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Mechanisms of heteroresistance to isoniazid and rifampin of *Mycobacterium tuberculosis* in Tashkent, Uzbekistan

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ABSTRACT: Heteroresistance of *Mycobacterium tuberculosis* (MTB) is defined as the coexistence of susceptible and resistant organisms to anti-tuberculosis (TB) drugs in the same patient. Heteroresistance of MTB is considered a preliminary stage to full resistance. To date, no mechanism causing heteroresistance of MTB has been proven.

Clinical specimens and cultures from 35 TB patients from Tashkent, Uzbekistan, were analysed using the Genotype MTBDR assay (Hain Lifescience, Nehren, Germany), which is designed to detect genetic mutations associated with resistance to rifampin and isoniazid. Cases of heteroresistance were further subjected to genotyping using mycobacterial interspersed repetitive unit-variable-number tandem repeat typing, spoligotyping and IS*6110* fingerprinting.

Heteroresistance to rifampin and/or isoniazid was found in seven cases (20%). In five of them, heteroresistance was caused by two different strains and in two by a single strain of the Beijing genotype. The latter cases had a history of relapse of their TB.

For the first time, two different mechanisms of heteroresistance in tuberculosis have been proven using a stepwise molecular-biological approach: 1) superinfection with two different strains, which is of interest for clinical infection control practitioners; and 2) splitting of a single strain into susceptible and resistant organisms. The latter mechanism is most likely to be related to poor treatment quality and could serve as a quality marker for tuberculosis therapy programmes in the future.

KEYWORDS: Beijing genotype, Genotype MTBDR, heteroresistance, MIRU-VNTR (mycobacterial interspersed repetitive unit-variable-number tandem repeat) typing, multidrug resistance, tuberculosis

Multidrug resistance (MDR) epitomises the increasing health problem of tuberculosis (TB) in the world. According to the fourth report on the Global Project on Anti-Tuberculosis Drug Resistance Surveillance [1], the world's highest rate of MDR-TB (60%) was observed in Tashkent, Uzbekistan.

MDR-TB is defined by resistance of the *Mycobacterium tuberculosis* (MTB) complex to at least isoniazid (INH) and rifampin (RMP). The majority of resistance to INH is caused by a mutation at codon 315 (S315T) of the *katG* gene [2]. Over 95% of cases of resistance to RMP are determined by one or more mutations in an 81-bp core region of the *rpoB* gene [2, 3].

Some TB patients harbour mixed populations of MTB organisms with or without resistance, a phenomenon which is referred to as heteroresistance [4]. Previous studies suggest that the relevance of heteroresistance in TB is highly underestimated [4]. To date, heteroresistance has been described for INH, RMP, ethambutol and streptomycin. It is detected using conventional drug susceptibility testing (DST) of several subcultures [5], or by simultaneous detection of wild-type (WT) and mutated sequences using PCR-based techniques, such as restriction fragment length polymorphism [6], sequencing [7] or ''line probe assays'' [8].

Heteroresistance of MTB is considered a preliminary stage to full resistance. Studies addressing the mechanisms underlying heteroresistance in TB are lacking so far. The aim of the present study was to analyse systematically the causes of heteroresistance to INH and RMP. The Genotype MTBDR assay (Hain Lifescience, Nehren, Germany) was used to identify such cases among TB patients of the directly observed treatment strategy (DOTS) centre in Tashkent, Uzbekistan.

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MATERIALS AND METHODS

Clinical specimens

Sputa of 35 pulmonary TB patients attending the DOTS centre in Tashkent were collected for the present study. These were sent to the supranational reference laboratory (SNRL) in Gauting, Germany, for DST in the frame of the Drug Resistance Survey in the city. The sputa comprised consecutive samples which were processed on February 10 (cases 1169–1185), February 22 (cases 1565–1579) and May 15, 2006 (cases 4070–4072). The investigator (H. Hoffmann) processing the samples was not aware of the clinical history of the patients. All sputa were spot specimens. They were produced spontaneously and collected under observation.

The clinical data and treatment history of patients are summarised in table 1. Patients' data were kept anonymous. The study was approved ethically by the Ministry of Health, Tashkent, Uzbekistan.

