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Delamanid susceptibility testing of *Mycobacterium tuberculosis* using the resazurin microtitre assay and the BACTEC™ MGIT™ 960 system

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Objectives: The objective of this study was to develop standardized protocols for rapid delamanid drug susceptibility testing (DST) using the colorimetric resazurin microtitre assay (REMA) and semi-automated BACTEC™ MGIT™ 960 system (MGIT) by establishing breakpoints that accurately discriminate between susceptibility and resistance of *Mycobacterium tuberculosis* to delamanid.

Methods: MICs of delamanid were determined by the MGIT, the REMA and the solid agar method for 19 pre-characterized strains. The MIC distribution of delamanid was then established for a panel of clinical strains never exposed to the drug and characterized by different geographical origins and susceptibility patterns. WGS was used to investigate genetic polymorphisms in five genes (*ddn*, *fgd1*, *fbiA*, *fbiB* and *fbiC*) involved in intracellular delamanid activation.

Results: We demonstrated that the REMA and MGIT can both be used for the rapid and accurate determination of delamanid MIC, showing excellent concordance with the solid agar reference method, as well as high reproducibility and repeatability. We propose the tentative breakpoint of 0.125 mg/L for the REMA and MGIT, allowing reliable discrimination between *M. tuberculosis* susceptible and resistant to delamanid. Stop codon mutations in *ddn* (Trp-88→STOP) and *fbiA* (Lys-250→STOP) have only been observed in strains resistant to delamanid.

Conclusions: We established protocols for DST of delamanid in the MGIT and REMA, confirming their feasibility in routine TB diagnostics, utilizing the same discriminative concentration for both methods. Moreover, taking advantage of WGS analysis, we identified polymorphisms potentially associated with resistance in two genes involved in delamanid activation.

Introduction

Despite the worldwide implementation of effective control programmes, TB continues to prevail as the most lethal infectious disease, with an estimated one-third of the global population being infected and 1.5 million deaths annually.[1] The TB epidemic is complicated further by the selection and transmission of *Mycobacterium tuberculosis* strains resistant to the most effective first- and second-line drugs.[1] Implementing the proper use of newly approved drugs is a life-saving priority. Delamanid (Deltyba™) is a novel anti-TB agent developed by Otsuka Pharmaceutical Co., Ltd (hereafter referred to as Otsuka) that received marketing authorization by the EMA, the Korean Ministry of Health and Welfare and the Japanese Ministry of Health, Labour and Welfare in 2014.[2] The compound has proved to be well tolerated, to accelerate culture conversion, to improve treatment outcome and to significantly lower mortality of MDR-TB patients.[3–5] Delamanid has been approved for the treatment of MDR-TB, defined by resistance to at least isoniazid and rifampicin. It is recommended by the WHO for treatment of MDR-TB cases otherwise not manageable with currently available regimens.[6] Delamanid derives from the nitro-dihydro-imidazooxazole class of compounds and specifically impairs the biosynthesis of methoxy- and keto-mycolic acids, which are components of the

mycobacterial cell wall.[7] It exerts its effects primarily on species of the *M. tuberculosis* complex, in both replicating and non-replicating stages. Its bactericidal activity against intracellular *M. tuberculosis* in human macrophages was confirmed in 2006 by Matsumoto *et al*.[7] Delamanid is equally potent against drug-susceptible and MDR *M. tuberculosis* strains with a range of reported *in vitro* MICs from 0.02 to 0.006 mg/L.[7,8] *In vivo*, the compound shows high therapeutic activity even at low dose.[7–10] Delamanid is a pro-drug, requiring metabolic activation by an F420-dependent mycobacterial nitroreductase encoded by the gene *Rv3547* (*ddn*). Co-factor F420 is synthesized and reactivated by a group of enzymes encoded by the genes *fgd1*, *fbiA*, *fbiB* and *fbiC*. Polymorphisms in any of these five genes have been shown to lead to *in vitro* delamanid resistance.[7,9,11] The rate of spontaneous mutations conferring delamanid resistance is estimated to be between 6.4×10^{-6} and 4.2×10^{-5}, which is higher than the average mutation rate of the *M. tuberculosis* genome, highlighting the need to improve delamanid drug susceptibility testing (DST) capacities.[9] Ten years after its discovery and more than a year after conditional approval, only a validated solid-medium-based DST is available, while a liquid-medium-based DST has not yet been established.

