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Revisiting susceptibility testing in MDR-TB by a standardized quantitative phenotypic assessment in a European multicentre study

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Objectives: Treatment outcome of MDR-TB is critically dependent on the proper use of second-line drugs as per the result of *in vitro* drug susceptibility testing (DST). We aimed to establish a standardized DST procedure based on quantitative determination of drug resistance and compared the results with those of genotypes associated with drug resistance.

Methods: The protocol, based on MGIT 960 and the TB eXiST software, was evaluated in nine European reference laboratories. Resistance detection at a screening drug concentration was followed by determination of resistance levels and estimation of the resistance proportion. Mutations in 14 gene regions were investigated using established techniques.

Results: A total of 139 *Mycobacterium tuberculosis* isolates from patients with MDR-TB and resistance beyond MDR-TB were tested for 13 antituberculous drugs: isoniazid, rifampicin, rifabutin, ethambutol, pyrazinamide, streptomycin, *para*-aminosalicylic acid, ethionamide, amikacin, capreomycin, ofloxacin, moxifloxacin and linezolid. Concordance between phenotypic and genotypic resistance was >80%, except for ethambutol. Time to results was short (median 10 days). High-level resistance, which precludes the therapeutic use of an antituberculous drug, was observed in 49% of the isolates. The finding of a low or intermediate resistance level in 16% and 35% of the isolates, respectively, may help in designing an efficient personalized regimen for the treatment of MDR-TB patients.

Conclusions: The automated DST procedure permits accurate and rapid quantitative resistance profiling of first- and second-line antituberculous drugs. Prospective validation is warranted to determine the impact on patient care.

Keywords: antibiotic susceptibility testing, DST, antituberculous drugs, TB eXiST, MGIT

Introduction

MDR-TB is a major problem in the global fight against TB.[1–3] MDR-TB is defined as resistance to at least isoniazid and rifampicin, the two cornerstones of the standard short-course chemotherapy.[4,5] Consequently, treatment of MDR-TB requires the use of second-line drugs such as fluoroquinolones and aminoglycosides.[6–8] Moreover, the treatment of XDR-TB, i.e. MDR-TB with additional resistance to fluoroquinolones and to amikacin, capreomycin or kanamycin, requires a hand-tailored regimen using the few remaining active drugs.

The treatment regimen for MDR- and XDR-TB is designed according to the results of drug susceptibility testing (DST), to ensure that the patient receives at least four drugs to which the *Mycobacterium tuberculosis* strain is susceptible.[6–8] Since administration of appropriate therapy is a major prognostic factor, accurate testing of second-line drugs is mandatory.[6,7,9–12] However, this testing is generally poorly standardized and often not quality controlled, which increases the risk of providing inadequate treatment.[13–16]

Several techniques, based on critical proportion and critical concentration, have been described for *M. tuberculosis* DST.[8,9,17–19] Procedures for determining isoniazid and rifampicin resistance are standardized and quality controlled, allowing for relatively accurate determination of resistance rates for MDR-TB surveillance at national and supranational levels.[2,3,14,15,20,21] However, for second-line drugs these procedures are in part unreliable.[13,15,22] In addition to poor reproducibility and extended laboratory turnaround times, currently established techniques provide no information on quantitative levels of drug resistance, limiting the possibility of individualized treatment.

Commercially available molecular tests have been recently implemented to detect mutations associated with resistance, especially to isoniazid and rifampicin.[23] In addition, reference laboratories use more extended sequencing of molecular targets to trace mutations associated with resistance.[24,25]

Mycobacterial growth indicator tube (MGIT) DST is a well-established, standardized, semi-automated liquid system that provides reproducible results for first-line antituberculous drugs on the basis of critical concentration testing using the Becton-Dickinson (BD) MGIT 960 instrumentation (BD, Sparks, MD, USA).[26] The suitability of the system for second-line DST has also been evaluated.[27] The TB eXiST (extended individual susceptibility testing) software (BD) for interpretation of DST on the basis of the MGIT instrumentation was developed to allow for continuous growth monitoring and more detailed analysis.[28] Here, we used the MGIT 960 instrumentation equipped with the TB eXiST software to establish quantitative DST for *M. tuberculosis*. The procedure was designed to combine the endpoints of proportion and concentration testing with continuous monitoring of bacterial growth. We validated the specificity of the resistance detected by assessing the concordance with genetic profiles of resistance. Quantitative DST provides results on quantitative measures of resistance, which can help in optimizing treatment regimens for MDR-TB patients.

Materials and methods

TB eXiST susceptibility testing protocol

The following antituberculous agents were tested: isoniazid, ethionamide, rifampicin, rifabutin, ethambutol, streptomycin, *para*-aminosalicylic acid, pyrazinamide, ofloxacin, moxifloxacin, amikacin, capreomycin and linezolid. Antituberculous drugs were purchased from Sigma (St Louis, MO, USA), except for pyrazinamide, linezolid and moxifloxacin, which were provided by BD, Pfizer (Singapore) and Merck (Whitehouse Station, NJ, USA), respectively. The drug concentrations selected for testing (Table 1) were based on literature data on drug resistance, distribution of the WT isolates [epidemiological cut-off (ECOFF)], critical concentrations and *in vivo* drug concentrations.[29–31] Stock solutions were prepared in-house according to the manufacturers' recommendations. A 10× solution was prepared in a volume of 200 μL and added to a 7 mL MGIT tube along with 0.8 mL of OADC (BD). The 100% inoculum for MGIT testing was prepared as follows. Briefly, strains were grown for an additional 2 days after MGIT reached positivity (growth units >400) and used as pure inoculum or strains were grown until 5 days after positivity and diluted to 1:5 in 7H9 supplemented with 10% OADC. After 10- and 100-fold dilutions in MGIT medium, 0.5 mL of the 100%, 10% and 1% inocula were distributed into the growth control tubes. The 1% inoculum was termed the growth control (GC). An equal volume of 0.5 mL of the 100% inoculum was distributed in all the drug-containing tubes.

