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Mechanisms of Heteroresistance to Isoniazid and Rifampin of *M. tuberculosis* in Tashkent, Uzbekistan

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Running title: Heteroresistance of MTB in Tashkent

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ABSTRACT

Heteroresistance of *Mycobacterium tuberculosis* (MTB) is defined as the co-existence of susceptible and resistant organisms to anti-tuberculosis (TB) drugs in the same patient. Heteroresistance of MTB is considered a pre-stage to full resistance. So far, no mechanism causing heteroresistance of MTB has been proven.

Clinical specimens and cultures from 35 TB patients from Tashkent, Uzbekistan, were analyzed using the Genotype MTBDR assay, which is designed to detect genetic mutations associated with resistance to rifampin and isoniazid. Cases of heteroresistance were further subjected to genotyping using MIRU-VNTR 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, 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 TB have been proven using a stepwise molecular-biological approach: super-infection with two different strains which is of interest for clinical infection control practitioners and splitting of one single strain in susceptible and resistant organisms. The latter one is most likely related to poor treatment quality and could serve as quality marker for TB therapy programs in future.

Key words: Beijing genotype, Genotype MTBDR, heteroresistance, multi-drug resistance, MIRU-VNTR typing, tuberculosis,

INTRODUCTION

Multi drug resistance (MDR) epitomizes 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 worldwide highest rate of MDR-TB (60 %) was observed in Tashkent, Uzbekistan.

MDR-TB is defined by resistance of the *Mycobacterium tuberculosis* complex (MTBC) 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 is determined by one or more mutations in a 81 bp core region of the *rpoB* [2, 3].

Some TB patients harbor mixed populations of *M. tuberculosis* (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]. So far, 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 like restriction fragment-length polymorphism (RFLP) [6], sequencing [7] or "line probe assays" [8].

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

MATERIAL AND METHODS

Clinical specimens

The sputa of 35 pulmonary TB patients of the DOTS center of Tashkent, Uzbekistan, were taken for the present study. They were sent to the supranational reference laboratory (SNRL) Gauting, Germany, for DST in the frame of the Drug Resistance Survey (DRS) in the Uzbek capital. The sputa comprised consecutive samples which were processed on February 10 (no. 1169 to 1185), February 22 (no. 1565 to 1579) and May 15, 2007 (no. 4070 to 4072). The author (H.H.) including the samples was not aware of the clinical history of the patients. All sputa were spot specimens. They were spontaneously produced and collected under observation.

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

Bacterial isolates

With few modifications, sputa were processed as recommended by the IUATLD [9] using decontamination by the N-acetyl-cystein (NALC)-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^{TM}, Becton-Dickenson, Heidelberg, Germany) in the BACTEC^{TM} MGIT 960 incubator 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 (Hain Lifesciences, Nehren, Germany) using 5 µl of DNA extracts and Taq polymerase (Invitrogen, Germany). The test detects gene mutations in the *rpoB* and *katG* genes and is based on multiplex PCR followed by reverse hybridization of amplicons to respective WT and mutation probes. PCR cycling using DNA extracted from sputa was adapted to the optimized method of Bang *et al*. [10].

Typing of *MTB*

Molecular typing of MTB was based on 24 different loci containing variable number of tandem repeats of mycobacterial interspersed repetitive units (MIRU-VNTRs) [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 done according to the standardized 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 (INH_r, RMP_r), ten (28%) were resistant to INH (INH_r, RMP_s) and 12 (34%) were susceptible to INH and RMP (INH_s, RMP_s) (table 2). There were no cases of resistance to RMP only (INH_s, RMP_r).

Genotype MTBDR was applied directly to decontaminated sputa and later-on to the bacterial cultures. When applied to cultures, we detected 19 isolates (54%) with mutations in the *katG*, and fourteen (40%) with mutations in the *rpoB* gene (table 2). The 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 hybridization signals were markedly weaker, yielding indeterminate test results.

