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Second-line drug susceptibility breakpoints for *Mycobacterium tuberculosis* using MODS assay

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Summary

Objective—The objective of this study was to establish breakpoint concentrations for the fluoroquinolones (MOX and OFX) and injectable second-line drugs (AMK, KAN and CAP) using the MODS assay.

In memoriam

Luz Caviedes was the Peruvian scientist who first recognised the potential for turning her observation of rapid growth of characteristic microscopic cords of MTB in liquid culture into a useful diagnostic test. She led the development of the non-commercial MODS assay into a reproducible rapid test for MDR-TB endorsed by the WHO. To our eternal sadness Luz passed away on 4th November 2012. Aside from her huge, open heart and immense humility she was a dear and generous friend to many and she will be forever missed.

Setting—A multinational study conducted between February 2011 and August 2012 in Peru, India, Moldova and South Africa.

Design—First phase determined the breakpoints to the fluoroquinolones and injectable second-line drugs (n = 58). Second phase evaluated the MODS second-line DST as an indirect test compared to MGIT DST (n = 89). The third (n = 30) and fourth (n = 156) phases determined reproducibility and concordance of the MODS 2nd line DST directly from sputum.

Results—Breakpoints for moxifloxacin (0.5 μg/ml), ofloxacin (1 μg/ml), amikacin (2 μg/ml), kanamycin (5 μg/ml) and capreomycin (2.5 μg/ml) were determined. In all phases the MODS results were highly concordant with MGIT DST. The few discrepancies suggest that the MODS breakpoint concentrations for some drugs may be too low.

Conclusion—The MODS second-line DST yielded comparable results to MGIT second-line DST, and is thus a promising alternative. Further studies are needed to confirm the accuracy of the drug breakpoints and the reliability of the MODS second-line DST as a direct test.

Keywords

tuberculosis; fluoroquinolones; aminoglycosides; susceptibility test; MODS

Introduction

Although the incidence of tuberculosis (TB) worldwide is declining, drug resistant TB is increasing, threatening global TB control. Countries that are most affected by the increase in TB drug resistance are often those least resourced to deal with this escalating problem.[1] Patients infected with multi-drug or extensively drug resistant TB (MDR/XDR-TB) strains require treatment regimens that include second-line anti-TB drugs. These treatment regimens also require extended treatment duration, and typically comprise aminoglycosides such as kanamycin (KAN) and amikacin (AMK); cyclic peptide, capreomycin (CAP); and fluoroquinolones such as ofloxacin (OFX) and moxifloxacin (MOX).[2]

The increasing prevalence of highly resistant *Mycobacterium tuberculosis* (MTB) strains demand that the diagnostic method of choice detect resistance to both first and second-line anti-TB drugs.[3] Furthermore, it should be rapid, inexpensive and easily implementable. Molecular and culture-based methods are available to detect drug resistant TB, but several do not meet all these criteria. Traditional agar-based methods (Löwenstein Jensen or Middlebrook 7H10/7H11 by either proportion or absolute concentration method) can take weeks to obtain results. Initially these tests set the standard for drug susceptibility testing (DST) but have largely been replaced by the liquid culture system BACTEC Mycobacterial Growth Indicator Tube (MGIT) 960 (Becton Dickinson, MD, USA). MGIT DST is currently the standard for phenotypic DST of first and second-line drugs[4,5] and performed following primary isolation in MGIT culture. MGIT DST is accurate and reproducible, but implementation demands advanced technical infrastructure not widely accessible in many resource poor countries.[6] Due to the slow growth of some drug resistant MTB, it can take one to two months from specimen receipt to delivery of results for all the drugs tested for MDR/XDR-TB strains.

Methods for molecular detection of gene mutations associated with drug resistance have been developed, and the line-probe assay (LPA) has been endorsed by the World Health Organization (WHO) for rapid screening for MDR-TB.[7] The GenoType MTBDRsl LPA (Hain Lifesciences, Nehren, Germany) for detection of genotypic resistance to the aminoglycosides, fluoroquinolones and ethambutol, has been shown to be a rapid DST method.[8] However, the technical expertise and infrastructure required may be too advanced to implement these tests in resource-limited settings with poor laboratory infrastructure. While the WHO endorsement of the GeneXpert System (Cepheid, CA, USA) addresses this issue, the assay detects only rifampicin (RIF) resistance.[9] The LPA and Xpert MTB/RIF assays are also more costly than traditional phenotypic methods.[10]

Non-commercial DST techniques, like the Microscopic Observation Drug Susceptibility (MODS) assay, can be implemented in resource poor settings with low cost and training.[11,12] The MODS assay can be performed directly with decontaminated sputum and does not require primary MTB isolation. A recent review found that the MODS assay was highly accurate for detection of RIF resistance, and slightly less sensitive for detection of isoniazid (INH) resistance.[13] While the potential for MODS to be used for DST of second-line drugs has been recently reported,[14] its application to date has been limited to INH and RIF.

