MAJOR ARTICLE

Use of Molecular Techniques to Distinguish between Treatment Failure and Exogenous Reinfection with *Mycobacterium tuberculosis*

Annika Krüüner,[1,3,4] Lea Pehme,[2] Solomon Ghebremichael,[3] Tuija Koivula,[3] Sven E. Hoffner,[3] and Marika Mikelsaar[1]

[1]Institute of Microbiology and [2]Department of Pulmonary Medicine, Tartu University, Estonia; and [3]Swedish Institute for Infectious Disease Control and [4]Microbiology and Tumor Biology Center, Karolinska Institute, Stockholm, Sweden

We investigated the means by which drug resistance emerges among drug-susceptible *Mycobacterium tuberculosis* strains during antituberculosis therapy. Patients who experienced failure of treatment for active pulmonary tuberculosis, who initially received diagnoses of infection with drug-susceptible *M. tuberculosis,* and who had had at least 3 isolates tested for drug susceptibility were selected from a 6-year period in the Estonian National Reference Laboratory archive. Eleven patients from whom 35 sequential isolates of *M. tuberculosis* had been obtained were recruited into the study. Their clinical data and treatment charts were analyzed and correlated with drug-susceptibility patterns and IS*6110* restriction fragment–length polymorphism (RFLP) profiles. Six patients excreted isogenic drug-susceptible *M. tuberculosis* strains, whereas, in the other 5 patients, the isolated strain shifted from a susceptible to a resistant phenotype. In all cases, this shift correlated to a shift in RFLP pattern, which showed reinfection with a new strain. Exogenous reinfection with drug-resistant *M. tuberculosis* may be misinterpreted as the emergence of drug resistance if molecular testing techniques are not used.

Mycobacterium tuberculosis is a successful pathogen in humans that infected approximately one-third of the world's population in 1993. Despite the effectiveness of antimicrobial chemotherapy, tuberculosis (TB) remains a leading infectious cause of death worldwide [1, 2]. The long survival of *M. tuberculosis* in the host mainly has been associated with the ineffectiveness of the immune system, particularly of macrophages, to clear the infection [3]. On the other hand, the emergence of resistance to anti-TB drugs has been considered another important mechanism by which *M. tuberculosis* adapts to host conditions when the host is receiving antimicrobial treatment. *M. tuberculosis* strains resistant to anti-TB drugs were found in all 58 geographic sites studied by the World Health Organization (WHO) between 1996 and 1999. The median prevalence of multidrug resistance among new cases of TB was 1.0%, but the prevalence in Estonia, one of the former Soviet republics, was much higher (14.1%) [4]. The highly irregular use of anti-TB treatment in Estonia, which does not correspond to the WHO's global treatment strategy, has been associated with high rates of multidrug-resistant (MDR) *M. tuberculosis* (i.e., *M. tuberculosis* that is resistant to at least isoniazid and rifampin) [4–9].

However, the role of inappropriate chemotherapy in failure to cure TB has, to our knowledge, not yet been proved by direct studies that assess the emergence of drug resistance during therapy for infection with drug-susceptible *M. tuberculosis.* Molecular testing methods could provide good tools [10–12] for investigating

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Reprints or correspondence: Dr. Annika Krüüner, Dept. of Microbiology, Tartu University, Ravila 19, 50411 Tartu, Estonia (annika.kruuner@kliinikum.ee).

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whether persistent TB is caused by genotypically isogenic strains.

The well-standardized and most widely applied molecular method for the study of *M. tuberculosis* complex isolates is IS*6110* restriction fragment–length polymorphism (RFLP) typing [13–15]. Likewise, for distinguishing between strains of *M. tuberculosis,* spoligotyping [16] has been successfully applied in addition to IS*6110* RFLP typing. Regardless of the molecular techniques and genetic markers available, few investigators have focused specifically on the simultaneous use of bacteriological, molecular/genetic, and clinical investigation methods to study the relationship between the quality of treatment and the development of resistance [17].

To investigate the means by which drug resistance evolves among drug-susceptible *M. tuberculosis* strains during anti-TB treatment, a retrospective case series including patients with TB who had experienced failure of treatment for active pulmonary TB and who initially had received diagnoses of infection with drug-susceptible *M. tuberculosis* were selected from the Estonian National Reference Laboratory (ENRL) archive for our study. The IS*6110* RFLP assay and spoligotyping were used, and the results were correlated with drug-susceptibility test results and treatment regimen and sociodemographic data.

PATIENTS, MATERIALS, AND METHODS

Patients. From January 1994 through December 1999, 1037 patients in Tartu, Estonia, with culture-confirmed pulmonary TB were identified by the ENRL. During the 6-year period, 194 sequential isolates from 86 patients were tested for drug susceptibility. In that study, the following inclusion criteria were set for the patients: initial diagnosis of pulmonary infection with drug-susceptible *M. tuberculosis,* testing of at least 3 sequential isolates for drug susceptibility, and an interval of ⩾2 months between testing of the first and second or the second and third isolates. Thirty-one patients had testing of at least 3 serial isolates for drug susceptibility. For 27 of those patients, the interval between testing of the first and second or second and third isolates was ⩾2 months; the first isolate from 14 of the 27 patients was susceptible to the drug tested. The first isolate from 2 of those 14 patients was not available for additional (molecular) testing, and for 1 patient, 2 sequential isolates were not genotyped. Therefore, 11 (78.6%) of 14 patients and 35 *M. tuberculosis* isolates were included for further analyses (table 1, figure 1).