Heteroresistance to INH and/or RMP

In seven cases (20 %), we got evidence of heteroresistance to either INH or RMP (fig. 1). In three cases with resistance to INH (1171, 1571 and 4071), the *katG* WT and the S315T-specific hybridization bands were simultaneously visible. In two of them (4071 and 1571), heteroresistance became visible in sputa and cultures, in one (1171) only after culturing.

In five cases, *rpoB* hybridization patterns indicated heteroresistance to RMP (fig. 1); one of them (4071) simultaneously showed heteroresistance to INH. In cases 1567, 4071 and 4072, heteroresistance to RMP became 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 co-existence 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, results of phenotypic DST corresponded to the mutated, i.e. resistant, organism.

Verification of heteroresistance

The co-existence of wild-type and mutated sequences was confirmed by sequencing of the *rpoB* and *katG* PCR products for all seven cases, respectively (fig. 2a). In order to check for reproducibility of hybridization patterns, we repeated the Genotype MTBDR and additionally performed the Genotype MTBDR plus 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 unspecific hybridization.

We assessed the relative proportion of WT and mutated organisms needed to allow for the simultaneous visualization of WT and mutation signals. Therefore, artificial heteroresistance was produced using different mixtures of susceptible and resistant bacteria followed by DNA extraction and Genotype MTBDR. Both, WT and mutation bands were simultaneous visible at ratios of the WT to the resistant strain of 1:1, 10:1 and 1:10 (fig. 2b). The hybridization signal of the respective bacterium of the lower concentration 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 at least 10 %.

Characterization of heteroresistant *MTB* isolates

We asked 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. To distinguish between these possibilities, 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, 4071) two distinct alleles were simultaneously detected 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 countries of Asia and the former Russian federation [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 being 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, we performed IS*6110* fingerprinting of cultures grown either in the absence or in the presence of RMP. 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 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). Comparing biological and clinical data we found 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 to experience 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, Uzbekistan, identified seven cases (20%) of heteroresistance. For the first time, two mechanisms of heteroresistance were proven, i.e. the co-existence 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”. We showed that the co-existence 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 super-infection of a patient already infected with one MTB strain with an additional one. For the three “new cases”, super-infection of a latent TB infection seemed to be the best explanation although the hypothesis that super-infection can trigger a re-activation of pre-existing latent TB has so far never been proven [16]. In contrast, super-infection 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 super-infection. Usbek TB hospitals might be risky sites of super-infection with resistant strains. This shall be investigated in future studies and may give valuable information for infection control practitioners.

Heteroresistance due to infection with single strains is most likely 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 incompliance 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 up to proportions detectable by the Genotype MTBDR. If so, the rate of heteroresistance with single strains could serve as an indicator for the quality of anti-TB treatment programs, which could be of help for public health practitioners.

In our study, heteroresistance due to infection with single strains was exclusively caused by Beijing genotype 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 this 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 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 our TB patients, a high rate of MDR-TB was observed and 40 % of them 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 have been included. Second, application of assays directly to the clinical specimens enhances the chance to detect heteroresistance [4, 24]. Third, the simultaneous testing of the clinical specimens and the bacterial cultures further improves the detection of heteroresistance.

In conclusion, heteroresistance is a so far underestimated phenomenon in TB especially in highly endemic areas. In our study, heteroresistance was primarily caused by co-infection with different *MTB* strains certainly favored by the high incidence of TB 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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Table 1

Patient description: clinical data and treatment history

Patients	clinical data	treatment history[#]
21 (60)	"new cases"	before sputum collection: no treatment after sputum collection: 2-3 months: INH/RMP/PZA/EMB 4 months: INH/RMP
8 (23)	"treatment failure"	before sputum collection: treatment like "new cases" after diagnosis of "treatment failure" and sputum collection: 2 months: INH/RMP/PZA/EMB/SM 1-2 months: INH/RMP/PZA/EMB 5 months: INH/RMP/EMB
6 (17)	"relapse"	before sputum collection: treatment like "new cases" after diagnosis of "relapse" and sputum collection: treatment like cases with "treatment failure"

The total number of patients was n = 35. Values represent numbers, with percentages in parentheses. [#]Treatment according to the DOTS protocol. DOTS: directly observed treatment strategy; INH: isoniazid; RMP: rifampin; PZA: pyrazinamide; EMB: ethambutol; SM: streptomycin.