Bacterial isolates

With few modifications, sputa were processed as recommended by the International Union Against Tuberculosis and Lung Disease [9] using decontamination by the *N*-acetylcysteine–NaOH method. After inoculation for growth detection, the leftover sediment was used for Genotype MTBDR testing. When liquid cultures turned positive, isolates were sub-cultured and DST was performed for first-line drugs including INH and RMP in Mycobacteria Growth Indicator Tubes (MGIT™; Becton-Dickinson, Heidelberg, Germany) in the BACTEC™ MGIT 960 incubator (Becton-Dickinson) following the protocol of the manufacturer.

Decontaminated specimens or cultured bacteria were inactivated at 100°C for 20 min followed by sonication. The suspension was centrifuged and the supernatant used for PCR. Amplification and sequencing of the *katG* and *rpoB* loci were performed using standard protocols with primers rpoB-f (5′-GGG AGC GGA TGA CCA CCC A-3′), rpoB-r (5′-GCG GTA CGG CGT TTC GAT GAA C-3′), katG-f (5′-CGG CGC ATG GCC ATG AAC GAC GTC-3′) and katG-r (5′-CCG GCA CCG GCG CCG TCC TTG-3′).

Genotype MTBDR assay

The Genotype MTBDR assay was carried out according to the manufacturer's recommendations using 5 μL of DNA extracts and Taq polymerase (Invitrogen, Karlsruhe, Germany). The test detects gene mutations in the *rpoB* and *katG* genes and is based on multiplex PCR followed by reverse hybridisation of amplicons to respective WT and mutation probes. PCR cycling using DNA extracted from sputa was adapted to the optimised method of BANG *et al.* [10].

Typing of MTB

Molecular typing of MTB was based on 24 different loci containing variable numbers of tandem repeats (VNTR) of mycobacterial interspersed repetitive units (MIRU) [11]. Multiplex PCRs and automated MIRU-VNTR analyses were performed as previously described [11, 12]. Extraction of genomic DNA from mycobacteria and IS*6110* fingerprinting were carried out according to the standardised protocol described elsewhere [13]. Spoligotyping was performed according to KAMERBEEK *et al.* [14].

RESULTS

Phenotypic and genotypic DST

According to conventional DST, 13 (37%) of the 35 isolates were MDR (INHr, RMPr), 10 (28%) were resistant to INH (INHr, RMPs) and 12 (34%) were susceptible to INH and RMP (INHs, RMPs; table 2). There were no cases of resistance to RMP only (INHs, RMPr).

Genotype MTBDR was applied directly to decontaminated sputa and later to the bacterial cultures. When applied to cultures, 19 isolates (54%) were detected with mutations in the *katG*, and 14 (40%) with mutations in the *rpoB* gene (table 2). Test results from the sputa were concordant with those from the cultures in all but three cases, which were subsequently identified as heteroresistant to INH or RMP. In four cases, *rpoB*-specific hybridisation signals were markedly weaker, yielding indeterminate test results.

Heteroresistance to INH and/or RMP

In seven cases (20%), there was evidence of heteroresistance to either INH or RMP (fig. 1). In three cases with resistance to

TABLE 1 Patient descriptions: clinical data and treatment history

Clinical data	Patients n (%)	Treatment history#	
		Before sputum collection	After sputum collection
New cases	21 (60)	No treatment	2–3 months: INH/RMP/PZA/EMB 4 months: INH/RMP
Treatment failure	8 (23)	Treatment like ''new cases''	After diagnosis of ''treatment failure'': 2 months: INH/RMP/PZA/EMB/SM 1–2 months: INH/RMP/PZA/EMB 5 months: INH/RMP/EMB
Relapse	6 (17)	Treatment like ''new cases''	After diagnosis of ''relapse'': treatment like cases with ''treatment failure''

The total number of patients was n=35. INH: isoniazid; RMP: rifampin; PZA: pyrazinamide; EMB: ethambutol; SM: streptomycin. #: treatment according to the directly observed treatment strategy protocol.