Clinical data. The WHO-recommended strategy of TB control, which includes directly observed treatment and short-course chemotherapy, was applied in Estonia only after 1997. To document the regularity (e.g., use of a standard treatment regimen and dosages and level of patient compliance) versus irregularity of the anti-TB treatment applied, we searched patient treatment charts and outpatient cards for the following clinical data: duration of the current TB episode, duration of each course of treatment and total treatment duration, conditions under which treatment was administered (i.e., hospital, ambulatory setting, direct observation), number and length of treatment interruptions, and the drugs included in each regimen. Also, the clinical outcome of the treatment was recorded. In addition, some unfavorable sociodemographic factors—unemployment, alcohol abuse, and prior imprisonment—were used for analyses.

M. tuberculosis *isolates.* All available samples that we analyzed were kept frozen at −70°C before additional testing. Clinical isolates were identified as *M. tuberculosis* by use of a DNA probe (ACCUProbe; GenProbe). Isolates were tested for susceptibility to rifampin (breakpoint, 2.0 μg/mL), isoniazid (breakpoint, 0.1 μg/mL), streptomycin (breakpoint, 6 μg/mL), and ethambutol (breakpoint, 7.5 μg/mL) by the Bactec radiometric method (Becton Dickinson Diagnostic Instrument Systems). Resistance was judged by comparison of the change in the growth index of the control with that of the test drugs, as recommended by the manufacturer [18].

Molecular techniques. IS*6110* RFLP and spoligotyping were used to assess the stability of serial isolates. For IS*6110* RFLP, *M. tuberculosis* isolates were subcultured onto Löwenstein-Jensen slopes at 37°C for a minimum of 4 weeks before DNA extraction. Chromosomal DNA was prepared by chloroform–isoamyl alcohol DNA extraction, and 4.5 μg of DNA from each isolate was restricted with *Pvu*II. Separation of *Pvu*II-restricted DNA by electrophoresis, Southern blot hybridization with a 245-bp PCR probe that recognized the right side of the restricted IS*6110,* and chemiluminescence detection was performed according to the standard method recommended for the DNA fingerprinting of *M. tuberculosis* [19].

Spoligotyping is used to detect the 43 known spacer sequences that intersperse the directly repeated sequences in the genomic direct repeat region. The spoligotyping method, which has a lower level of discrimination than does IS*6110* RFLP typing [20], was used as an additional tool for determining relationships among the serial isolates. Altogether, 20 isolates (preferably 2 isolates from each patient, the first isolate and 1 of the serial isolates) were selected for spoligotyping. The procedure was performed according to standard protocols [16].

RFLP profiles were analyzed by means of a computer program (Gelcompar, version 4.1; Applied Maths) by use of the Dice coefficient for similarity calculations and the unweighted pair-group method with arithmetic averages for clustering, as described elsewhere [21]. IS*6110* RFLP fingerprinting and spoligotyping were done at the Swedish Institute for Infectious Disease Control in Stockholm.

Statistical analyses. Data analysis was performed by use of the SigmaStat program for Windows, version 2.0 (Jandel).

Table 1. Drug susceptibility patterns of *Mycobacterium tuberculosis* isolates from sequential sputum samples from patients with tuberculosis.

Group, patient, isolate	Culture date	RFLP pattern	Drug resistance status[a]
1			
1			
3291/95	31 Oct 1995	nB1	Susceptible
2868/97[b]	24 Sep 1997	ND	Susceptible
822/98	19 Feb 1998	nB1	Susceptible
2			
3329/96	10 Dec 1996	nB2	Susceptible
3806/97	25 Nov 1997	nB2	Susceptible
3967/98	07 Oct 1998	nB2	Susceptible
3			
253Võ/97	10 Apr 1997	nB3	Susceptible
4162/97	16 Dec 1997	nB3	Susceptible
784/98	17 Feb 1998	nB3	Susceptible
4			
3711/95	05 Nov 1995	B1	Susceptible
447/97	13 Feb 1997	B1	Susceptible
1001/98	05 Mar 1998	B1	Susceptible
722/99	18 Feb 1999	B1	Susceptible
5			
413/96	08 Feb 1996	nB4	Susceptible
2230/97	22 Jul 1997	nB4	Susceptible
2778/98	02 Jul 1998	nB4	Susceptible
6			
1210/96	17 Apr 1996	nB5	Susceptible
1676/97	22 May 1997	nB5	Susceptible
3203/98	06 Aug 1998	nB5	Susceptible
2125/99	25 May 1999	nB5	Susceptible
2			
1			
2013/94	19 May 1994	nB6	Susceptible
2494/95	29 Nov 1995	nB6	Susceptible
3325/97	28 Oct 1997	B2	Resistant to S, H, R, and E
2			
1349/97	25 Apr 1997	nB7	Susceptible
2050/98	10 May 1998	B3	Resistant to S, H, R, and E
3783/99	06 Oct 1999	B3	Resistant to S, H, and R

(continued)

Subject variables were examined by use of the χ^2 test of association for categorical variables and the *t* test for continuous variables. $P < .05$ was considered to be statistically significant.