In our study, we aimed: (i) to investigate the distribution of MICs of clinical isolates of different geographical origins that showed various patterns of susceptibility to the most important anti-TB drugs and that have never been exposed to delamanid; (ii) to provide standardized protocols for delamanid DST using the resazurin microtitre assay (REMA) and the semi-automated BACTEC™ MGIT™ 960 system (MGIT) with the pure substance; (iii) to determine the breakpoint of delamanid DST in both REMA and MGIT, defined as the concentration that allows discrimination of susceptible from resistant isolates in an assay determining MICs by exposing the bacteria to a series of declining delamanid concentrations; and (iv) to confirm phenotypic results by the identification of genetic polymorphisms in the five genes of the F420 biosynthesis pathways involved in delamanid activation.

Materials and methods

Study design and strains

The study was carried out in parallel in two different WHO TB Supranational Reference Laboratories (SRLs; München-Gauting and Milan), both having received ethics approval for the study. The two laboratories have been previously validated by Otsuka for their capacity to perform agar-based DST of delamanid using the proportion method protocol developed by the company. The study design included three different work packages: (i) validation of MICs determined using the REMA and MGIT against the standard agar-based protocol (provided by Otsuka) on a panel of 19 pre-characterized strains with known levels of delamanid resistance (kindly provided by Otsuka); (ii) determination of MIC distribution of delamanid in both the REMA and the MGIT in 194 (REMA) and 139 (MGIT) clinical isolates never exposed to the drug; and (iii) WGS of study strains performed in order to correlate MICs to genetic polymorphisms in the five genes involved in the mechanism of delamanid activation.

The strains originated from four different WHO geographical regions. All strains were checked for absence of contamination before their use. We used delamanid concentrations ranging from 32.0 to 0.0005 mg/L in the REMA and from 16.0 to 0.0005 mg/L in the MGIT in order to determine solid bell-shaped probabilistic MIC distributions in both systems. Susceptibility to delamanid was further confirmed by the agar proportion method. The reproducibility and repeatability of the REMA and MGIT were evaluated by repeated testing of clinical isolates. To evaluate repeatability, strains were tested in quadruplicate, starting from the same bacterial suspension, on the same day by a single operator. To evaluate reproducibility, a batch of clinical isolates from each SRL was blindly cross-checked with the respective other SRL by different operators, using different delamanid stock solutions and fresh bacterial suspensions. Reproducibility and repeatability were calculated as the percentages of MIC values having a >2-fold dilution difference.

Antimicrobial agents

Pure delamanid powder was provided by Otsuka and dissolved in DMSO (Sigma–Aldrich, St Louis, MO, USA). Stock solutions were aseptically prepared at concentrations of 8.0 and 16.0 mg/L for the REMA and MGIT, respectively; they were considered self-sterilizing and were stored in aliquots between −70 and −80°C. Working solutions were prepared by further diluting aliquots of stock solutions in DMSO for DST using the MGIT and the agar proportion method, and in modified Middlebrook 7H9 broth for the REMA. The final test concentrations used were obtained by serial 2-fold dilutions from 16.0 to 0.0005 mg/L for the MGIT and 7H11 and from 32.0 to 0.0005 mg/L for the REMA. Frozen aliquots of stock solutions were thawed once and discarded afterwards; working solutions were not stored. The shelf life of delamanid stock solutions, stored at −80°C, was determined to be at least 6 months by repeating delamanid DST of the *M. tuberculosis* H37Rv (ATCC 27294) reference strain at the beginning and end of the study, in both the REMA and the MGIT.

