISTICS OF PATIENTS DIAGNOSED WITH MULTI-DRUG-RESISTANT TUBERCULOSIS BETWEEN 1993 AND 1998

	Single DNA Fingerprint, % (*n* = 37)	Multiple DNA Fingerprints, %* (*n* = 10)	p Value
Male	51	50	1.00
Mean age, yr	30.8	36.8	0.22
Disease classification			
Pulmonary TB	95	100	1.00
Primary TB	5	0	
Treatment history			
New	51	40	0.72
Retreatment	49	60	
HIV status			
Negative	46	50	0.08
Positive	0	10	
Unknown	54	40	
Smear at diagnosis			
Positive	54	80	0.26
Negative	35	20	
Unknown	11	0	
Outcome			
Culture negative	59	60	0.68
Culture positive	22	10	
Death	14	20	
Unknown	5	10	

Definition of abbreviation: TB = tuberculosis.

* One patient for whom the sputum culture was identified as laboratory error by strain-specific polymerase chain reaction method was omitted from this group.

adherence or default, the underlying drug-susceptible strain population reemerged as the dominant population. This suggests a mechanism of selection in the absence of antibiotic pressure. The reason for the observed "overgrowth" by the drug-susceptible population remains unknown. We propose that this could reflect a difference in the level of "fitness" between the drug-susceptible and drug-resistant populations present in these patients. Similar observations were made when drug-susceptible and drug-resistant populations were cultured *in vitro* and in macrophage cell lines (28, 29). The reemergence of the drug-susceptible population demonstrates that the initial period of therapy in these patients was insufficient to enable complete sterilization. This supports the need to ensure adherence over the full course of therapy.

Third, we observed the reemergence of the underlying drug-susceptible strain population when the antibiotic pressure was changed by the introduction of second-line therapy. It is well known that second-line antibiotics have lower bactericidal activities when compared with first-line antibiotics and therefore we propose a mechanism of selection due to a reduced antibiotic pressure. This study supports the suggestion by Post and coworkers (30) that the treatment of patients with MDR tuberculosis may require antibiotics which target both drug-susceptible and drug-resistant subpopulations.

Comparison of the clinical parameters between patients infected with a single strain or multiple strains did not identify significant differences. However, because this study was not designed to detect a difference between these patient groups and precludes the period of disease before diagnosis, we cannot make definite conclusions on the influence of mixed infection on the disease presentation, disease progression, or treatment outcome. Most important, this study demonstrates the inability of routine culture-based drug-susceptibility methods to identify the presence of underlying drug-resistant strains, which is of concern for the patient and their contacts. Diagnostic delays could prolong the implementation of an appropriate treatment regimen and thereby extend the window of opportunity for transmission. Furthermore, inappropriate therapy during the period in which drug-susceptibility testing is being done could enhance the risk of the drug-resistant strain acquiring additional resistance mutations.

We acknowledge that the conclusions drawn from this study are limited by the small number of patients, although, with this study, we did not aim to provide a measure of the frequency of these processes but rather to demonstrate the mechanisms and the fact that they are able to occur in an epidemic setting. It is, however, warranted that our hypothesis generated on the basis of a limited number of observations be confirmed in different geographic settings.

In conclusion, this study describes the first molecular analysis of the population structure of drug-resistant and drug-susceptible *M. tuberculosis* strains in serial sputum cultures during the course of MDR tuberculosis. We have demonstrated that mixed infection is an important mechanism underlying changing drug-susceptibility patterns in a high-incidence region. Drug-susceptibility patterns change through the presence or absence of antibiotic pressure, which determines the dominant growth of the coinfecting strain. The inability to accurately determine resistance patterns in cases of mixed infection may exacerbate delays in the diagnosis of drug-resistant tuberculosis, which could have implications for the individual patient and the spread of drug-resistant strains.

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