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Can Molecular Methods Detect 1% Isoniazid Resistance in *Mycobacterium tuberculosis*?

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Patients may harbor both drug-susceptible and -resistant bacteria, representing heteroresistance. We studied mixtures of isoniazid-resistant and -susceptible *Mycobacterium tuberculosis* strains. Conventional drug susceptibility testing was the most sensitive method of detection, whereas the line probe assay and sequencing were not able to detect the clinically relevant 1% proportion of resistant bacteria.

Patients with tuberculosis (TB) usually harbor susceptible *Mycobacterium tuberculosis* but may also have a small proportion of drug-resistant isogenic variants that develop spontaneously during replication. For isoniazid (Inh) resistance, mutations are introduced at rates of approximately 1 mutation per 10^8 to 10^{10} divisions (1). The mutations are found in several genes but mainly in the *katG* gene and in the *inhA* promoter (2). An increased proportion of resistant variants in a susceptible main population of isogenic or nonisogenic bacteria is called "heteroresistance" in this paper. Standard short-course chemotherapy with combinations of several drugs prevents the selection of drug-resistant bacteria during treatment. The proportion of patients infected with heteroresistant tuberculosis is largely unknown.

In the 1960s, the criteria for defining clinically relevant drug resistance were set from clinical and bacteriological studies. By consensus, a cutoff of 1% for Inh was decided. This is the proportion of resistant bacteria above which therapeutic success had been demonstrated to be unlikely (3, 4). Therefore, phenotypic drug susceptibility testing (DST) aims to determine if 1% or more of the bacterial population in clinical specimens is drug resistant.

In recent years, commercial genotypic methods have become available for detection of mutations that may confer resistance. Among these, it is only with the line probe assay (LPA) GenoType MTBDR*plus* that resistance to Inh can be detected by analysis of the *katG* and *inhA* genes (5). The LPA uses PCR covering areas where mutations associated with resistance are common. It is followed by hybridization of target sequences to a membrane strip; the hybrids are visualized as bands. The test detects specific mutations as well as unspecified mutations, the latter by the absence of at least one wild-type band.

In June 2008, a WHO Expert Consultation concluded that sufficient information was available to recommend the use of LPAs for detection of multidrug resistance (MDR) in *M. tuberculosis* isolates and smear-positive sputum specimens (http://www.who.int/tb/features_archive/policy_statement.pdf). The newly published European Union Standards for Tuberculosis Care recommends also that rapid testing for Inh and rifampin should be performed on primary specimens when MDR TB is suspected (6).

These assays are important for the global scaling up of detection of MDR and extensively drug-resistant (XDR) *M. tuberculosis*. However, little is known of the sensitivity of these methods for detection of small proportions of drug-resistant bacteria. Furthermore, to facilitate the DSTs, laboratories may preincubate the bacteria in different ways before they test them. The influence of these procedures on the DSTs, if the patient has TB caused by heteroresistant bacteria, is unclear.

The aim of the present study was therefore to evaluate the ability of the phenotypic and genotypic DST methods to detect resistance in mixtures of Inh-resistant and Inh-susceptible bacteria. The influence of various common pretest conditions on the test outcome with regard to culture media, incubation time, and presence of CO_2 was also studied.

Two strains each of the spoligotype families Haarlem and Beijing were obtained from the World Health Organization—Tropical Disease Research (TDR) *M. tuberculosis* Strain Bank in Antwerp. Haarlem strain TB-TDR-063 was Inh susceptible, and TB-TDR-187 was Inh resistant. Beijing strain TB-TDR-077 was Inh susceptible, and TB-TDR-082 was Inh resistant. The strains were tested with the GenoType MTBDR*plus* assay before the experiments were performed. The susceptible strains from both families had wild-type DNA in *katG* and *inhA*. Both Inh-resistant strains had the *katG* S315T mutation, had lost a wild-type band, and were *inhA* wild type. The freeze-dried strains were subcultured on Löwenstein-Jensen slants and in Dubos with 0.045% Tween 80 (both from SSI Diagnostika, Hilleroed, Denmark) with 0.4 mg/ml Inh (BD, Franklin Lakes, NJ) diluted in water for the resistant strains and incubated at 37°C. After 2 weeks of incubation, the bacterial concentrations in liquid media were adjusted to equal densities at 580 nm by adding Dubos-Tween. Subsequently, mixtures of 99% plus 1%, 95% plus 5%, 90% plus 10%, and 50% plus 50% Inh-susceptible and Inh-resistant isolates of the Haar-