TABLE 2 Phenotypic and genotypic susceptibility testing

	DST in MGIT	Genotype MTBDR	
		Cultures[#]	Sputum
INHs, RMPs	12 (34)	14 (40)	13 (37)
INHr, RMPr	13 (37)	12 (34)	9 (26)
INHr, RMPs	10 (28)	7 (20)	7 (20)
INHs, RMPr	0 (0)	2 (6)	2 (6)
Not interpretable	0 (0)	0 (0)	4 (11)

The total number of samples was n=35. Data are presented at n (%). DST: drug susceptibility testing; MGIT: Mycobacterial Growth Indicator Tube; INHs: susceptible to isoniazid (INH); RMPs: susceptible to rifampin (RMP); INHr: resistant to INH; RMPr: resistant to RMP. [#]: Using Genotype MTBDR (Hain Lifescience, Nehren, Germany), the following mutations were identified: 19 cases with mutations in *katG*, of whom 18 had mutation S315T and one a missing *katG* wild-type band; 14 cases with mutations in *rpoB*, eight of them with S531L, two with D516V, and one with H526Y and three with missing *rpoB* wild-type bands.

INH (1171, 1571 and 4071), the *katG* WT and the S315T-specific hybridisation bands were visible simultaneously. In two of them (4071 and 1571), heteroresistance was visible in both sputa and cultures and in one (1171) it was seen only after culturing.

In five cases, *rpoB* hybridisation patterns indicated heteroresistance to RMP (fig. 1); one of them (case 4071) simultaneously showed heteroresistance to INH. In cases 1567, 4071 and 4072, heteroresistance to RMP was evident both in sputum and culture. In case 1177, the WT band and two mutation bands (S531L plus H526Y) were detected in the sputum, consistent with the coexistence of three different organisms. The S531L-specific band disappeared after culturing. In case 1575, all *rpoB* WT signals were detectable in sputum whereas WT band 3 (codons 521–525) disappeared in culture, suggesting overgrowth of the WT by mutated organisms. In all seven cases of heteroresistance to either drug, the results of phenotypic DST corresponded to the mutated, *i.e.* resistant, organism.

Verification of heteroresistance

The coexistence of WT and mutated sequences was confirmed by sequencing of the *rpoB* and *katG* PCR products for all seven