Mycobacterial suspensions

The mycobacterial suspensions for the REMA and the proportion method were prepared from 21- to 28-day-old Lowenstein–Jensen (LJ) subcultures. Using a sterile loop, *M. tuberculosis* colonies were transferred to a tube containing 6–10 glass beads in modified 7H9 broth [Middlebrook 7H9 broth containing 0.1% casitone and 0.5% glycerol with 10% OADC (oleic acid, dextrose, catalase) supplement (Becton Dickinson, Sparks, MD, USA)]. Clumps were disrupted by vortexing for 60 s in the presence of the beads and the suspension was allowed to sediment for 20 min. Part of the supernatant was transferred to a new tube. The suspension was adjusted to a turbidity equivalent to that of a 0.5 McFarland standard and the suspension was diluted 10^{-2} to 10^{5} cfu/mL (suspension A) and 10^{-4} to 10^{3} cfu/mL (suspension B) in 7H9 broth.

Agar proportion method

MIC determination in an agar-dilution proportion method assay on Middlebrook 7H11 agar enriched with 10% OADC supplement was performed in polystyrene plates containing serially diluted drug concentrations and two drug-free plates as controls to monitor the 1% proportion of inhibition. One-hundred microlitres of suspension A was inoculated on delamanid-containing media and 100 μL of suspension B on control plates. All plates were incubated at 37°C with 5% CO_2 until colonies became visible to the naked eye on control plates (minimum 21 days). Assays were considered valid when control plates showed 50–200 colonies. The MIC was defined as the lowest delamanid concentration that inhibited 99% of growth.

REMA

The assay was carried out as previously described.[12] One-hundred microlitres of suspension A was used as inoculum per well and 100 μL of serial 2-fold dilutions of delamanid working solution was added to each well of a sterile, polystyrene 96-well flat-bottom plate (Becton Dickinson). A drug-free growth control and a *M. tuberculosis*-free sterility control of the medium were included in each plate. Two-hundred microlitres of sterile water was

added to all outer perimeter wells to avoid evaporation during incubation. Plates were then covered with self-adhesive membranes and incubated at 37°C. After 7 days, the assay was stained by adding 32 μL of a freshly prepared solution of resazurin (alamarBlue®, Sigma–Aldrich, Taufkirchen, Germany) in 3.7% Tween 80. The plates were re-incubated for an additional 24 h at 37°C. A change in colour from blue (oxidized state) to pink (reduced state) indicated growth of the bacteria, and the MIC was defined as the lowest concentration of drug that prevented this change in colour. Each lot of REMA plates was quality assured by confirming whether the MIC of *M. tuberculosis* H37Rv ATCC 27294 fell within the range 0.016–0.002 mg/L.

DST in MGIT

MIC values were determined at both study centres using the MGIT connected to a BD EpiCenter™ Microbiology Data Management System (Becton Dickinson) equipped with TBeXIST modules for quantitative DST. For each strain, a bacterial suspension (M) was prepared from MGIT subcultures according to the manufacturer's instructions (BACTEC™ MGIT™ 960 System User's Manual: Becton Dickinson Document Number MA-0117). For DST, 0.1 mL of delamanid at the respective concentration dissolved in DMSO (final DMSO concentration 1.19%) and 0.5 mL of suspension M were added to each MGIT tube supplemented with 0.8 mL of OADC. One drug-free control tube per test series was inoculated with 0.5 mL of a 10^{-2} dilution of suspension M (representing 1% of the bacterial concentration in the drug-containing tubes) after the addition of 0.1 mL of pure DMSO and 0.8 mL of OADC. The delamanid MIC in MGIT was defined as the lowest drug concentration that kept growth <100 growth units at the time when the BACTEC™ 960 reader indicated 400 growth units for the drug-free control.

DNA isolation and WGS

Genomic DNA was extracted from cultured *M. tuberculosis* isolates using the cetyl trimethylammonium bromide (CTAB) method as previously described.[13] Prior to submission for WGS, DNA was quality assessed and quantified using the Qubit dsDNA BR assay (Life Technologies, Paisley, UK). Paired-end libraries of read length 101 bp were prepared using the Nextera XT DNA Sample Preparation kit (Illumina Inc., San Diego, CA, USA) and sequenced on an Illumina HiSeq 2500 platform according to the manufacturer's instructions. Strains showing a coverage of at least 20× were considered for SNP analysis. Total variant calling was performed by using the PhyResSE web tool.[14]

Results

Determination of delamanid MIC and genetic characterization of a panel of reference strains

Delamanid MIC values of 19 pre-characterized control strains with known levels of delamanid resistance identified five resistant strains (MIC >0.2 mg/L) and 14 susceptible strains (MIC <0.2 mg/L) with the agar proportion method, REMA and MGIT (100% concordance of all three methods; Table S1, available as Supplementary data at *JAC* Online). WGS analysis of the 19 strains revealed the presence of indels and non-synonymous mutations affecting the *ddn* gene of the 5 delamanid-resistant strains (Table S2).