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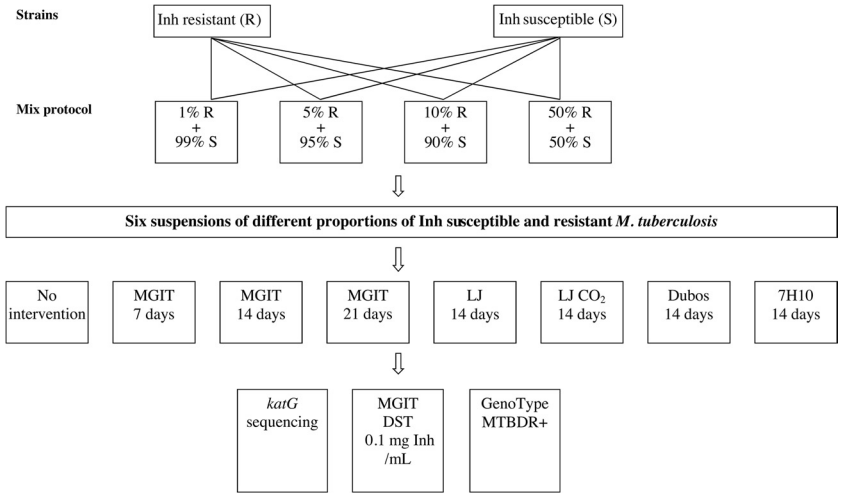

FIG 1 Study design for the Inh-resistant and -susceptible strains of *M. tuberculosis*—the same designs were applied for the Haarlem and the Beijing strains.

lem and Beijing strains, respectively, were prepared by combining suspensions of susceptible and resistant bacteria belonging to the same family as shown in Fig. 1. Phenotypic DSTs and molecular analyses were performed using either the mixtures and strains (henceforward denoted "suspensions") immediately or after different interventions. Each of the suspensions was inoculated into Dubos (500 μl), MGIT (500 μl), two Lowenstein-Jensen (LJ) medium slants (150 μl), and Middlebrook 7H10 (BD) (150 μl) and incubated at 37°C. The MGIT tubes were incubated for 7, 14, and 21 days. One of the LJ slants was incubated at 37°C in a 5% CO_2 atmosphere and the other at 37°C in a normal atmosphere; both were incubated for 14 days.

One or two colonies grown on solid medium were subjected to a vortex procedure with glass beads in 4 ml MGIT for 2 to 3 min, and the suspension was allowed to sediment for 20 min. The supernatant was transferred to new sterile tubes and handled as bacteria grown in liquid media, as described previously (5).

The GenoType MTBDR*plus* (Hain Lifescience, Nehren, Germany) analysis was carried out according to the manufacturer's instructions (5).

The *katG* sequencing was carried out with 5 μl lysate in PCR amplification with a total volume of 50 μl (PCR buffer, 2 mM $MgCl_2$, 0.2 μM deoxynucleoside triphosphate [dNTP], 0.2 μM primers katG-F and katG-R [7] [DNA Technology A/S, Risskov, Denmark], 2.5 U GoTaq Flexi [Promega, Madison, WI], and water to reach a volume of 45 μl). Thermocycling was done with initial denaturation at 95°C for 7 min, followed by 40 cycles of 94°C for 30 s, 59°C for 60 s, 72°C for 60 s, and final extension at 72°C for 10 min and cooling to 4°C on a GS1 thermal cycler (G-Storm, Somerset, United Kingdom). Sequencing was carried out with a BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) using amplification primers and analyzed on an ABI 3730 Genetic Analyzer (Applied Biosystems) and evaluated with Sequencing Analysis 5.3.1 (Applied Biosystems) and Sequencer 5.0 (Gene Codes, Ann Arbor, MI).