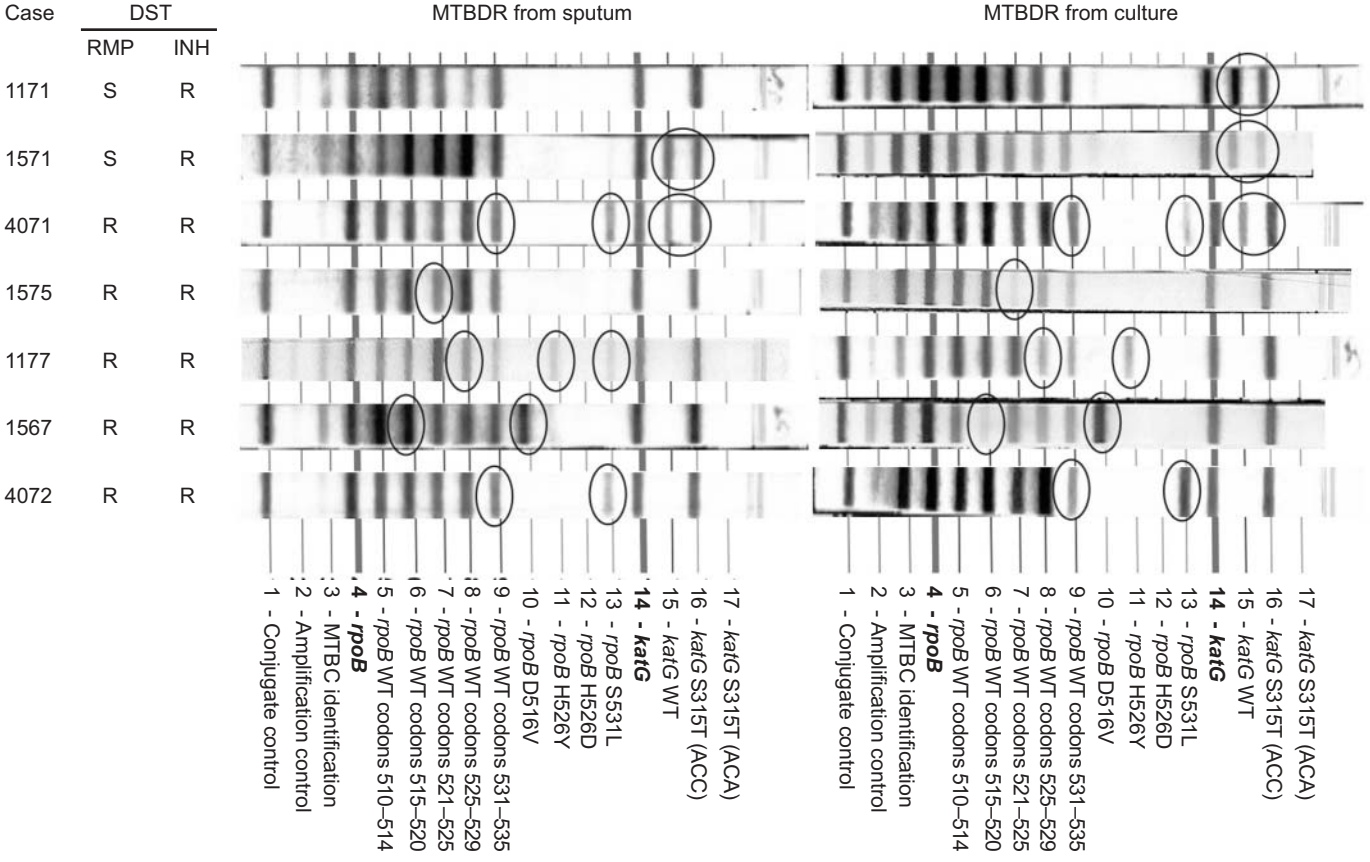

FIGURE 1. Heteroresistance in seven tuberculosis patients as detected by Genotype MTBDR (Hain Lifescience, Nehren, Germany). Genotype MTBDR was applied directly to the sputum and later to cultures. Simultaneous detection of wild-type (WT) and mutation-specific bands for *katG* and/or *rpoB* became visible in all seven cases (circled).The missing WT band in case 1575 corresponded to mutation S522L as shown by sequencing. The results of conventional drug susceptibility testing (DST) are indicated. RMP: rifampin; INH: isoniazid; S: susceptible; R: resistant; MTBC: *Mycobacterium tuberculosis* complex.

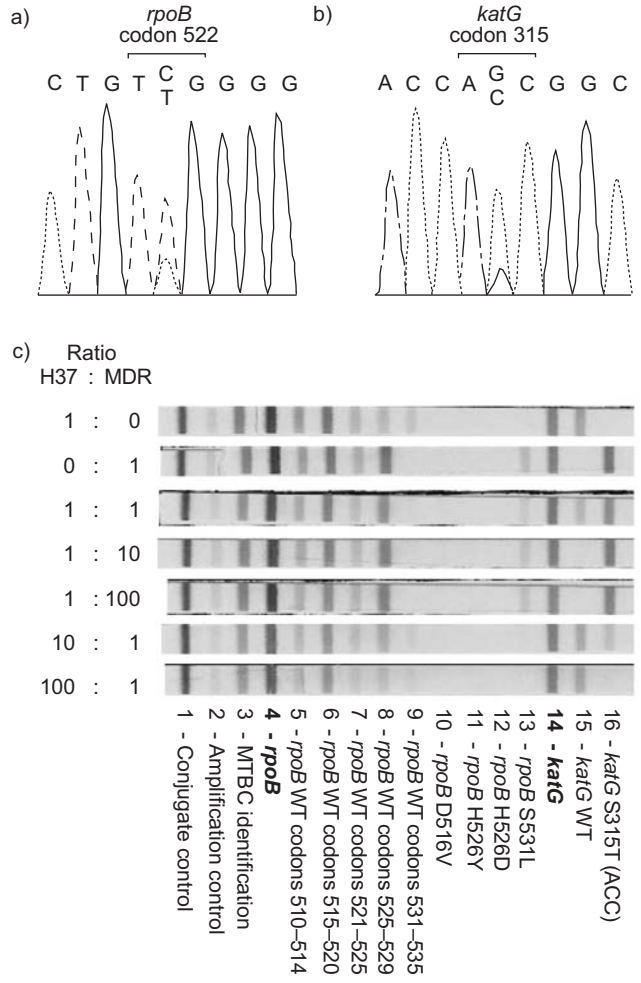

FIGURE 2. Tests for specificity of Genotype MTBDR (Hain Lifescience, Nehren, Germany) results. a and b) Heteroresistance was confirmed by sequencing. Example sequencing results showing a) both the wild-type (WT) sequence (TCG) and the mutation (TTG) at codon 522 of *rpoB* (case 1575) as well as b) WT (AGC) and mutation (ACC) at codon 315 of *katG* (case 1571). c) Artificial heteroresistance was generated using different mixtures of the susceptible reference strain H37Rv (sensitive to rifampin (RMP) and isoniazid (INH)) and a multidrug-resistant (MDR) strain (resistant to RMP and INH) derived from the supranational reference laboratory quality-assessment strain collection. The mixtures were tested with the Genotype MTBDR. MTBC: *Mycobacterium tuberculosis* complex.