Distribution of MICs in clinical strains

The 194 study isolates were recovered from clinical samples of patients originating from Europe (83), Africa (44), America (2) and Asia (65). The susceptibility patterns of the strains included 56 (29%) strains that were pan-susceptible to first-line anti-TB drugs (DS-TB), 17 (9%) with drug resistances not fulfilling MDR criteria (non-MDR), 63 (32%) MDR, 32 (16.5%) pre-XDR (defined as MDR-TB plus resistance to either a fluoroquinolone or one of the second-line injectables) and 26 (13.5%) XDR (defined as MDR-TB plus resistance to a fluoroquinolone and at least one second-line injectable). Testing the isolates with doubling concentrations of delamanid (from 0.0005 to 32.0 mg/L in the REMA and from 0.0005 to 16.0 mg/L in the MGIT), we observed bell-shaped MIC distributions in both the REMA and the MGIT. The modal values for the REMA and MGIT were 0.004 and 0.016 mg/L, respectively, and the epidemiological cut-off (ECOFF) values 0.03 and 0.06 mg/L (Figure 1). The curve of MGIT MIC values was slightly skewed to higher values compared with the curve of REMA MICs, but this difference was not significant in the rank sum test (Wilcoxon–Mann–Whitney *U*-test, $P=0.65$), and there was 100% agreement with regard to resistance and susceptibility to delamanid when selecting any breakpoint between 0.125 and 0.25 mg/L, i.e. close to the reference breakpoint proposed by Otsuka for the agar proportion method (Table 1). Delamanid-susceptible isolates showed MICs between 0.03 and 0.0005 mg/L in REMA and between 0.06 and 0.002 mg/L in MGIT. We identified four resistant strains (one XDR and three MDR *M. tuberculosis* strains) with MIC values >32.0 and >16.0 mg/L in the REMA and MGIT, respectively. All resistances to delamanid were phenotypically confirmed by the proportion method on Middlebrook 7H11 agar.

Repeatability and reproducibility of REMA and MGIT testing

Delamanid MIC values of 14 and 3 *M. tuberculosis* strains were determined in quadruplicate in the REMA and MGIT, respectively. MIC values deviated maximally by one titre step from the average MIC of each isolate, proving excellent repeatability of both assays (Tables S3 and S4). The reproducibility of MIC values was evaluated by cross-checking the results in the respective other SRL of five and 28 clinical isolates in REMA and in MGIT, respectively. Four and 21 isolates yielded identical MIC values in the REMA and MGIT, respectively. MIC values of the other eight isolates differed maximally by one titre step (Tables S5 and S6).

Genome analysis

One-hundred-and-fifty-nine strains reached read coverages of >20× (mean coverage 71.2×) and were considered for SNP analysis, obtaining >98% alignment relative to the *M. tuberculosis* H37Rv reference genome (GenBank ID NC_000962.3). WGS data were submitted as fastq files to the Sequence Read Archive of the National Center for Biotechnology Information (accession number SRP068011). Ten *M. tuberculosis* complex lineages were identified by WGS analysis, with the Beijing family as the most represented (Figure S1). Genome analysis of the 155 delamanid-susceptible strains revealed the presence of 11 different non-synonymous substitutions affecting the five genes *ddn*, *fgd1*, *fbiA*, *fbiB* and *fbiC* in 35 (22.6%) strains (Table 2). Nine different synonymous mutations were detected in genes *fgd1*, *fbiA* and *fbiC* (Table 2). Analysis of the four delamanid-resistant strains revealed the presence of the stop codon mutations Trp-88→STOP and Lys-250→STOP in *ddn* (three strains) and *fbiA* (one strain), respectively (Table 3). These mutations were not observed in any of the 155 delamanid-susceptible strains. All the resistant strains belonged to the Beijing family.