RESULTS

Sociodemographic and clinical data. The 11 patients with TB could be divided into 2 groups. Group 1 included 6 patients with TB that was characterized by continuous excretion of drug-susceptible *M. tuberculosis* isolates for >10 months, despite anti-TB treatment. Group 2 included 5 patients with TB characterized by continuous excretion of *M. tuberculosis* isolates and a shift from susceptible to resistant strains (table 1). The majority of patients in both groups were male (5 of 6 in group 1 and 4 of 5 in group 2). The mean age was 47 years (range, 36–67 years) in group 1 and 43 years (range, 29–66 years) in group 2 ($P = .5$). The majority of patients (all 6 patients in group 1 and 3 patients in group 2) were alcohol abusers ($P = .1$). In addition,

Table 1. ***(Continued.)***

Group, patient, isolate	Culture date	RFLP pattern	Drug resistance status[a]
3			
3958/94	07 Nov 1994	nB8	Susceptible
2712/96	10 May 1996	B2	Resistant to S, H, R, and E
3319/97	28 Oct 1997	B2	Resistant to S, H, R, and E
4			
3001/96	11 Nov 1996	nB9	Susceptible
2276/97	28 Jul 1997	nB9	Susceptible
4190/97	17 Dec 1997	B4	Resistant to S, H, R, and E
5			
3599/94	13 Oct 1994	B5[c]	Susceptible
2875/98	08 Jul 1998	B2	Resistant to S, H, R, and E
4925/99	21 Dec 1999	B2	Resistant to S, H, R, and E

NOTE. B, Beijing genotype; E, ethambutol; H, isoniazid; nB, non-Beijing genotype; RFLP, restriction fragment–length polymorphism; R, rifampin; S, streptomycin.

[a] "Susceptible" indicates that the isolate was susceptible to H, R, S, and E.

[b] Isolate was not available for RFLP typing.

[c] Isolate was not available for spoligotyping.

4 patients in group 1 and all 5 patients in group 2 were unemployed ($P = .4$). In both groups, 1 patient had previously been imprisoned. With respect to the manifestation of TB and a history of TB, the patients in the 2 groups did not differ ($P = .5$ and $P = 1.0$, respectively) (table 2).

Drug regimens, duration of therapy, and outcome for patients. Patients in both groups received initial daily therapy in hospitals. For group 1, the median duration of treatment was 45 days (range, 18–63 days); in group 2, it was 38 days (range, 28–52 days) ($P = .3$; table 2). The duration of the TB episode varied from 19 to 60 months (mean, 37.4 months) for group 1 and from 14 to 66 months (mean, 42.9 months) for group 2 ($P = .5$). There was no difference in the treatment duration between the groups (range, 5.2–19.3 months, and median, 7.6 months for group 1; range, 3.0–36.9 months, and median, 24.5 months, for group 2; $P = .2$) (table 3).

Treatment regimens were regularly changed in both groups. Therapy was changed 3–9 times (median, 4 changes) for patients in group 1 and 6–17 times (median, 7 changes) for patients in group 2 ($P = .1$). No standard regimens were applied during the therapy. In both groups, the daily doses of drugs used in the treatment regimens were consistent with WHO recommendations (table 3). The number of treatment interruptions of >2 months during the therapy was similar in both groups (range, 2–5 interruptions, and mean, 2.5 interruptions for patients in group 1; range, 2–5 interruptions, and mean, 3.2 interruptions for patients in group 2; $P = .3$); a treatment interruption of >2 months was considered to indicate poor compliance.

The treatment outcome did not differ statistically significantly between group 1 and group 2. Three patients in group 1 were cured, and 3 died. One patient in group 2 was cured, 3 patients died, and treatment was stopped for 1 patient because of a lack of active drugs remaining in the regimen (the isolate from that patient demonstrated resistance to 9 drugs).

Bacterial isolates. The interval between the obtaining of samples for the first and the last isolate from each patient varied from 313 to 1201 days (mean, 828 days) for group 1 and from 370 to 1850 days (mean, 1091 days) for group 2 ($P = .3$) (figure 1). Twenty-six *M. tuberculosis* isolates obtained from 11 patients with TB were susceptible to rifampin, isoniazid, streptomycin, and ethambutol, and 8 *M. tuberculosis* isolates from 5 patients were MDR (i.e., resistant to at least isoniazid and rifampin) (table 1).