Strains were submitted to a screening protocol in order to distinguish WT strains from strains suspected of resistance. This protocol consisted of testing all drugs at the lowest drug concentration (Table 1) used as a screening concentration, which corresponds to an optimized critical or ECOFF concentration. Strains were submitted to a quantitative testing protocol for those drugs for which they were suspected to be resistant on the basis of screening concentration testing. For quantitative testing, three to four concentrations per drug were used, except for pyrazinamide where only one concentration was tested (Table 1).

MDR-TB cases and participating centres

Patients living in a European country at the time of diagnosis, whatever their country of birth, were included if their *M. tuberculosis* strain was MDR. Each centre included between 10 and 30 randomly chosen MDR cases diagnosed in 2009; however, if there were <10 cases, cases from 2010 were also included (Table S1, available as Supplementary data at *JAC* Online). Clinical *M. tuberculosis* strains were subjected to resistome analysis, i.e. a search for resistance-associated gene mutations by using commercial line probe assays followed by PCR sequencing when necessary. The gene regions studied are listed in Table 2.

Expert laboratories from nine European countries (Belgium, France, Germany, Italy, the Netherlands, Portugal, Spain, Sweden and Switzerland) participated in the study. This protocol received agreement from the institutional ethics review boards of each centre. At least one representative of each centre participated at the meetings organized: (i) at the beginning of the study to agree on the protocol; (ii) following the calibration and proficiency testing to optimize the protocol; and (iii) several times during the study, i.e. when phenotypic (culture-based) and genotypic (gene mutation-based) results became available. Each centre followed its established procedures for quality assurance. A steering committee (E. C., M. V. and E. C. B.) met several times between the group meetings and analysed the data. The interlaboratory reproducibility of the protocol was assessed by comparing results obtained in each of the participant laboratories for a set of 10 *M. tuberculosis* control strains: 5 strains for calibration (strain susceptibility was known to the participant) and 5 strains for evaluating the proficiency testing (strain susceptibility was unknown to the participant). These strains were provided by the National Center for Mycobacteria, Institute of Medical Microbiology, University of Zurich. In addition, each centre tested three to five WT pansusceptible clinical strains isolated in their country. The calibration panel included three strains monoresistant to isoniazid (two with a *katG* S315T mutation and one with an *inhA* c−15t mutation conferring resistance also to ethionamide), one strain resistant to isoniazid (*katG* S315T) and streptomycin (*rrs* a523g), and one strain resistant to rifampicin and rifabutin

(*rpoB* H526Y). The proficiency testing included two strains with resistance to isoniazid (one *inhA* c−15t and one *katG* S315T), one strain resistant to rifampicin and rifabutin (*rpoB* S531L), one strain resistant to isoniazid (*katG* S315T) and to streptomycin (*rpsL* K43R), and one strain resistant to isoniazid (*katG* S315T) and to ethambutol (*embB* M306V).

Results

General features of TB eXiST DST

Drug resistance was initially detected by testing growth at the screening drug concentration. Resistance was subsequently quantified using higher drug concentrations. Since TB eXiST results are available in real time, the growth was recorded in parallel in control tubes and in drug-containing tubes. Figure 1 shows examples of growth curves for the three control tubes that contained 100%, 10% and 1% of the inoculum, resulting in three parallel curves separated by a theoretical mean of 3.5 days, which is the approximate time for an *M. tuberculosis* bacterial population to multiply by 10-fold. When growth was observed in the drug-containing tube before the GC (1% inoculum) was positive, this indicated that >1% of the population was growing in the presence of the drug, i.e. as per proportion testing the strain is considered resistant at the corresponding drug concentration. By studying bacterial growth at several drug concentrations, we observed that the quantitative levels of resistance differed significantly for the different antituberculous drugs and strains studied (Figure 1).

The time to result, calculated as the time elapsed until the GC became positive, was <30 days in all centres. Statistics, calculated in three of the nine centres, showed mean and median values of time to results between 10 and 11 days with a range of 8–30 days. For most isolates, the time to positivity in the drug-containing tubes was shorter than that of the GC and equal to that of the 100% inoculum, since for most strains and drugs the resistant proportion was close to 100%. The mean and median time to obtain resistance testing results was calculated as 4 and 5 days (range 3.5–13.5 days). Since growth curves were observed in a real-time manner, resistance could be suspected as rapidly as 5 days after inoculation (Figure 1b and c).

All laboratories performed the calibration and proficiency testing. A high rate of concordance (88%–97% with regard to strain and drug) was observed. Adjustments were necessary for the preparation of the stock solutions and we compared the results of quantitative resistance with regard to strains with the same genotype. This led to the exclusion of some of the data, for which we suspected a problem of drug preparation: rifabutin (19 results were excluded), *para*-aminosalicylic acid (15 results were excluded) and ethionamide (15 results were excluded).