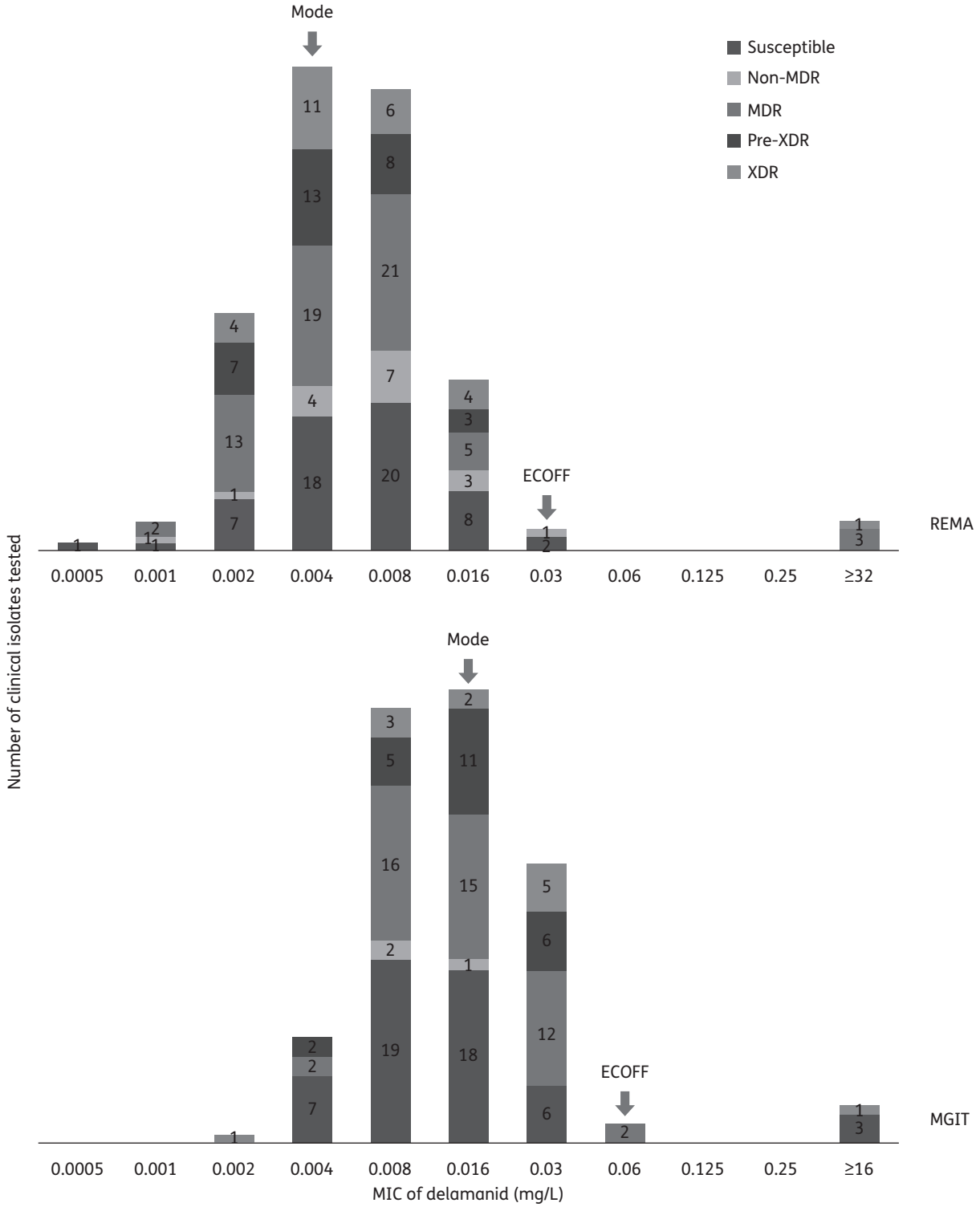

Figure 1. Distribution of delamanid MICs (mg/L) in the REMA and MGIT for a panel of 194 and 139 clinical isolates, respectively. Strains were stratified based on their susceptibility patterns. ECOFF and modal values are indicated.