Within group 1, 5 patients had a follow-up isolate with an IS*6110* RFLP pattern that was identical to the pattern of the first isolate (figure 2). One patient had a second isolate with an RFLP pattern that slightly differed from that of the first isolate (1-band difference; loss of 1 band), whereas a third isolate from that patient was indistinguishable from the first. In group 2, 5 patients had follow-up isolates with RFLP patterns that clearly differed from the pattern of the first isolate, and, in 2 cases, the patterns of the first and second isolates differed from that of the third. In all such cases, these changes have been considered, after the possibility for laboratory cross-contamination was ruled out, to reflect the appearance of a new *M. tuberculosis* strain. In addition, 3 of the MDR *M. tuberculosis* isolates obtained from patients in group 2 (patients 1, 3, and 5) had the same RFLP pattern.

Twenty *M. tuberculosis* isolates were characterized by spoli-

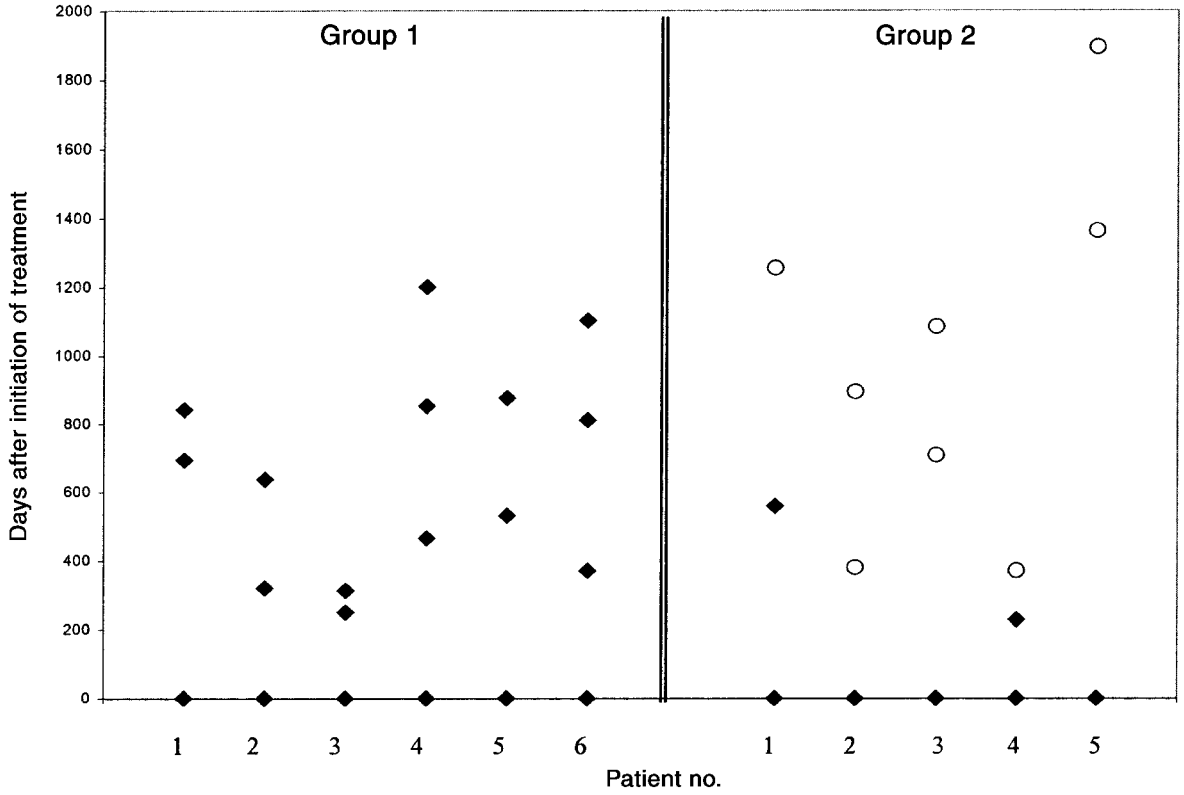

Figure 1. Time intervals between sequential isolates obtained from patients with tuberculosis in groups 1 and 2. *Diamonds,* drug-susceptible isolates of *Mycobacterium tuberculosis; circles,* multidrug-resistant *M. tuberculosis* isolates with the Beijing genotype.

gotyping. In group 1, the spoligotypes of isolates obtained from the same patient did not vary during the study period. In all patients in group 2, the spoligotypes of the first and second isolates differed. All MDR *M. tuberculosis* isolates identified in group 2 had an identical spoligotype, one that belongs to the Beijing genotype [22]. The interval between the point at which the drug-susceptible *M. tuberculosis* isolates and the point at which MDR isolates were recovered from samples varied from 142 to 1364 days (mean, 617 days).