Table 2.

Phenotypic and genotypic susceptibility testing

		Genotype MTBDR applied to	
	DST in MGIT	cultures*	sputum
INH_s, RMP_s	12 (34)	14 (40)	13 (37)
INH_r, RMP_r	13 (37)	12 (34)	9 (26)
INH_r, RMP_s	10 (28)	7 (20)	7 (20)
INH_s, RMP_r	0 (0)	2 (6)	2 (6)
Not interpretable	0 (0)	0 (0)	4 (11)

The total number of samples comprised n = 35. Values are numbers, with percentages in parentheses. DST = drug susceptibility testing; MGIT = mycobacterial growth indicator tube; INH_r = resistant to INH; RMP_r = resistant to RMP; INH_s = susceptible to INH; RMP_s = susceptible to RMP. *Using Genotype MTBDR, the following mutations were identified: 19 cases with mutations in *katG*, of these 18 with mutation S315T and one with a missing *katG* WT band; 14 cases with mutations in *rpoB,* eight of them with S531L, two with D516V, and one with H526Y, three with missing *rpoB* WT bands.

Table 3.

Genotype MTBDR using 64 strains of the SNRL external quality assessment

	MTBDR	DST
INH_s	20	15
INH_r	44	49
Heteroresistance	0	
Total	64	64
RMP_s	27	33
RMP_r	37	31
Heteroresistance	0	
Total	64	64

Values represent numbers. SNRL: supra-national reference laboratory; DST: drug susceptibility testing; INH_r: resistant to INH; RMP_r: resistant to RMP; INH_s: susceptible to INH; RMP_s: susceptible to RMP;

Table 4. MIRU-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
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
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
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
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
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
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
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
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
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
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
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
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
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
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
H37Rv	2	4	103	1	3	2	2	5	3	2	3	3	x	5	x	2	x	2	x	1	x	x	2

A total of 24 different MIRU-VNTR loci were analyzed 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 highlighted. VNTR: variable number of tandem repeats; MIRU: mycobacterial interspersed repetitive units; C: culture; S: sputum; x: not interpretable due to the absence of a specific amplification product.

Table 5.

Typing results and clinical data of the seven cases with heteroresistant TB

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 MIRU-VNTR typing, spoligotyping and fingerprinting were summarized and compared to clinical data. *Case 4071 had a spoligo-pattern which was potentially consistent with a mixture of a Beijing genotype plus a non-Beijing strain.

Figure legends

Figure 1

Heteroresistance in seven TB patients as detected by Genotype MTBDR. Genotype MTBDR was applied directly to the sputum and later-on to cultures. Simultaneous detection of WT and mutation-specific bands for *katG* and/or *rpoB* became visible in all seven cases (highlighted by circles).The missing WT band in case 1575 corresponded to mutation S522L as shown by sequencing. Results of conventional DST are indicated. S: susceptible; R: resistant; DST: drug susceptibility testing. WT: wild-type.