Discussion

The introduction of new antimicrobials on the market should be accompanied by simultaneous establishment of standardized and reproducible protocols for *in vitro* DST in order to avoid early development of drug resistance and transmission of resistant strains. In the particular case of TB, there is a paucity of new compounds in the development pipeline, and all efforts should be made

to protect the two new anti-TB drugs now available, i.e. bedaquiline and delamanid, from early development of drug resistance.[15,16] Our finding of four isolates displaying spontaneous resistance to delamanid emphasizes the urgency of DST before any clinical use of the drug in treatment regimens, in order to prevent selection and further transmission of delamanid-resistant strains, as previously seen with other anti-TB drugs.[1] Furthermore, adjusting the treatment strategy to the *in vitro* susceptibility patterns not only improves the treatment outcome at the individual level but also decreases the risk of transmission and further spreading of drug-resistant strains in the community. DST in liquid media yielding results in <10 days on average is 3 weeks faster than the agar-based proportion method, which means earlier detection of drug resistance, earlier adjustment of treatment and earlier interruption of transmission of drug-resistant forms of TB.[17–19] For these reasons, we established standard protocols for delamanid DST in both the MGIT and the REMA and validated them by cross-referencing and comparison with the proportion method, which was previously proposed by Otsuka and validated in a multicentre approach that included the two SRLs involved in this study (D. M. Cirillo and H. Hoffmann, unpublished data). Our results confirmed that the MGIT and REMA can be used for accurate determination of delamanid MIC, with reproducibility and repeatability of 100% and excellent concordance with the solid agar reference method. The fact that the bell-shaped MGIT MIC distribution curve was slightly closer to higher MIC values compared with the REMA curve might be explained by the fact that the MGIT is a modified proportion method using Middlebrook 7H9 broth, which does not provide exact MIC values, while the REMA is a microdilution assay designed for precise MIC determination.[12] Despite these slight differences, both assays clearly distinguished susceptible from resistant strains. None of the study isolates had MIC values between 16.0 and 0.06 mg/L in either assay. Thus, any concentration between these two extremes might be eligible as a breakpoint MIC for the distinction between susceptibility and resistance to delamanid. Following the rules of EUCAST, breakpoints should be defined rather conservatively, i.e. close to the ECOFF.[20] Additionally to the MIC distribution of the relevant species, the

Table 1. Correlation of drug susceptibility results obtained for 112 clinical strains tested in the MGIT and REMA applying any breakpoint between 0.125 and 0.25 mg/L, i.e. close to the reference breakpoint proposed by Otsuka for the agar proportion method for the determination of susceptibility and resistance

	REMA DST		
MGIT DST	resistance	susceptibility	total
Resistance	4	0	4
Susceptibility	0	108	108
Total	4	108	112[a]

[a]Among 139 clinical isolates tested in the MGIT, 27 did not give a valid result in the REMA.

Table 2. List of SNPs identified in the candidate genes *ddn*, *fgd1*, *fbiA*, *fbiB* and *fbiC* by WGS analysis of 155 delamanid-susceptible *M. tuberculosis* strains

Gene	Genome position	Nucleotide substitution	Amino acid change	Number of strains with the SNP
ddn (Rv3547)	3987057	C→T	Arg-72→Trp	2
	3987092	G→C	Glu-83→Asp	1
fbiA (Rv3261)	3640879	C→T	Leu-113→Leu	1
	3640901	A→G	Gln-120→Arg	3
	3640974	T→G	Pro-144→Pro	3
	3641447	C→T	Thr-302→Met	2
	3641289	A→G	Glu-249→Glu	1
fbiB (Rv3262)	3642874	T→G	Lys-447→Arg	1
	3642877	A→G	Lys-448→Arg	1
	3642192	T→C	Phe-220→Leu	8
fbiC (Rv1173)	1303095	G→A	Leu-55→Leu	2
	1303131	T→C	Asp-67→Asp	1
	1303476	G→C	Leu-182→Leu	2
	1303747	A→G	Thr-273→Ala	5
	1304972	C→T	Thr-681→Ile	1
	1305363	G→A	Leu-811→Leu	1
fgd1 (Rv0407)	491247	T→C	Tyr-155→Tyr	3
	491668	A→G[a]	Lys-296→Glu[a]	1
	491591	A→T[a]	Lys-270→Met[a]	12
	491742	T→C	Phe-320→Phe	72

For each SNP the amino acid substitution with the genome position, the corresponding codon number and the number of strains harbouring the polymorphism are indicated.
[a]SNPs previously described as lineage-specific mutations of Haarlem (K270M) and *M. africanum* WA2 (K296E) genotypes.