For the phenotypic DST, the Bactec MGIT960 (BD) with a 1/100-diluted control and 0.1 μg Inh/ml was used (8). All analyses were interpreted as unknown samples in the same way as in routine situations.

Compared to the molecular methods, the ability to detect heteroresistance was better with conventional DST. It was possible to detect the expected 1% proportion of the resistant bacteria with MGIT DST irrespective of the strain. The various pretest incubations did not negatively affect the ability to detect drug resistance. Growth on different culture media occasionally increased detection on 7H10, Dubos, and MGIT with both molecular methods. Different times of incubation gave different detection results, as growth for 21 days occasionally increased the detection limits for both molecular methods. Growth with or without CO_2 did not change the results obtained with any of the methods (Table 1).

The experiment has not been repeated, due to the complexity of this study and the high number of tests done, and we encourage other centers to verify our findings.

This study showed that the ability to detect heteroresistance to Inh with LPA and sequencing of the *katG* gene is significantly lower than with conventional DST ($P < 0.001$, Fisher's exact test). Furthermore, common subcultivation techniques do not substantially influence the detection of resistance. To our knowledge, this is the first study to test the performance of the molecular methods GenoType MTBDR*plus* and automatic cycle sequencing for drug susceptibility testing when mixtures of resistant and susceptible bacteria are present. For both the Beijing and the Haarlem strains investigated, we found that we were unable to detect 1% resistant

TABLE 1 Isoniazid resistance detected with drug susceptibility testing using Bactec MGIT, MTBDR*plus*, and sequencing with suspensions, each containing various proportions of resistant and susceptible Haarlem or Beijing *M. tuberculosis* strains[a]

Suspension result	No. of suspensions with indicated detection result		
	MGIT	LPA	Seq
100% R	16	16	16
50% R + 50% S	16	16	16
10% R + 90% S	16	16	4
5% R + 95% S	16	16	2
1% R + 99% S	16	3	0
100% S	0	0	0

[a] The suspensions were analyzed before and after subculture under different conditions. LPA, MTBDR*plus*; Seq, sequencing; R, resistant; S, susceptible.

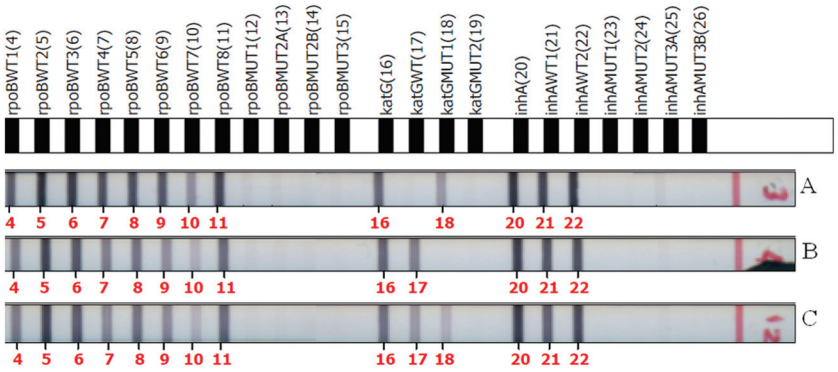

FIG 2 Examples of GenoType MTBDR*plus* with suspensions containing various proportions of Inh-susceptible (S) and Inh-resistant (R) Haarlem or Beijing bacteria. (A) Data represent 100% resistance (R) for ΔWT (wild type; band 17 is missing) and MUT1 (band 18) strains. (B) Data represent 100% sensitivity (S) for all WT (bands 16 and 17) strains. (C) 50% R for WT (bands 16 and 17) and MUT1 (band 18) strains, i.e., heteroresistance.

bacteria, which is the cutoff proportion for conventional drug susceptibility testing in liquid media for Inh, with either MTBDR*plus* or sequencing of *katG* (3, 4). However, with the LPA it was generally possible to detect 5% resistant bacteria with the S315T mutation. Due to the construction of the kit, the LPA is unable to detect the unspecified mutations in the heteroresistant suspensions. In patients with Inh-heteroresistant TB, only strains with the specific mutations represented in the MTBDR*plus* kit may be detected. The other mutations, normally detected by the absence of wild-type bands, will probably not be found in samples with heteroresistance (Fig. 2).