cases, respectively (fig. 2a and b). In order to check for reproducibility of hybridisation patterns, the Genotype MTBDR assay was repeated and supplemented with the Genotype MTBDRplus with all seven sputa and cultures from the cases with heteroresistance. The results were concordant with the first ones (data not shown). Furthermore, analysis of 64 MTB cultures of the SNRL quality-assessment strains with the Genotype MTBDR did not show cases of heteroresistance (table 3). It is therefore unlikely that simultaneous detection of WT and mutation bands was due to nonspecific hybridisation.

To assess the relative proportions of WT and mutated organisms needed to allow for the simultaneous visualisation of WT and mutation signals, artificial heteroresistance was produced using different mixtures of susceptible and resistant bacteria followed by DNA extraction and Genotype MTBDR. WT and mutation bands were simultaneously visible at ratios of the WT to the resistant strain of 1:1, 10:1 and 1:10 (fig. 2c). The hybridisation signal of the lower-concentration strain disappeared when the ratio was 1:100 or lower. Thus, Genotype MTBDR seems to be a reliable and specific method to detect heteroresistance of MTB, provided that the relative proportion of the organisms is $\geqslant$10%.

TABLE 3 Genotype MTBDR results using 64 strains of the supranational reference laboratory external quality assessment

	Genotype MTBDR	DST
INHs	20	15
INHr	44	49
Heteroresistance	0	
Total	64	64
RMPs	27	33
RMPr	37	31
Heteroresistance	0	
Total	64	64

Data are presented as n. DST: drug susceptibility testing; INHs: susceptible to isoniazid (INH); INHr: resistant to INH; RMPs: susceptible to rifampin (RMP); RMPs: resistant to RMP.

Characterisation of heteroresistant MTB isolates

To distinguish whether the occurrence of heteroresistance to INH and/or RMP originated from infection with different MTB strains or from infection with a single strain separated into two lineages of organisms, MIRU-VNTR typing was applied to sputum samples and cultures. In case of infection with two strains, two distinct MTB genotypes should be detectable in sputum and/or culture. In four cases of heteroresistance (1171, 1571, 1575 and 4071), two distinct alleles were detected simultaneously at two or more loci (table 4) suggesting the presence of two different genotype strains. In cases 1177, 1567 and 4072, only single genotypes were seen.

In order to estimate the relevance of the Beijing genotype among cases of heteroresistance, spoligotyping was performed from cultures. Beijing genotype strains are predominantly found in Asia and the former Soviet Union [15]. Spoligotyping of cases 1177, 1567 and 4072 yielded patterns corresponding to the Beijing genotype (fig. 3a). Spoligo-patterns of cases 1171, 1571 and 4071 could not be assigned to known patterns but showed a mixture of strong and faint signals, consistent with mixtures of two different strains.

Although MIRU-VNTR typing suggested the presence of single strains in the cases with Beijing genotypes, the presence of two different Beijing genotype strains could not be excluded. Therefore, IS*6110* fingerprinting of cultures grown either in the absence or in the presence of RMP was performed. In case of infection with two Beijing strains, different patterns should be obtained from the resistant organisms selected on RMP and the mixed organisms grown in drug-free medium. IS*6110* typing gave identical patterns with strains 1177 and 1567 but slight

TABLE 4 Mycobacterial interspersed repetitive unit (MIRU)-variable-number tandem repeat (VNTR) typing results