DISCUSSION

On the basis of our investigation of sequential isolates obtained from patients who experienced failure of treatment for active pulmonary TB, we propose that highly irregular treatment and unfavorable sociodemographic factors do not always lead to the emergence of drug resistance in drug-susceptible *M. tuberculosis* strains, even during a long treatment period. We have followed up 11 patients selected from patients in the ENRL TB register whose treatment failed during a 6-year period. The 11 patients were similar in most respects; they had similar sociodemographic status and met a full scale of preconditions, such as having had no standard regimens administered as therapy, having had no supervision of drug administration, and having exhibited poor treatment compliance. We found that 6 patients excreted isogenic drug-susceptible *M. tuberculosis* strains for >10 months. In contrast, in 5 patients, a strain shift occurred concurrently with the appearance of multidrug resistance.

Treatment failure is defined by the WHO as the maintenance of or reversion to positive results of a smear and/or culture ⩾5 months after the commencement of treatment in a patient receiving treatment or conversion from negative results before initiation of treatment to positive results of a smear and/or a culture after the second month of treatment [23]. Therefore, testing of specimens from patients with TB before initiation of treatment (sample 1) and at the end of the second (sample 2) and fifth (sample 3) months of treatment is recommended. We aimed to obtain at least 3 sequential isolates from the same TB episode from each patient and to maintain an interval between isolates of ⩾2 months. In our TB laboratory register, during the 6-year period, there were 14 patients who fulfilled all inclusion criteria (i.e., initial diagnosis of TB caused by drug-susceptible organism, testing of at least 3 sequential isolates for drug susceptibility, and an interval between the isolates of ⩾2 months). Out of all possible candidates, a clear majority (close to 80%) were recruited into our study.

In the present study, the standard and most widely applied

Table 2. Sociodemographic and clinical data for patients with tuberculosis (TB) who met the study inclusion criteria.

Group, patient	Age, years/sex	Risk factor for TB infection			Manifestation of TB		Previous history of TB	Treatment initiated at hospital	Duration of first hospitalization, days	No. of drugs in initial therapeutic regimen	Inclusion of isoniazid and rifampin in initial therapy
		Unemployment	Alcohol abuse	Prior imprisonment	Pulmonary	Extrapulmonary					
1											
1	44/M	Yes	Yes	No	Yes	Yes; pleural	No	Yes	63	2	No
2	36/M	Yes	Yes	No	Yes	No	No	Yes	47	4	Yes
3	50/F	Yes	Yes	No	Yes	No	No	Yes	45	3	Yes
4	47/M	Yes	Yes	Yes	Yes	No	Yes	Yes	42	2	No
5	38/M	No	Yes	No	Yes	Yes; pleural	No	Yes	56	4	Yes
6	67/M	No	Yes	No	Yes	Yes; pleural	No	Yes	18	4	Yes
2											
1	56/M	Yes	No	No	Yes	No	No	Yes	28	2	No
2	29/F	Yes	No	No	Yes	No	No	Yes	27	3	Yes
3	45/M	Yes	Yes	No	Yes	No	No	Yes	52	3	Yes
4	44/M	Yes	Yes	Yes	Yes	Yes; pleural	Yes	Yes	50	4	Yes
5	40/M	Yes	Yes	No	Yes	No	No	Yes	31	4	Yes

Table 3. Treatment regimens for 11 patients with tuberculosis (TB).

Group, patient	Duration of TB episode, months	Duration of treatment, months	Treatment regimen (duration in months)[a]	Daily doses of drugs used in the treatment regimens, mg					
				R	H	S	Z	E	K
1									
1	38.3	13.4	HE (2.1)[b]/HZE (1.7)/HZEK (2.0)[c]/ZEK (0.1)/ZEKR (0.3)/ZK (0.5)/ZE (1.7)[b]/ZE (3.3)[c]/HZE (0.9)[c]/RZE (0.8)	450/600	300/450	—	1600/2000	1200/1600/2000	500
2	30	5.5	HRZS (1.6)/RZ (0.2)[c]/HRZS (1.0)[c]/HRZES (2.7)	450/600	300	1000	1600/2000	1600	—
3	19	5.2	HRS (0.2)/HRZS (1.5)/HRZE (0.5)[c]/HRZE (1.4)[c]/HRZE (1.6)	450/600	300	1000	1000/1600/2000	1200/2000	—
4	60	>9.3	ZE (1.4)/HZE (ND)[c]/HZE (ND)[c]/HRZE (7.9)	600	300	—	1200/2000	1200/1600	—
5	39.9	19.3	HRZS (2.0)[c]/ZE (2.0)[c]/RZE (0.2)/HRZS (1.4)/HRZE (2.0)[c]/HRZ (1.0)[c]/ HRZEK (3.2)/HRZE (7.5)	450/600	300/600	1000	1500/1600/2000	800/1500/1600	1000
6	38.1	5.9	HRZE (0.6)/RZE (2.5)[c]/HRZS (1.1)[b]/HRZS (1.2)[c]/HRZES (0.5)	450/600	300	500/1000	1500/1600/2000	1200/1600	—
2									
1[d]	52.9	36.9	RZ (0.6)/HRZ (0.9)/HZ (0.3)/HZE (0.3)/Z (0.3)/ZE (1.5)/ZE (7.6)/HZE (0.1)/ZE (3.2)/HE (0.7)/HZE (3.0)[c]/HZ (2.0)[c]/HRZ (3.9)/HRZE (1.7)/ HRZ (0.5)/HRZE (0.9)/ZE (1.2)/ZE (8.2)	600	300/600	—	450/1000/1200/1500/2000	800/1200/1600/	—
2[d]	44	34.3	HRZS (1.8)/HRZE (2.4)[c]/HRZE (2.0)/HRZE (2.3)[c]/HZ (4.0)/HZ (9.0)/E (1.7)[c]/HZES (4.4)/ZE (6.7)	600	300	1000	2000	1600/2000	—
3	38.5	12.4	HRZS (1.7)[c]/HRZ (1.9)/RZ (0.3)[c]/HRE (0.3)[c]/HZE (2.0)[b]/HZE (1.0)[c]/ HRZE (1.9)[c]/ZE (3.3)	600	300/600	1000	1200/1500/1600/2000	1200/1600/2000	—
4	13.8	3.0	HRZS (0.4)/RZES (0.4)/HRZE (0.2)/HRZES (1.2)/RZE (0.3)[c]/HRZE (0.5)	600	300	1000	2000	1200/1600	—
5[d]	65.6	25.1	HRZS (1.1)[c]/HRE (9.0)/HE (3.7)[c]/HRZE (1.3)/HRZ (0.8)/ZEK (2.8)/ZEK (1.7)[c]/HRZES (2.8)[c]/HZE (1.9)	600	300	1000	1000/1200/2000	1200/1600	1000