Assessment of resistance for clinical strains

Testing growth at the screening concentration, i.e. the lowest drug concentration, distinguished susceptible strains from resistant strains (Table 1). Among the 139 MDR strains studied, resistance to fluoroquinolones and to amikacin or capreomycin was observed for 10 strains, corresponding to an XDR rate of 7.2%. Resistant strains were submitted to quantitative testing for confirmation of resistance by retesting at the screening drug concentration and for quantitative determination of the level of resistance by studying growth at two to three additional higher drug concentrations, except for pyrazinamide for which we tested only a single concentration. The resistance patterns are presented in Table 1.

All strains studied were MDR strains; however, various levels of resistance were observed for isoniazid and rifampicin. For isoniazid, four levels of resistance were observed: low-level resistance with growth at 0.1 mg/L, but no growth at 1 mg/L (11 strains); two intermediate levels of resistance with growth at 0.1 and 1 mg/L, but no growth at 3 and 10 mg/L (51 strains) or growth at 0.1, 1 and 3 mg/L, but no growth at 10 mg/L (58 strains); and high-level resistance with growth at all drug concentrations (19 strains). For rifampicin, the resistance was high level for 84% of the strains. Cross-resistance to rifabutin was observed for 98% of the strains, although the levels of resistance were different for the two rifamycins.

In total, 720 resistant characters were observed, with a resistant character being defined as one drug resistance observed in one isolate. Low-level resistance, i.e. resistance to the screening concentration, but susceptibility at higher drug concentrations, was observed in 16% of the test results and at varying rates according to the drug, from 5% (rifampicin) to 73% (ethambutol). Conversely, high-level resistance, i.e. resistance to the highest drug concentration tested, was observed in 49% of the isolates, at rates between 2% (ethambutol) and 84% (rifampicin) according to the drug. Antituberculous drugs could be divided into three groups: predominantly high levels of resistance were observed for rifampicin, rifabutin, amikacin; low levels of resistance were mainly observed for ethambutol; and various levels of resistance were observed for isoniazid, streptomycin, capreomycin, *para*-aminosalicylic acid, ethionamide, ofloxacin and moxifloxacin.

Genotypic investigation of resistance

Data on the number of resistant strains in which we found at least one mutation involved in resistance are presented in Table 2. When molecular assays did not detect a mutation in a resistant strain, we repeated the molecular analysis on bacteria grown in the drug-containing tubes in order to avoid false test results due to heteroresistance, i.e. coexistence of susceptible and resistant bacteria.[32,33] Genetic analysis was not done for *para*-aminosalicylic acid resistance since the underlying genetic mechanisms are still a matter of debate[34] and not for linezolid for which no resistant strains were observed. Overall, we observed resistance mutations in >80% of the resistant strains, except for ethambutol. Since the same mutation can confer cross-resistance, we also compared the phenotypes of isoniazid and ethionamide resistance in the case of *inhA* mutations, rifampicin and rifabutin resistance in the case of *rpoB* mutations, amikacin and capreomycin resistance in the case of a mutation in the *rrs* 1400 region and ofloxacin and moxifloxacin resistance in the case of *gyrA* mutations.

katG mutations were the most prevalent mutation (85%) in strains resistant to isoniazid, with S315T being the most frequent *katG* mutation (96%) and associated with an intermediate level of resistance (for the frequency of genotypes see Table 2, for genotype–phenotype relationships see Table 3 and for mutations in strains with low-level resistance see Table 4). Mutations in the *inhA* promoter region were associated with low-level isoniazid resistance. *rpoB* mutations at position 531 were the most

prevalent rifampicin resistance mutation (98/139, 71%) and were associated with high-level resistance to rifampicin and rifabutin. Altogether, 21 patterns of *rpoB* mutations were observed at 13 different codons, reflecting the diversity of the MDR strains in the nine European countries. We confirmed that *rpoB* mutations occurred also frequently at position 516 (12 strains) and at position 526 (14 strains). Mutations at position 526 were mostly associated with high-level resistance to rifampicin and rifabutin (11/14 for rifampicin and 9/11 for rifabutin). In contrast, mutations at position 516 were mostly associated with low-level resistance to rifabutin (8/10 resistant strains) or even no resistance (1/11 strains tested). Some, but not all, mutations are shown in Table 3. Resistance to ethionamide was associated with mutations in *inhA* (55%), in *ethA* (61%) or both (16%). Mutations in