Sample	VNTR 0424	VNTR 0577	MIRU 04	MIRU 40	MIRU 10	MIRU 16	VNTR 1955	VNTR 2163b	VNTR 2165	VNTR 2401	MIRU 26	MIRU 31	VNTR 3690	VNTR 4052	VNTR 4156	MIRU 02	MIRU 23	MIRU 39	MIRU 20	MIRU 24	MIRU 27	VNTR 2347	VNTR 2461	VNTR 3171
1177-C	4	4	2	3	3	3	x	6	4	x	5	5	3	x	x	2	x	3	x	1	3	4	2	3
1177-S	4	4	2	3	x	3	x	6	4	x	5	5	3	x	x	2	x	3	x	1	3	4	2	3
1567-C	4	4	2	3	3	3	x	6	5	x	5	5	3	x	x	2	x	3	2	1	3	4	2	3
1567-S	4	4	2	3	x	3	x	6	5	4	5	5	3	x	x	2	x	3	x	1	3	4	2	3
4072-C	4	4	2	3	3	3	x	6	4	4	5	5	3	x	x	2	x	3	x	1	3	4	2	3
4072-S	4	4	2	3	x	3	x	x	4	4	5	5	3	x	x	2	x	3	x	1	3	4	2	3
1575-C	3	5	2	3	x	2	3	**2**	4	4	1	2	3	x	x	**1**	x	x	x	1	3	x	2	x
1575-S	x	5	2	3	x	2	x	**5**	4	4	1	2	3	x	x	**2**	x	2	x	1	3	4	2	3
4071-C	2	4	2	**3+4**	**2+3**	**1+3**	**2+6**	**4+6**	3	2	**1+5**	3	**3+4**	x	x	2	x	**2+3**	2	1	3	4	2	3
4071-S	x	4	2	3	3	3	x	x	4	4	5	5	3	x	x	2	5	3	x	1	3	4	2	3
1171-C	**2**	4	2	**2**	3	**1**	**2**	**2**	**2**	**2**	**5**	**2**	**2**	4	x	x	x	x	x	1	x	2	2	3
1171-S	**4**	4	2	**3**	3	**3**	**5**	**6**	**4**	**4**	**6**	**5**	**3**	x	x	2	x	3	x	1	3	2	2	3
1571-C	2	2	2	**3+4**	4	3	**3**	2	2	**1+2**	**5+6**	2	2	x	x	1	x	2	x	x	x	x	2	x
1571-S	2	**2+3**	2	**3+4**	4	3	**4**	**2+3**	**2+3**	1	**5+6**	2	2	x	x	x	x	2	2	1	3	x	2	x
H37Rv	2	4	35[#]	1	3	2	2	5	3	2	3	3	x	5	x	2	x	2	x	1	x	x	3	3

A total of 24 different MIRU-VNTR loci were analysed using automated fluorescence-based genotyping. The detection of more than one copy number at a specific locus or the detection of different copy numbers in DNA samples derived from sputum and cultured material are shown in bold. C: culture; S: sputum; x: not interpretable due to the absence of a specific amplification product. [#]: variant allele of locus MIRU 04.

differences with strain 4072 (fig. 3b) suggesting infection with two different Beijing strains in the latter case.

Evaluation of clinical data showed that in three cases of heteroresistance, TB had been newly diagnosed whereas four cases had a history of treatment failure or relapse (table 5). Comparison of biological and clinical data showed that all ''new cases'' were infected with two different strains. Both cases with single strains were ''relapses''. Notably, all three cases with Beijing genotype strains showed a history of relapse, whereas among the four non-Beijing cases, three were new and one had experienced ''treatment failure'' (table 5). Thus, the risk of relapse or treatment failure was higher in cases with Beijing genotype strains.

DISCUSSION

In the present study, molecular investigation of 35 TB patients from Tashkent, identified seven cases (20%) of heteroresistance. For the first time, two mechanisms of heteroresistance were proven, *i.e.* the coexistence of two different MTB strains and the segregation of single strains into resistant and susceptible organisms. These mechanisms were linked to the clinical entities new cases, treatment failures and relapses. The present study showed that the coexistence of two different strains prevailed in new cases, while segregation of single strains prevailed in treatment failures and relapses.

Heteroresistance due to infection with two different strains is theoretically explained by superinfection of a patient already infected with one MTB strain with an additional one. For the three new cases, superinfection of a latent TB infection seemed to be the best explanation, although the hypothesis that superinfection can trigger a reactivation of pre-existing latent TB has so far never been proven [16]. In contrast, superinfection of patients with active TB has repeatedly been demonstrated, particularly with resistant Beijing genotype strains [17, 18]. This mechanism could explain the heteroresistance due to infection with two strains in patients with a history of relapse or treatment failure.

A high TB incidence certainly increases the risk of superinfection. Uzbek TB hospitals might be risky sites of superinfection with resistant strains. This will be investigated in future studies and may give valuable information for infection-control practitioners.