Table 3. Mutation analysis of the four delamanid-resistant *M. tuberculosis* strains

		ddn (Rv3547)			*fbiA* (Rv3261)		
Strain	Resistance pattern	genome position[a]	nucleotide substitution	amino acid change	genome position[a]	nucleotide substitution	amino acid change
Delamanid_R1	MDR	3987106	G→A	Trp-88→STOP		WT	WT
Delamanid_R2	MDR	3987106	G→A	Trp-88→STOP		WT	WT
Delamanid_R3	MDR	3987106	G→A	Trp-88→STOP		WT	WT
Delamanid_R4	XDR		WT	WT	3641290	A→T	Lys-250→STOP

The four strains did not show SNPs in the genes *fgd1*, *fbiB* and *fbiC*.
[a]With reference to *M. tuberculosis* H37Rv genome GenBank number NC_000962.3.

following data should be considered when defining the breakpoint of a new antimicrobial agent: (i) proposed dosages of the agent being assessed; (ii) proposed indications for the agent; (iii) proposed target organisms; (iv) pharmacokinetics and pharmacodynamics; and (v) clinical trial data.[21] Though the safety of delamanid was proved at dosages of up to 400 mg daily, early bactericidal activity was found optimal in dosages of 200–300 mg per day.[10] Delamanid is licensed at the dosage of 100 mg twice daily, i.e. at the lower limit of dosages with proven activity. With this, average plasma concentrations reach 0.372–0.562 mg/L. This is just two to three titre steps above the ECOFF concentration observed in our study and previous studies.[7,22] Though the tissue concentrations of the compound were almost 3-, 4- and 10-fold higher (1.563, 2.033 and 5.193 mg/L, measured 8 h after administration of the drug) in lungs, kidneys and livers, respectively, of rats, these concentrations have not yet been confirmed in human tissues (N. Hittel, Otsuka Novel Products GmbH, Munich, Germany, personal communication).[9] The only licensed indication for the use of delamanid is the treatment of MDR-TB patients.[9] This patient group by definition leaves few options for combination regimens with other active compounds. Recently, we identified an XDR-TB patient with bedaquiline resistance who had subsequently developed additional delamanid resistance upon treatment with a delamanid-containing regimen.[23] Thus, the lower the MIC breakpoint of delamanid, the higher the probability that the drug contributes with sufficient efficiency to the treatment of the patient and that resistance can be prevented. In view of these data, we decided to propose the most conservative cut-off. Thus, both the optimal tentative breakpoint MIC for the REMA and the most accurate tentative critical concentration in the MGIT would be 0.125 mg/L. Very few anti-TB drugs are characterized by a comparably sharp demarcation of resistance. Isoniazid, rifampicin, aminoglycosides and fluoroquinolones have MIC distributions in *M. tuberculosis* resembling that of delamanid, but all drugs are characterized by a relatively high number of low-level resistant isolates.[24–27] Low-level resistance results from genetic polymorphisms leading to amino acid changes in the target proteins and increased MICs of the respective anti-TB drugs, but these do not reach the levels of the defined MIC breakpoint. Recently, the group of Armand van Deun[28] proved that low-level resistance to rifampicin might lead as frequently to unfavourable treatment outcome as full resistance. So far, we have only found clear resistance or susceptibility and no evidence of low-level resistance to delamanid. This might change when delamanid has been in use for a longer period of time and more strains with delamanid resistance-inducing genetic polymorphisms have been selected. We propose that the critical concentration in the MGIT should be the first concentration above the ECOFF in order to detect low-level resistance, which might develop in relation to increased use of delamanid in MDR-TB treatment. The tentative MIC breakpoint in the REMA is proposed to be two titre steps above the ECOFF in order to allow more inter-laboratory variation, which is intrinsic to this test; this test is less standardized than the MGIT and might lead to false resistant results if the breakpoint is set too conservatively.