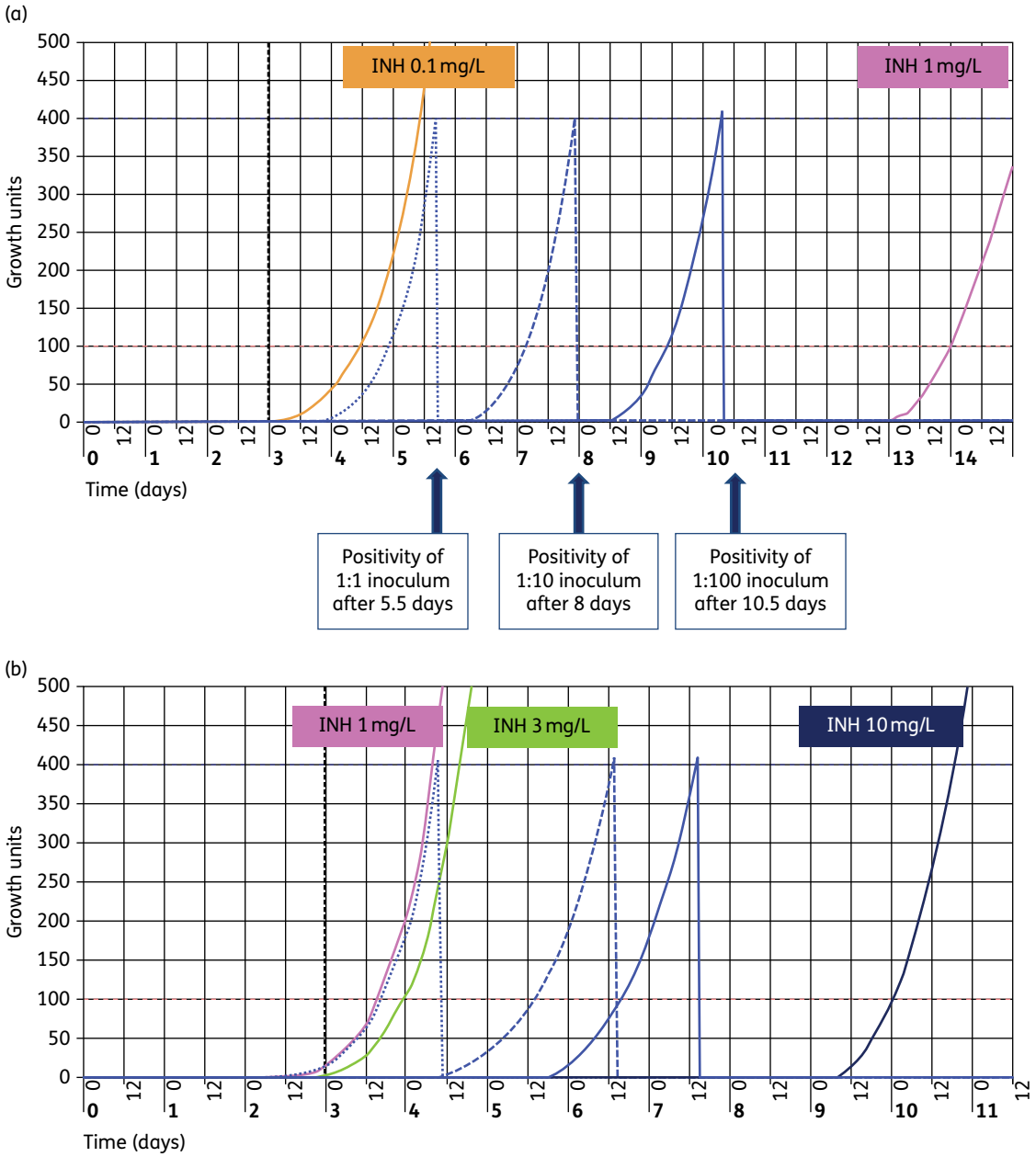

Figure 1. Representative results for quantitative resistance testing using the TB eXiST software. Growth in control tubes appears as blue lines; dotted line for 1:1; discontinuous line for 1:10 and continuous line for 1:100 (GC). (a) Low-level resistance to isoniazid (*inhA* c−15t): resistance to isoniazid at 0.1 mg/L (orange line) observed for 100% of the inoculum, but susceptibility at 1 mg/L (pink line). (b) Intermediate resistance to isoniazid (*katG* S315T): resistance to isoniazid observed at 1 mg/L (pink line) and at 3 mg/L (green line) for 100% of the inoculum, but susceptibility at 10 mg/L (dark blue line). (c) Homogeneous high-level resistance to ofloxacin (mutation *gyrA* D94A): resistance observed at 1 mg/L (pink line), 2 mg/L (green line) and 10 mg/L (dark blue line). (d) Heteroresistance for ofloxacin and intermediate level of resistance (*gyrA* A90V): resistance observed at 1 mg/L (pink line) and 2 mg/L (green line) for between 10% and 1% of the inoculum. See the text for interpretation of positive and negative growth results. INH, isoniazid; OFX, ofloxacin.