As molecular tests have been recommended by the WHO for use at the global level (http://www.who.int/tb/features_archive/policy_statement.pdf), it is important to consider the implications of a decreased ability to detect resistant bacteria in a heteroresistant patient sample. When testing patient specimens, either directly on a smear-positive specimen or after culturing, the detection of wild-type DNA in *katG* and *inhA* genes should be interpreted with care, as low proportions of Inh resistance in a mixture may be present, even though wild-type DNA is detected, as we showed in this study (Table 1), or Inh resistance can be caused by mutations in other genes (9). Therefore, if only molecular tests are done, either phenotypic DST and/or repeated molecular testing after some time is indicated in cases of lack of response to treatment. Furthermore, as the LPA or sequencing may be used during the course of treatment to test for development of resistance, it is important to keep in mind that resistance-related mutations might not be detected unless more than 1% of the bacteria are resistant to Inh.

Automatic cycle sequencing of the *katG* gene showed that a proportion of more than 10% resistant bacteria was required for detection of resistance by sequencing, making this method less sensitive. However, when minute peaks are also considered mutations instead of noise, the analytic sensitivity increases (Fig. 3). Pyrosequencing might detect lower levels of resistance than Sanger sequencing; it has previously been shown to be able to detect mixtures of wild-type and resistant strains of virus with a ratio of 20/80 (10) but, to our knowledge, has not been tested on heteroresistant mycobacteria.

Laboratories may use different culture media or culture conditions to ensure enough bacterial growth before DST. We tested the ability to detect resistance after culturing the different strains and mixtures on several media for different time periods and with or without incubation in CO_2. There were only small differences for some of the parameters, findings that may have been due to slight variations in inocula. From the data generated, it seems that, in general, laboratory practices do not influence the ability to detect resistance. Our findings are limited to Inh susceptibility testing and to only one mutation in *katG*. Other drugs, genes, and mutations might give different results.

In conclusion, the molecular tests in the study are useful for finding resistant TB in patient samples, but it appears that they are less efficient than conventional culture-based DST in finding resistance in samples with heteroresistant bacteria. Using the molecular methods, we could not detect 1% Inh-resistant bacteria in a mixture of susceptible and resistant *M. tuberculosis*, a result which we could obtain by using phenotypic DST with MGIT. With the LPA, however, it was possible to detect *M. tuberculosis* Inh resistance when 5% or more of the bacterial population was resistant. Automated cycle sequencing seems to be less sensitive than the LPA in detecting heteroresistance. Different laboratory practices to culture isolates prior to DST did not have an impact on detection of resistance with any method.

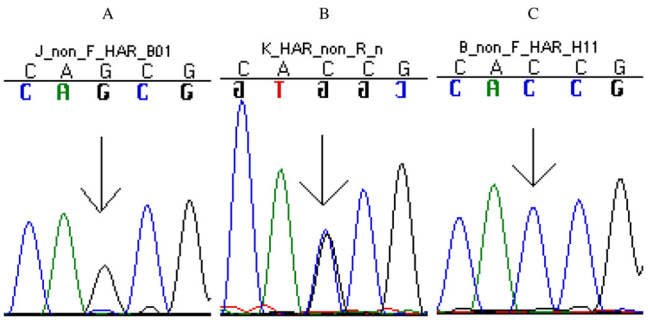

FIG 3 Examples of *katG* sequencing chromatograms of suspensions containing various proportions of Inh-susceptible (S) and Inh-resistant (R) Haarlem bacteria, showing that heteroresistance was difficult to detect. (A) 10% R, no mutation. (B) 50% R, heteroresistance. (C) 100% R (the S315T resistance mutation only).

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