The stop mutations in the *ddn* and *fbiA* genes only occurred, however, in the four delamanid-resistant strains, which had never been exposed to the drug. In our previously reported case a mutation had been selected in the *fbiA* gene (D49Y) during delamanid treatment.[23] The FbiA and FbiB enzymes modify flavin F0 further to F420. F0 is synthesized by FbiC.[29,30] Delamanid is intracellularly activated by the F420-dependant nitroreductase Ddn (Rv3547).[9] F420 is recycled into the reduced form by a glucose-6-phosphate dehydrogenase (Fgd1). Polymorphisms in one of those enzymes might lead to low- or high-level resistance. These very preliminary data suggest that amino acid changes in Ddn and FbiA seem to be particularly relevant. Of note, polymorphisms have been described in all five genes without indication of delamanid resistance. We observed two of those polymorphisms that occurred in the *fgd1* gene and had been previously described as potential lineage-specific polymorphisms of the Haarlem [K270M, observed in 12 study strains (7.6%), all belonging to the Haarlem genotype] and West African 2 [K296E, one *Mycobacterium africanum* 2 (0.58%)] genotypes.[31,32] The other nine variants identified in this study were harboured in the genes *ddn*, *fbiA*, *fbiB* and *fbiC* and have not been previously described in *M. tuberculosis*. Our findings may have great implications for the development of molecular resistance tests for delamanid in the near future, which might further accelerate the turn-round time for delamanid DST. Moreover, with the increasing use of delamanid, there should be a strong recommendation to monitor the onset of drug resistance to delamanid by testing strains serially collected from patients under treatment. The establishment of a repository of strains collected from patients during treatment will provide a precious resource for future studies on mechanisms of resistance to delamanid. Interestingly, taking advantage of WGS, we found that all four strains resistant to delamanid belonged to the Beijing family. Our data did not show any

statistically significant association between resistance to delamanid and the lineage of the strains, despite our finding that all delamanid-resistant strains identified in this study belonged to the Beijing lineage. This hypothesis was further supported by the identification of lineage-specific SNPs not related to delamanid resistance in strains belonging to the Haarlem and West African 2 lineages, which were phenotypically susceptible to delamanid. Even though clinical strains resistant to delamanid showed resistance to other anti-TB drugs, we did not observe any statistically significant association between delamanid resistance and the MDR/XDR phenotype for the strains included in the study.

Recently, Keller *et al.*[22] proposed a DST protocol for delamanid in the MGIT, but the study had two major weaknesses: (i) the sample size of strains (25 *M. tuberculosis* isolates) was rather small; and (ii) the authors used delamanid derived from mortared pills instead of pure substance, as recommended by WHO.[10] Thus, the authors were not able to propose a tentative breakpoint for delamanid DST. The major strengths of our work are the use of a pure compound, the large collection of strains originating from four different continents and demonstrating a large variety of susceptibility patterns, the evaluation of the tests performed independently by two different Supranational Reference Laboratories, and the availability of WGS data for a representative majority of strains. Nonetheless, we acknowledge some limitations. During the initial validation phase using pre-characterized strains provided by Otsuka, the laboratories were blinded to the exact MIC but not to their resistance status (tested by the agar proportion method); however, the validity of the susceptibility testing has been re-confirmed by blinded cross-checks of the results in the respective other SRL. The two laboratories performing the tests are both included in the SRL network, with proven high capacity to perform both phenotypic and genotypic tests, which might therefore not represent the average capacity of TB diagnostic laboratories in high-prevalence countries. Therefore, the robustness of our DST protocol in the MGIT should be re-confirmed by other laboratories and further validated under routine diagnostic conditions.

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Transparency declarations

None to declare.

The protocol of the study was designed by the investigators and Otsuka was not involved in the design and data evaluation.

Supplementary data

Tables S1 to S6 and Figure S1 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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