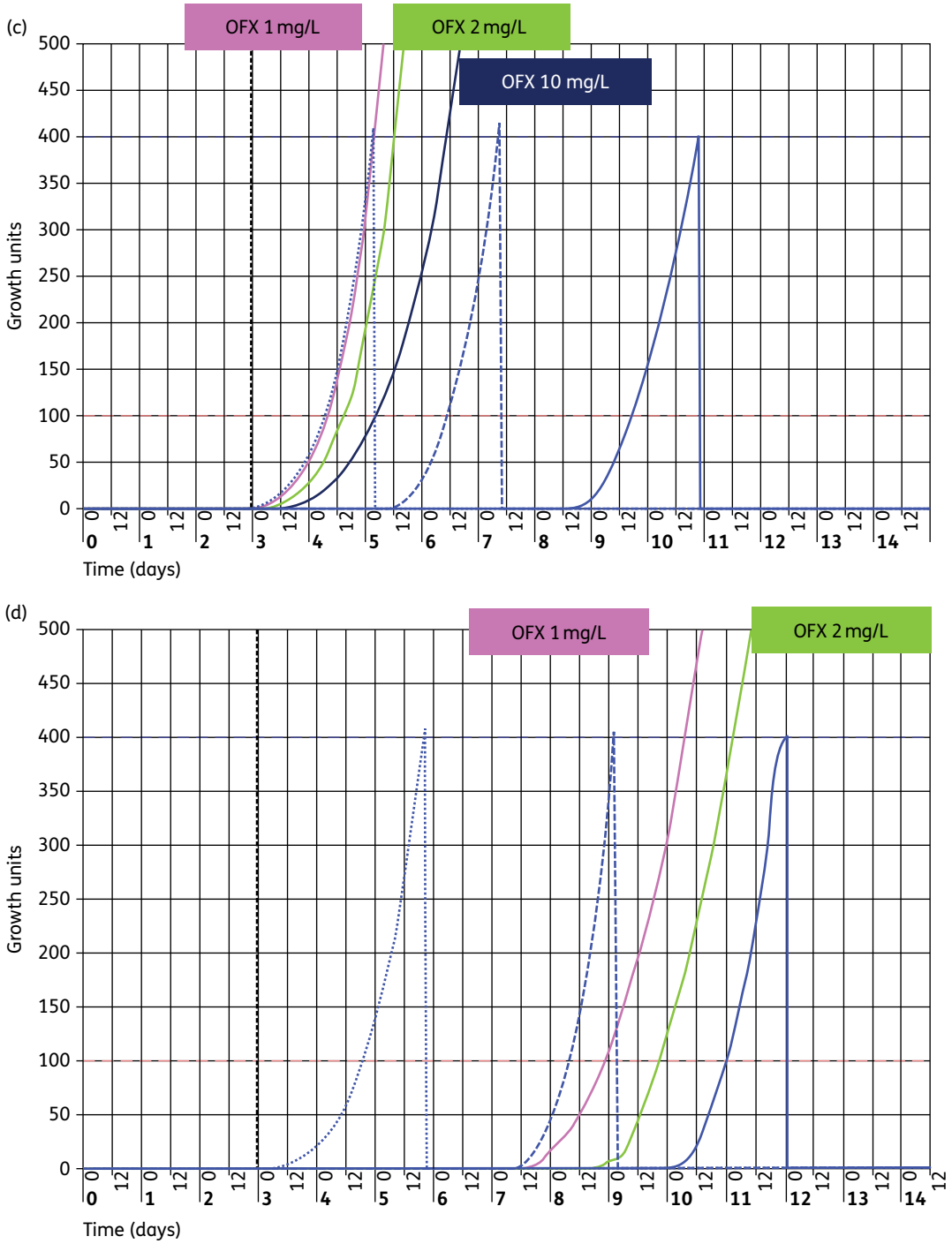

Figure 1. *Continued*

ethA were various with deletions and stop codons introduced at different sites of the gene sequence.

Phenotypic resistance to pyrazinamide, i.e. growth at the concentration of 100 mg/L, was highly associated with mutations in *pncA*: various missense mutations for 53 strains, deletion mutations for 4 strains and insertion mutations for 7 strains. In addition, pyrazinamidase production was negative in 31/33 (94%) resistant strains subjected to this biochemical test. This shows that both altered *pncA* gene and absence of pyrazinamidase production confirmed the resistant character. *embB* mutations at the hot-spot 306 codon were observed in 70% of the resistant strains, indicating either that the remaining strains harbour an unknown mechanism of resistance or that the screening concentration does not reliably discriminate between susceptible and resistant strains. However, most of the strains devoid of *embB* mutations showed only a low level of resistance. We noticed that mutations

Table 1. Phenotypic resistance observed for the 139 MDR strains of *M. tuberculosis* and detailed for the level of resistance (except pyrazinamide) by quantitative DST

Drug (list of concentrations tested in mg/L)	No. of resistant strains[a]	Low-level resistance[b]	Intermediate-level resistance[c]	High-level resistance[d]
Isoniazid (**0.1**, 1, 3, 10)	139	11 (8%)	109 (78%)	19 (14%)
Rifampicin (**1**, 4, 20)	139	7 (5%)	15 (11%)	117 (84%)
Rifabutin (**0.1**, 0.4, 2)	118	11 (9%)	23 (19%)	84 (71%)
Ethambutol (**5**, 12.5, 50)	56	41 (73%)	14 (25%)	1 (2%)
PAS (**4**, 16, 64)	11	4 (36%)	3 (27%)	4 (36%)
Pyrazinamide (**100**)	65	NT	NT	NT
Streptomycin (**1**, 4, 20)	107	17 (16%)	22 (21%)	68 (64%)
Ethionamide (**5**, 10, 25)	57	10 (18%)	10 (18%)	37 (65%)
Amikacin (**1**, 4, 20)	22	4 (18%)	0	18 (82%)
Capreomycin (**2.5**, 5, 25)	24	4 (17%)	20 (83%)	0
Ofloxacin (**1**, 2, 10)	25	3 (12%)	19 (76%)	3 (12%)
Moxifloxacin (**0.25**, 0.5, 2.5, 7.5)	22	3 (14%)	18 (82%)	1 (5%)
Linezolid (**1**, 4, 16)	0	0	0	0
Total resistant characters	720	115 (16%)	253 (35%)	352 (49%)

PAS, *para*-aminosalicylic acid; NT, not tested.
[a]Phenotypic resistance was determined by growth at the screening concentration (in bold). This concentration was repeated in the quantitative DST along with higher concentrations of the drug.
[b]Low-level resistance was defined as resistance at the screening concentration, but susceptibility at the intermediate drug concentration.
[c]Intermediate-level resistance was defined as resistance at the screening concentration and the intermediate concentration, but susceptibility at the highest concentration.
[d]High-level resistance was defined as resistance at all concentrations tested.

M306V and M306I were not associated with the same level of resistance to ethambutol: 42% (10/24) of the strains harbouring the mutation M306V were resistant at 12.5 mg/L, but only 7% (1/14) of those with the mutation M306I.