NOTE. E, ethambutol; H, isoniazid; K, kanamycin; ND, not documented; R, rifampin; S, streptomycin; Z, pyrazinamide.

[a] Each change in treatment is marked by a slash (/).

[b] Treatment was stopped for >1 month.

[c] Treatment was stopped for >2 months.

[d] Second-line drugs were used during the course of treatment but are not included here.

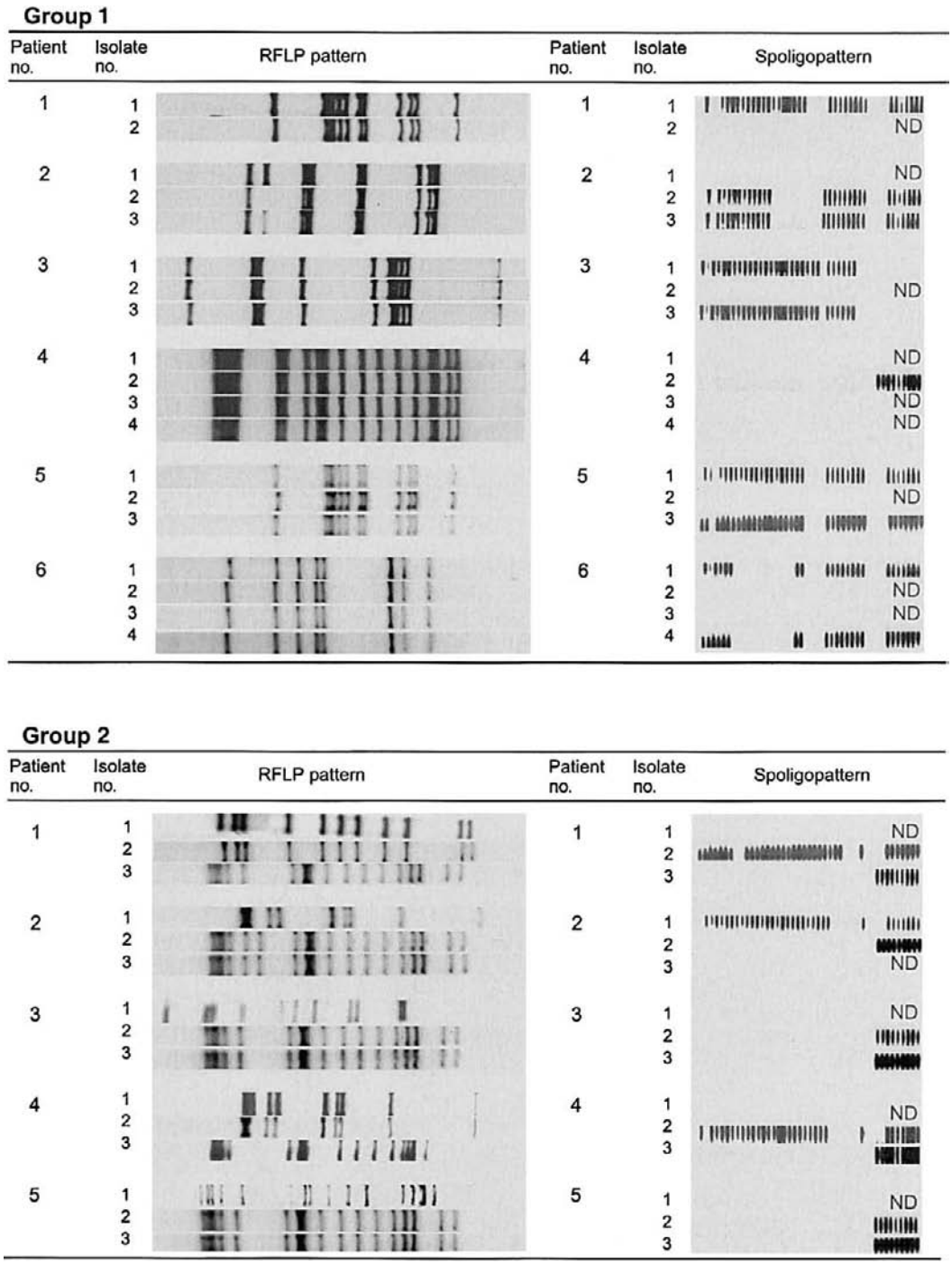

Figure 2. DNA fingerprints of *Mycobacterium tuberculosis* isolates related to patient groups 1 and 2. ND, not determined; RFLP, restriction fragment–length polymorphism.

molecular technique for distinguishing between strains in the *M. tuberculosis* complex, IS*6110* RFLP typing, which has a high degree of discrimination and stability [20], was used. The most unexpected result was that exogenous reinfection with a new *M. tuberculosis* strain during the same TB episode took place in 5 patients. By combining RFLP with spoligotyping, it was possible to classify these *M. tuberculosis* complex isolates as having the Beijing genotype. Although the spread of a drug-resistant Beijing clone has been followed in wide, community-based studies [24], to the best of our knowledge, a causative association with clinical treatment regimens and failure of such regimens has not been demonstrated.

Different studies have indicated that exogenous reinfection may occur after successful treatment [25–27]. However, limited

data are available with regard to the rate at which superinfection will occur during the same episode of TB. Niemann et al. [28] have described a patient with TB who had double infection with a drug-resistant strain and an MDR strain. In our study, exogenous reinfection with the Beijing family of MDR *M. tuberculosis* was found in 5 immunocompetent patients with pulmonary TB. In addition, in all these cases, the physicians considered the emergence of drug resistance to be a result of unsupervised drug administration, poor patient compliance with therapy, and errors in medical prescriptions of drug regimens. It was demonstrated by molecular techniques that this was not the case.