Heteroresistance due to infection with single strains is most probably explained by segregation into susceptible and resistant organisms under the selective pressure of insufficient anti-TB therapy. Numerous reports have described the evolution of resistance due to inadequate therapy [19]. Ineffective therapy can result from noncompliance of the patient, poor pharmaceutical quality of the drugs, or pre-existing resistances of the pathogens. Notably, both patients with heteroresistance due to single strains had a history of relapse. Thus, positive selective pressure of inadequate therapy could have amplified the resistant organisms to proportions detectable by the Genotype MTBDR assay. If so, the rate of heteroresistance with single strains could serve as an indicator for the quality of anti-TB treatment programmes, which could be of help for public health practitioners.

In the current study, heteroresistance due to infection with single strains was caused exclusively by Beijing genotype

FIGURE 3. Spoligotyping and fingerprinting analysis. a) Spoligotyping of cultures from the seven cases of heteroresistance identified three cases with Beijing genotypes. The spoligo-pattern of case 1575 corresponds to spoligotype H4. b) IS*6110* fingerprinting of cases 1177, 1567 and 4072 performed from cultures grown in the absence or in the presence of rifampin (RMP). As control, the IS*6110* fingerprint from strain H37Rv is shown.

TABLE 5 Typing results and clinical data of the seven cases with heteroresistant tuberculosis

Sample	Infection with single or two strains	Genotype(s)	Clinical data
1177	Single strain	Beijing	Relapse
1567	Single strain	Beijing	Relapse
4072	Two strains	Beijing/Beijing	Relapse
1575	Two strains	H4/unknown	Treatment failure
4071	Two strains	Unknown/unknown[#]	New case
1171	Two strains	Unknown/unknown	New case
1571	Two strains	Unknown/unknown	New case

Data from mycobacterial interspersed repetitive unit-variable-number tandem repeat typing, spoligotyping and fingerprinting were summarised and compared with clinical data. [#]: case 4071 had a spoligo-pattern that was potentially consistent with a mixture of a Beijing genotype plus a non-Beijing strain.

strains. Single nucleotide polymorphisms in mismatch-repair genes enable the Beijing genotype to acquire resistance-associated mutations more easily than other genotypes [20]. This observation and the predominance of the Beijing genotype in Central Asian countries might explain why only Beijing genotype strains have been observed in the present patient group. Furthermore, heteroresistance due to infection with Beijing strains was invariably linked to the history of relapse. This is in line with previous studies reporting an association between relapses or treatment failures and the Beijing genotype in Vietnam [21] and Singapore [22].

The rate of 20% of heteroresistance in TB is similar to the finding by RINDER *et al.* [6], who reported a rate of 17%. Other studies have reported significantly lower rates [8, 23, 24]. This discrepancy may depend on several factors. First, the group of TB patients and the study site influence the rate of heteroresistance. Among the current TB patients, a high rate of MDR-TB was observed and 40% had previous failure of therapy or relapses. Similarly, in the study of RINDER *et al.* [6], only DNA samples from patients with risk factors such as prior anti-TB treatment were included. Secondly, application of assays directly to the clinical specimens enhances the chance of detecting heteroresistance [4, 24]. Thirdly, the simultaneous testing of clinical specimens and bacterial cultures further improves the detection of heteroresistance.

In conclusion, to date heteroresistance is an underestimated phenomenon in tuberculosis, especially in highly endemic areas. In the current study, heteroresistance was primarily caused by co-infection with different *Mycobacterium tuberculosis* strains, certainly favoured by the high incidence of tuberculosis in Tashkent. Further studies with larger numbers of patients are needed to estimate the epidemiology and the clinical impact of heteroresistance.

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REFERENCES

1 World Health Organization. Anti-tuberculosis Drug Resistance in the World. Report No. 4. The WHO/IUATLD Global Project on Anti-tuberculosis Drug Resistance Surveillance 2002–2007. Geneva, World Health Organization, 2008.
2 Ramaswamy S, Musser JM. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. *Tuber Lung Dis* 1998; 79: 3–29.
3 Heep M, Brandstatter B, Rieger U, *et al.* Frequency of *rpoB* mutations inside and outside the cluster I region in rifampin-resistant clinical *Mycobacterium tuberculosis* isolates. *J Clin Microbiol* 2001; 39: 107–110.
4 Rinder H. Hetero-resistance: an under-recognised confounder in diagnosis and therapy? *J Med Microbiol* 2001; 50: 1018–1020.
5 Adjers-Koskela K, Katila ML. Susceptibility testing with the manual mycobacteria growth indicator tube (MGIT) and the MGIT 960 system provides rapid and reliable