Resistance to streptomycin was associated either with a mutation in the *rrs* 530 region or in the *rpsL* gene encoding the S12 ribosomal protein (position 43 or 88, numbering system of *Escherichia coli*). For 23 streptomycin-resistant strains without *rrs* or *rpsL* mutations, we found a *gidB* mutation in 16 strains. This was more often associated with low-level resistance. For amikacin resistance, we observed the mutation a1408g in the *rrs* gene in all but four resistant strains, the latter showing resistance at a low level only. Capreomycin resistance was observed as cross-resistance with amikacin mediated by the *rrs* a1408g mutation and also as isolated capreomycin resistance with mutation in *tlyA* in five strains with no *rrs* mutation.

Resistance to fluoroquinolones correlated with *gyrA* mutations. However, the correlation was stronger for moxifloxacin than for ofloxacin, since three strains without *gyrA* and *gyrB* mutations showed a low level of resistance to ofloxacin, but susceptibility to moxifloxacin. The main problem for fluoroquinolone molecular resistance detection was heteroresistance, since in 72% (18/25) the *gyrA* mutation was evidenced only when molecular retesting was done on bacteria grown in drug-containing medium.

Discussion

The lack of standardization and reproducibility in DST of *M. tuberculosis* hinders the control of TB disease and facilitates the spread of drug-resistant organisms. This is especially true for second-line agents. In this multicentre study, we evaluated the feasibility and reliability of a standardized and automated DST procedure able to quantify levels of drug resistance to first- and second-line antituberculous drugs. The results of our DST measurements were validated by assessing the concordance with resistance-associated gene mutations.

The primary objective of DST is to distinguish resistant strains from susceptible strains, since resistance is prognostic of treatment failure and relapse.[35] Historically, two principles were followed in DST: critical drug concentration and critical proportion.[13,36,37] Several DST methods, including newly developed ones such as microscopic observation drug susceptibility, nitrate reductase assay and colorimetric tests, have since been described, all based on, but also limited by the principles of, critical proportion and critical drug concentration.[17,18,38] Unfortunately, DST is done differently not only in different parts of the world, but even within Europe. In Europe alone, three methods are mainly used for DST of *M. tuberculosis*: proportion testing, the absolute concentration method and the resistance ratio. As a result of the different methods used, it is difficult to compare the results from one laboratory with those from another laboratory, even for the same patient and the same strain. In addition, for most drugs and techniques, only a single drug concentration is tested while the quantitative levels of resistance may be important for treatment.

We aimed to improve DST for MDR *M. tuberculosis* by increasing the accuracy of resistance detection as well as that of susceptibility assessment. Since exhaustive testing is required for MDR-TB cases, we invested into a DST technique compatible with automation, standardization and rapid time to result. Automated procedures offer significant advantages in terms of personnel handling, standardization and quality control.

The DST procedure we developed follows a first step in which bacterial growth is assessed in the presence of a 'screening drug

Table 2. Phenotypic and genotypic resistance per antituberculous drug among the 139 MDR strains of *M. tuberculosis* tested

Drug	Phenotypic resistance[a]		Genotypic resistance		
	no. of resistant strains	resistance rate (%)	no. of strains with at least one mutation	rate of phenotypically resistant strains with mutation (%)	Gene and codon screened (no. of strains with mutation[b]; %[c])
Rifampicin	139	100	139	100	*rpoB* codons 511–533[d] (139; 100)
Rifabutin	118	100	118	100	*rpoB* codons 511–533[d] (118; 100)
Isoniazid	139	100	135	97	*katG* codon 315[e] and others (115; 85) *inhA* promoter (30; 22) *inhA* gene[f] (15; 11)
Ethionamide	57	46	49	86	*ethA* (30; 61) *inhA* promoter and *inhA* gene[f,g] (27; 55) *ethR* (0; 0)
Ethambutol	56	40	39	70	*embB* codon 306[e] (39; 100)
Pyrazinamide	65	47	64	98	*pncA* (64; 100%)
Streptomycin	107	77	98	92	*rpsL* codons 43–88[d] (68; 69) *rrs* region 530[d] (17; 17) *gidB*[h] (16; 16)
Amikacin	22	16	18	82	*rrs* region 1400[d]–1495 (18; 100)
Capreomycin	24	17	23	96	*rrs* region 1400[d]–1495 (18; 78) *tlyA*[i] (5; 22)
Ofloxacin	25	18	22	88	*gyrA* codons 88[e]–94 (22; 100) *gyrB*[j] (0; 0)
Moxifloxacin	22	16	22	100	*gyrA* codons 88[e]–94 (22; 100) *gyrB*[j] (0; 0)
PAS	11	9	NA	NA	NA
Linezolid	0	0	0	0	NA

PAS, *para*-aminosalicylic acid; NA, not applicable (for PAS resistance, gene mutations have not been established; for linezolid, no resistant strains were observed).
[a]Phenotypic resistance at the screening concentration.
[b]Strains may have mutations in several genes (see the text for details).
[c]Percentage of strains with mutation at this codon or in this gene.
[d]Numbering system of *E. coli*.
[e]Numbering system of *M. tuberculosis*.
[f]Sequence of the structural *inhA* gene.
[g]Sixty-seven out of 139 were studied for the entire *inhA* gene sequence.
[h]*gidB* was sequenced for 18 of the 23 streptomycin-resistant strains without mutation in both *rrs* and *rpsL*.
[i]*tlyA* was sequenced for the six capreomycin-resistant strains without mutation in *rrs*.
[j]*gyrB* was sequenced in eight ofloxacin-resistant strains among which three strains were without mutation in *gyrA*.