Treatment of patients with TB who are infected with drug-susceptible *M. tuberculosis* strains has been successful. According to Mitchison and Nunn [29], treatment failures among patients who were infected initially with drug-susceptible bacilli are rare among patients who have been treated with regimens of ⩾3 drugs that include rifampin. In contrast to those findings, our study has demonstrated that viable organisms can persist (as demonstrated by culture positivity during therapy) in sputum for several months or years, despite the demonstration of drug susceptibility in vitro and administration of appropriate doses of drugs for chemotherapy. However, this persistence was associated with marked irregularity of TB therapy.

Mitchison [30] has described the emergence of drug resistance during short-course chemotherapy with multiple drugs, solely due to irregularity in administration of drugs, because of the occurrence of several cycles of killing (when the drugs are taken) and regrowth (when drug-taking stops). During each of these cycles, selection of mutants that are resistant, relative to the susceptible bacterial population, is possible. However, the drug-susceptible *M. tuberculosis* strains obtained from 6 patients in our study did not develop resistance, despite administration of highly irregular therapy. This might be the result of longer intervals during which the treatment was stopped; thereafter, during the regrowth period, the proportion of susceptible bacilli could quickly increase again.

Our findings show that highly irregular treatment and unfavorable sociodemographic factors do not always contribute to the emergence of drug resistance in drug-susceptible *M. tuberculosis,* despite a long treatment period. Regardless, the host did not benefit from the drug susceptibility of infecting *M. tuberculosis;* one-half of our patients died.

The detection of superinfection with a new *M. tuberculosis* strain during the treatment of an episode of active TB is possible only through the use of molecular epidemiology tools. The results obtained by use of these tools make it clear that patients who are treated in >1 hospital setting can become infected with a new and more dangerous strain of *M. tuberculosis* if infectious patients are not isolated; on the other hand, it may also be that these particular strains are extremely virulent. This type of nosocomial spread of TB is obscured by the ability to diagnose TB earlier in the course of the disease, and, therefore, the extent of the problem remains largely unknown.