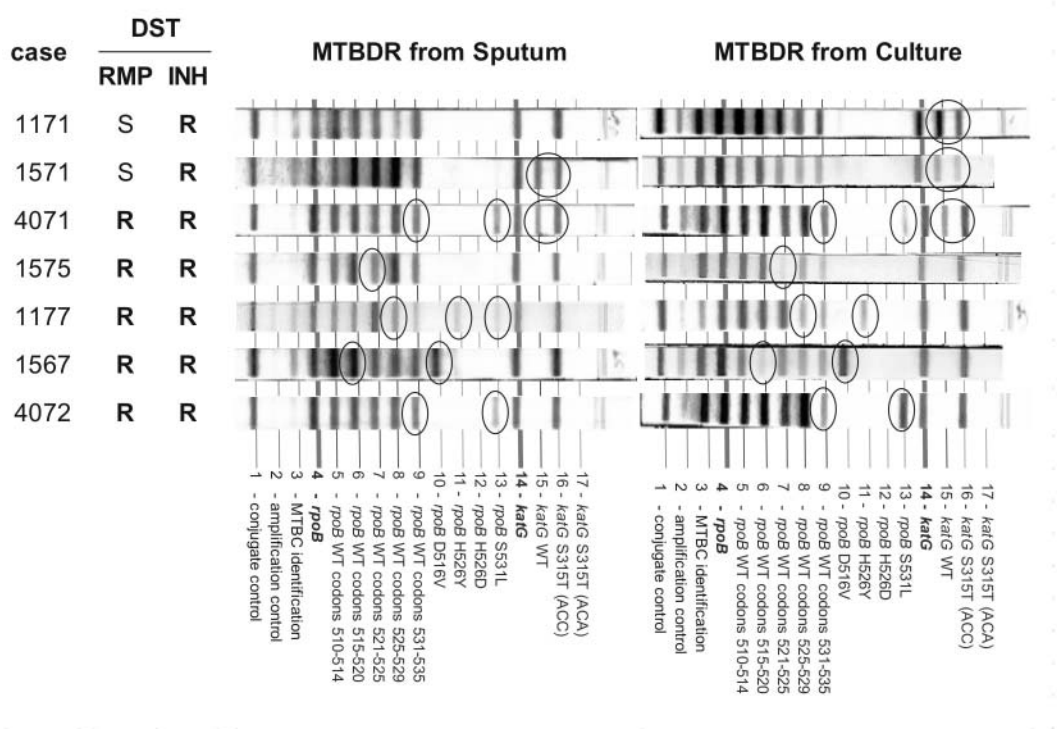
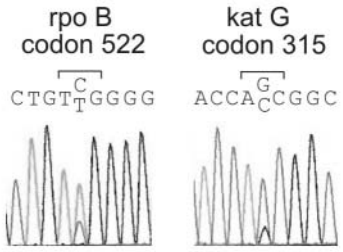


Figure 2

Tests for specificity of Genotype MTBDR results. a) Heteroresistance was confirmed by sequencing. Exemplary sequencing results showing both the WT

sequence (TCG) and the mutation (TTG) at codon 522 of *rpoB* (case 1575) (*left*) as well as WT (AGC) and mutation (ACC) at codon 315 of *katG* (case 1571) (*right*). **b)** Artificial heteroresistance was generated using different mixtures of the susceptible reference strain H37Rv (RMP_s, INH_s) and a MDR strain (RMP_r, INH_r) derived from the SNRL quality assessment strain collection. The mixtures were tested with the Genotype MTBDR. WT: wild-type; INH_r: resistant to INH; RMP_r: resistant to RMP; INH_s: susceptible to INH; RMP_s: susceptible to RMP; MDR: multi-drug resistant; SNRL: supra-national reference laboratory.



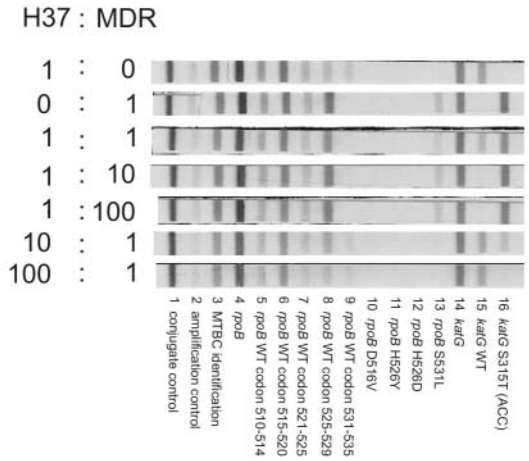


Figure 3

Spoligotyping and fingerprinting analysis. a) Spoligotyping of cultures from the seven cases of heteroresistance identified three cases with Beijing genotypes. 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 RMP, respectively. As control, the IS*6110* fingerprint from strain H37Rv is shown. RMP: rifampin.