concentration' that is close to the ECOFF described recently for isoniazid, rifampicin, ethambutol and fluoroquinolones.[29–31] This concentration is the smallest MIC value for the WT strains, i.e. susceptible strains will not grow at this concentration. The second step was limited to strains resistant at the screening drug concentration. This second step confirmed the resistance by retesting the screening concentration and in addition provided a quantitative assessment of resistance by testing different drug concentrations. Knowing the level of resistance has two advantages: it gives information on the putative mechanism of resistance and it may have a significant clinical impact. Recent studies showed that an increase in the dosage of isoniazid or rifampicin may improve MDR outcome.[39,40] A low level of resistance can also be overcome by the use of a more potent drug such as the use of moxifloxacin instead of ofloxacin in case of defined *gyrA* mutations.[41,42] We also confirmed that some strains resistant to rifampicin are only low-level resistant to rifabutin and harboured a specific *rpoB* mutation at codon 516.[43,44] Although the clinical efficacy of rifabutin in these cases remains to be demonstrated, the lack of effective drugs in some MDR cases may benefit from rifabutin if still active. A low level of resistance to ethionamide, as we observed in the majority of strains, may allow the use of an ethionamide booster in the future.[45,46]

In addition to quantitative DST determination, we also searched for resistance-associated mutations with the view to confirm that most of the strains categorized as resistant were not WT strains and to increase our knowledge on the resistance levels these mutations confer. Hot-spot mutations were found as expected in *rpoB* at codon 531 for strains resistant to rifampicin, in *katG* at codon 315 for strains resistant to isoniazid, in *gyrA* at codon 90 or 94 for strains

Table 3. Genotype–phenotype relationship of the most prevalent resistance gene mutations

Drug	Mutation[a]	Low-level resistance[b]	Intermediate-level resistance[c]	High-level resistance[d]
Isoniazid	*katG* S315T (*n*=99)	1/99	92/99	6/99
	inhA promoter (*n*=6)	5/6	1/6	0/6
Rifampicin	*rpoB* S531L (*n*=98)	1/98	2/98	95/98
	rpoB D516V (*n*=12)	2/12	9/12	1/12
	rpoB H526L (*n*=3)	2/3	0/3	1/3
	rpoB H526Y (*n*=4)	0/4	1/4	3/4
	rpoB H526D (*n*=6)	0/6	0/6	6/6
Rifabutin	*rpoB* S531L (*n*=87)	0/87	17/87	70/87
	rpoB D516V (*n*=9)	6/9	2/9	1/9
	rpoB H526L (*n*=2)	1/2	0/2	1/2
	rpoB H526Y (*n*=3)	0/3	0/3	3/3
	rpoB H526D (*n*=5)	0/5	0/5	5/5
Ethambutol	*embB* M306I (*n*=14)	13/14	1/14	0/14
	embB M306V (*n*=24)	14/24	10/24	0/24
Ethionamide	*inhA* promoter (*n*=8)	2/8	2/8	4/8
	ethA (*n*=21)	4/21	3/21	14/21
Streptomycin	*rrs* a523c (*n*=7)	0/7	2/7	5/7
	rrs c526t (*n*=8)	3/8	4/8	1/8
	rpsL K43R (*n*=55)	0/55	2/55	53/55
	rpsL K88R (*n*=9)	0/9	5/9	4/9
	gidB[e] (*n*=16)	9/16	7/16	0/16
Amikacin	*rrs* a1408 mutation (*n*=18)	0/18	0/18	18/18
	rrs a1408 WT (*n*=4)	4/4	0/4	0/4
Capreomycin	*rrs* a1408 mutation (*n*=18)	3/18	15/18	0/18
	tlyA[f] (*n*=5)	1/5	4/5	0/5
Ofloxacin	*gyrA* A90V (*n*=3)	0/3	3/3	0/3
	gyrA S91P (*n*=5)	0/5	5/5	0/5
	gyrA D94G (*n*=7)	0/7	6/7	1/7
Moxifloxacin	*gyrA* A90V (*n*=3)	2/3	1/3	0/3
	gyrA S91P (*n*=5)	0/5	4/5	1/5
	gyrA D94G (*n*=7)	0/7	7/7	0/7

[a]Only single mutations were considered and mutations observed in less than three strains are not shown.
[b]Low-level resistance was defined as resistance at the screening concentration, but susceptibility at the intermediate drug concentration.
[c]Intermediate-level resistance was defined as resistance at the screening concentration and the intermediate concentration, but susceptibility at the highest concentration.
[d]High-level resistance was defined as resistance at all concentrations tested.
[e]Eighteen out 23 streptomycin-resistant strains with no mutation in *rrs* and *rpsL* were tested for *gidB*.
[f]The six capreomycin-resistant strains WT for *rrs* 1400 were tested for *tlyA*.

resistant to ofloxacin and moxifloxacin, in *rpsL* at codons 43 and 88 for strains resistant to streptomycin and in *rrs* at nucleotide a1408 for strains resistant to amikacin. Resistance mutations were scattered in genes coding for *ethA* and *pncA* as described previously, since many different mutations can confer a loss or decrease in the function of these proteins, which activate ethionamide and pyrazinamide, respectively.[47,48] Although some of the latter mutations have not been described yet, we consider it unlikely that they are related to simple gene polymorphisms.