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References

1. Dolin PJ, Raviglione MC, Kochi A. Global tuberculosis incidence and mortality during 1990–2000. Bull World Health Organ **1994**; 72:213–20.
2. Styblo K, Raviglione MC. Tuberculosis, public health aspects. In: Dulbecco R, ed. Encyclopedia of human biology. 2nd ed. Vol 8. San Diego, CA: Academic Press, **1997**:537–58.
3. Michele TM, Ko C, Bishai WR. Exposure to antibiotics induces expression of the *Mycobacterium tuberculosis sigF* gene: implications for chemotherapy against mycobacterial persistors. Antimicrob Agents Chemother **1999**; 43:218–25.
4. Espinal MA, Laszlo A, Simonsen L, et al. Global trends in resistance to antituberculosis drugs. N Engl J Med **2001**; 344:1294–303.
5. Krüüner A, Sillastu H, Danilovitsh M, et al. Drug resistant tuberculosis in Estonia. Int J Tuberc Lung Dis **1998**; 2:130–3.
6. Lambregts-van Weezenbeek CSB, Veen J. Control of drug-resistant tuberculosis. Tuberc Lung Dis **1995**; 76:455–9.
7. Cohn D, Bustreo F, Raviglione M. Drug resistance in tuberculosis: review of worldwide situation and WHO's global surveillance project. Clin Infect Dis **1997**; 24(Suppl 1):S121–30.
8. Goble M, Iseman MD, Madsen LA, Waite D, Ackerson L, Horsburgh CR. Treatment of 171 patients with pulmonary tuberculosis resistant to isoniazid and rifampicin. N Engl J Med **1993**; 328:527–32.
9. Nunn P, Felten M. Surveillance of resistance to antituberculosis drugs in developing countries. Tuberc Lung Dis **1994**; 75:163–7.
10. Yeh RW, Ponce DL, Agasino CB, et al. Stability of *Mycobacterium tuberculosis* DNA genotypes. J Infect Dis **1998**; 177:1107–11.
11. De Boer AS, Borgdorff MW, de Haas PE, et al. Analysis of rate of change of IS*6110* RFLP patterns of *Mycobacterium tuberculosis* based on serial isolates. J Infect Dis **1999**; 180:1238–44.
12. Cave MD, Eisenach KD, Templeton G, et al. Stability of DNA fingerprint patterns produced with IS*6110* in strains of *Mycobacterium tuberculosis.* J Clin Microbiol **1994**; 32:262–6.
13. Thierry D, Brisson NA, Vincent LF, et al. Characterization of a *Mycobacterium tuberculosis* insertion sequence, IS*6110,* and its application in diagnosis. J Clin Microbiol **1990**; 28:2668–73.
14. Van Embden JD, Cave MD, Crawford JT, et al. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. J Clin Microbiol **1993**; 31:406–9.
15. Van Soolingen D, de Haas PEW, Kremer K. Restriction fragment length polymorphism (RFLP) typing of mycobacteria. Protocol. Bilthoven, The Netherlands: National Institute of Public Health and the Environment, **1999**.
16. Kamerbeek J, Schouls L, Kolk A, et al. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol **1997**; 35:907–14.
17. Niemann S, Rüsh-Gerdes S, Richter E, Thielen H, Heykes-Uden H, Diel R. Stability of IS*6110* restriction fragment length polymorphism patterns of *Mycobacterium tuberculosis* strains in actual chains of transmission. J Clin Microbiol **2000**; 38:2563–7.
18. Siddiqi SH, Libonati JP, Middlebrook G. Evaluation of a rapid radiometric method for drug susceptibility testing of *Mycobacterium tuberculosis.* J Clin Microbiol **1981**; 13:908–13.
19. Kremer K, van Soolingen D, Frothingham R, et al. Comparison of

methods based on different molecular epidemiological markers for typing of *Mycobacterium tuberculosis* complex strains: interlaboratory study of discriminatory power and reproducibility. J Clin Microbiol **1999**; 37:2607–18.
20. Van Soolingen D. Molecular epidemiology of tuberculosis and other mycobacterial infections: main methodologies and achievements. J Intern Med **2001**; 249:1–26.
21. Heersma HF, Kremer K, van Embden JD. Computer analysis of IS*6110* RFLP patterns of *Mycobacterium tuberculosis.* Methods Mol Biol **1998**; 101:395–422.
22. 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**; 33:3234–8.
23. Migliori GB, Raviglione MC, Schaberg T, et al. Tuberculosis management in Europe. Eur Respir J **1999**; 14:978–92.
24. Krüüner A, Hoffner SE, Sillastu H, et al. Spread of drug-resistant pulmonary tuberculosis in Estonia. J Clin Microbiol **2001**; 39:3339–45.
25. Small PM, Shafer RW, Hopewell PC, et al. Exogenous reinfection with multidrug-resistant *Mycobacterium tuberculosis* in patients with advanced HIV infection. N Engl J Med **1993**; 328:1137–44.
26. Van Rie A, Warren R, Richardson M, et al. Exogenous reinfection as a cause of recurrent tuberculosis after curative treatment. N Engl J Med **1999**; 341:1174–9.
27. De Boer AS, van Soolingen D. Recurrent tuberculosis due to exogenous reinfection [letter]. N Engl J Med **2000**; 342:1050–1.
28. Niemann S, Richter E, Rüsch-Gerdes S, et al. Double infection with a resistant and a multidrug-resistant strain of *Mycobacterium tuberculosis.* Emerg Infect Dis **2000**; 6:548–51.
29. Mitchison DA, Nunn AJ. Influence of initial drug resistance on the response to short-course chemotherapy of pulmonary tuberculosis. Am Rev Respir Dis **1986**; 133:423–30.
30. Mitchison DA. How drug resistance emerges as a result of poor compliance during short course chemotherapy for tuberculosis. Int J Tuberc Lung Dis **1998**; 2:10–5.