verification of multidrug-resistant tuberculosis. *J Clin Microbiol* 2003; 41: 1235–1239.
6 Rinder H, Mieskes KT, Loscher T. Heteroresistance in *Mycobacterium tuberculosis*. *Int J Tuberc Lung Dis* 2001; 5: 339–345.
7 Karahan ZC, Akar N. Restriction endonuclease analysis as a solution for determining rifampin resistance mutations by automated DNA sequencing in heteroresistant *Mycobacterium tuberculosis* strains. *Microb Drug Resist* 2005; 11: 137–140.
8 de Oliveira MM, da Silva Rocha A, Cardoso Oelemann M, *et al.* Rapid detection of resistance against rifampicin in isolates of *Mycobacterium tuberculosis* from Brazilian patients using a reverse-phase hybridization assay. *J Microbiol Methods* 2003; 53: 335–342.
9 International Union Against Tuberculosis and Lung Disease. Priorities for Tuberculosis Bacteriology Services in Low-income Countries. 2nd Edn. Paris, International Union Against Tuberculosis and Lung Disease, 2007.
10 Bang D, Bengard Andersen A, Thomsen VO. Rapid genotypic detection of rifampin- and isoniazid-resistant *Mycobacterium tuberculosis* directly in clinical specimens. *J Clin Microbiol* 2006; 44: 2605–2608.
11 Supply P, Allix C, Lesjean S, *et al.* Proposal for standardization of optimized mycobacterial interspersed repetetive unit-variable-numer of tandem repeat typing of *Mycobacterium tuberculosis*. *J Clin Microbiol* 2006; 44: 4498–4510.
12 Supply P, Lesjean S, Savine E, Kremer K, van Soolingen D, Locht C. Automated high-throughput genotyping for study of global epidemiology of *Mycobacterium tuberculosis* based on mycobacterial interspersed repetetive units. *J Clin Microbiol* 2001; 39: 3563–3571.
13 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: 406–409.
14 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: 907–914.
15 van Soolingen D, Quian L, de Haas PEW, *et al.* Predominance of a single genotype of *Mycobacterium tuberculosis* in countries of East Asia. *J Clin Microbiol* 1995; 33: 3234–3238.
16 Kaufmann SHE, McMichael AJ. Anulling a dangerous liaison: vaccination strategies against AIDS and tuberculosis. *Nat Med* 2005; 11: Suppl. 4, S33–S44.
17 Warren RM, Victor TC, Streicher EM, *et al.* Patients with active tuberculosis often have different strains in the same sputum specimen. *Am J Respir Crit Care Med* 2004; 169: 610–614.
18 Cox HS, Orozco JD, Male R, *et al.* Multidrug-resistant tuberculosis in Central Asia. *Emerg Infect Dis* 2004; 10: 865–872.
19 Woodford N, Ellington MJ. The emergence of antibiotic resistance by mutation. *Clin Microbiol Infect* 2006; 13: 5–18.
20 Rad ME, Bifani P, Martin C, *et al.* Mutations in putative mutator genes of *Mycobacterium tuberculosis* strains of the W-Beijing family. *Emerg Infect Dis* 2004; 9: 838–845.
21 Lan NT, Lien HT, Tung le B, Borgdorff MW, Kremer K, van Soolingen D. *Mycobacterium tuberculosis* Beijing genotype and risk for treatment failure and relapse, Vietnam. *Emerg Infect Dis* 2003; 9: 1633–1635.
22 Sun YJ, Lee AS, Wong SY, Paton NI. Association of *Mycobacterium tuberculosis* Beijing genotype with tuberculosis in Singapore. *Epidemiol Infect* 2006; 134: 329–332.
23 Mäkinen J, Marttila HJ, Marjamäki M, Viljanen MK, Soini H. Comparison of two commercially available DNA line probe assays for detection of multidrug-resistant *Mycobacterium tuberculosis*. *J Clin Microbiol* 2006; 44: 350–352.
24 Miotto P, Piana F, Penati V, Canducci F, Migliori GB, Cirillo DM. Use of genotype MTBDR assay for molecular detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* clinical strains isolated in Italy. *J Clin Microbiol* 2006; 44: 2485–2491.