To date, evidence is accumulating that the term 'resistance' in *M. tuberculosis* is not a biological entity, but quite heterogeneous and composed of quantitatively different levels of drug resistance. Most significantly, the quantitatively different resistance levels—low, moderate and high levels of resistance—are associated with different genetic mutations.[31,49] This biological diversity of resistance is not recognized by the current critical concentration or ECOFF testing, but requires measures of quantitative resistance determination. Through this multicentre study, we established and evaluated a standardized protocol for quantitative DST. The procedure developed integrates the established principles of critical concentration and critical proportion, combined with quantitative measures of drug resistance, automated detection and continuous monitoring, exploring drug susceptibility in the dimensions of concentration, proportion and time. Work still needs to be done to resolve some discrepancies between phenotypic and genotypic tests as in the case of ethambutol. In addition, group 4 and 5 antituberculous drugs[8] and new ones, such as bedaquiline and delamanid, will need to be tested.[50,51] Hopefully, a more thorough description of resistance in clinical strains will allow better exploitation of available antituberculous agents in the treatment

Table 4. Genotypic resistance detailed for the *M. tuberculosis* strains with a low level of resistance

Resistance phenotype (antibiotic concentration in mg/L)	No. of strains with low-level resistance	Mutations (no. of strains per genotype)[a]
R to INH (0.1), S to (1)	11	*inhA* promoter: c−15t (5), t−8c (1) *katG*: S315T (1), T149C (1) WT for *katG* and *inhA* (3)
R to RIF (1), S to (4)	7	*rpoB*: D516V (2), delN519 (1), H526L (2), S531L (1), L533P+N518D (1)
R to RFB (0.1), S to (0.4)	11	*rpoB*: L511P+D516G (1), D516V (6), D516Y (1), delN519 (1), S522L (1), H526L (1)
R to EMB (5), S to (12.5)	41	*embB*: M306V (14), M306I (13), D328G+G406A (1), WT (13)
R to STR (1), S to (4)	17	*rpsL*: WT (17) *rrs*: c523g (1), c526t (3), WT (13) *gidB*[b]: L16R+G73R (1), E40D (1), G69D (1), L74stop (1), L79W (1), A80P (1), S100F (1), A139V (1), deletion (1), WT (1)
R to ETH (5), S to (10)	10	*inhA* promoter: t−8c (1), c−15t (1), WT (8) *ethA*: M1R (1), I105L (1), deletion (2), WT (6)
R to AMK (1), S to (4)	4	*rrs*: WT (4)
R to CAP (2.5), S to (5)	4	*rrs*: a1408g (3) *tlyA*: V64G (1)
R to OFX (1), S (2)	3	*gyrA*: WT (3)
R to MXF (0.25), S (0.5)	3	*gyrA*: D89N (1), A90V (2)

INH, isoniazid; RIF, rifampicin; RFB, rifabutin; EMB, ethambutol; ETH, ethionamide; STR, streptomycin; AMK, amikacin; CAP, capreomycin; OFX, ofloxacin; MXF, moxifloxacin; R, resistant; S, susceptible.
[a]Only single mutations are listed, unless indicated otherwise. See Table 2 for numbering systems.
[b]*gidB* was tested for 10 of the 17 low-level streptomycin-resistant strains.

of drug-resistant TB disease, by tailoring therapy according to the resistance characteristics of individual strains. The time has come for personalized medicine in the treatment of drug-resistant TB disease.

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Supplementary data

Table S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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Supplementary data

Table S1. Number of Multidrug-resistant *M. tuberculosis* strains per center and resistance rates to antituberculous agents.

Antibiotic (screening concentration in mg/L)	Laboratory									**Total strains R (%)**
	B	**CH**	**E**	**F**	**G**	**I**	**N**	**P**	**S**	
	N=15	**N=15**	**N=7**	**N=16**	**N=14**	**N=19**	**N=30**	**N=10**	**N=13**	
INH (0.1)	15	15	7	16	14	19	30	10	13	139 (100 %)
RIF (1.0)	15	15	7	16	14	19	30	10	13	139 (100 %)
RFB (0.1)	15	14	7	16	13	ND	30	10	13	118 (98 %)
EMB (5.0)	11	7	6	3	7	1	14	4	3	56 (40%)
PZA (100)	6	12	4	4	9	9	7	7	7	65 (47%)
ETH (5.0)	ND	6	4	3	9	11	9	10	5	57 (46%)
STR (1.0)	13	13	5	10	12	13	23	10	8	107 (77%)
AMK (1.0)	7	1	0	1	1	3	3	4	2	22 (16%)
CAP (2.5)	7	1	0	0	1	4	4	5	2	24 (17%)
PAS (4.0)	ND	1	1	0	4	0	2	2	1	11 (9%)
OFX (1.0)	3	2	0	2	3	5	4	6	0	25 (18%)
MXF (0.25)	3	2	0	2	2	3	4	6	0	22 (16%)
LNZ (1.0)	0	0	0	0	0	0	0	0	0	0 (0%)

ND, no data since the testing for this antibiotic encountered technical problems

B, Belgium; CH, Switzerland; E, Spain; F, France; G, Germany; I, Italy; N, Netherlands; P, Portugal; S, Sweden.

INH, isoniazid; RIF, rifampicin; RFB, rifabutin; EMB, ethambutol; PZA; pyrazinamide; ETH, ethionamide; STR, streptomycin; AMK, amikacin; CAP, capreomycin; PAS, para-amino salicylic acid; OFX, ofloxacin; MXF, moxifloxacin; LNZ, linezolid.