The objective of this study was to establish the breakpoint concentrations of the fluoroquinolones (MOX and OFX) and injectable drugs (AMK, KAN and CAP) for the MODS assay. Using MDR/XDR isolates the drug concentrations that separated drug susceptible and drug resistant isolates were determined. Subsequently, we examined the accuracy of these second-line drug breakpoints by comparison to MGIT DST results from isolates and sputum specimens of TB patients at high risk for drug resistance in India, Moldova and South Africa.

Materials and methods

Setting and experimental design

This multinational study was conducted in four phases between February 2011 and August 2012. The critical concentrations (breakpoints) for each of the five drugs was determined in the Laboratorio de Investigación de Enfermedades Infecciosas (UPCH). Validation of the breakpoints was performed in the three laboratories that comprise the Global Consortium for Drug-resistant TB Diagnostics (GCDD). The University of California San Diego (UCSD) Institutional Review Board (IRB) and the IRBs that represent the GCDD laboratories in India, Moldova and South Africa approved the study.

MGIT DST was performed according to manufacturer's instructions using the WHO recommended critical concentrations.[15] The KAN critical concentration was 2.5 μg/ml.[16] The MODS assay was performed as described previously.[17] To ensure consistency and accuracy of results, the reference strain MTB H37Rv (ATCC 27294) was included each time the MGIT DST and MODS assay were performed. All work was carried out in Biosafety Level 2 laboratories according to international standards. [18] Preparation of the test inocula was done inside Class II biological safety cabinets. Sealed MODS plates were placed in

polyethylene Ziploc bags that were also sealed with tape. Plates were never removed from the sealed bags, even when reading.

Phase I: Determining breakpoints

A representative group of sixty-one MDR/XDR-TB clinical isolates were selected from the multinational collection of more than 500 highly drug-resistant characterized MTB isolates archived at UCSD. These isolates were all resistant to INH and RIF and 42 isolates were resistant to at least one injectable drug and 51 were resistant to at least one fluoroquinolone. MGIT DST was completed at UCSD on all of these isolates using standard WHO protocols and WHO recommended critical concentrations. Any discrepancies were confirmed using standard methodology. Selected isolates were sent to UPCH and the sensitivity and specificity for each drug was determined by comparing the MODS results at various drug concentrations (Table 1) with the standardized UCSD MGIT DST results.

Phase II: Validation using isolates

Each laboratory selected 10 susceptible and 10 resistant MTB isolates (at least five resistant to fluoroquinolones and injectables) from in-house or external strain banks. Five separate runs (5 isolates/run) were conducted in which the 20 isolates were tested at least once; one pan-susceptible isolate was tested five times, one resistant isolate tested twice. The pan-susceptible reference strain MTB H37Rv was also tested in each run. Thus, a total of 30 tests were performed by each laboratory (n = 90). MGIT positive cultures were used to prepare inocula for the indirect MODS assay. The inoculum was a 1:1000 dilution of a 1 McFarland standard suspension of the positive MGIT culture in 5 ml of Middlebrook 7H9 broth with Oleic Acid Dextrose Citrate; 900 μl of the inoculum was added to each well.

Phase III: Validation using sputa

Each laboratory selected 10 smear-positive sputum specimens for direct MODS second-line DST. Additional specimens were tested when the MODS assay was contaminated (n=1) or control wells failed to grow MTB (n=3). Sputum was decontaminated using a final concentration of 1% sodium hydroxide. Following neutralization with phosphate buffer, smear microscopy and MGIT culture were performed.[19,20] The MODS was set-up on three consecutive days using the same sediments. MGIT DST was performed on the positive cultures.

Phase IV: Evaluation of MODS second-line DST using sputa

One hundred and fifty six sputum specimens were prospectively collected from subjects enrolled in the GCDD study in India (n = 50), Moldova (n = 50) and South Africa (n = 56). Subjects provided a sputum sample (‘spot’) at enrollment and returned the following day with an early morning sample. In the laboratory, the spot and early morning specimens were combined to produce a pooled sample with volume of at least 7.5 ml. Samples were decontaminated and prepared for testing as described above.

Statistical analysis

Statistical analysis to determine the percent concordance of the MODS second-line DST as compared to the standard MGIT DST was performed using AcaStat software (Version 8.1.9) (AcaStat Software, VA, USA). ROC curves were constructed with GraphPad Prism (Version 5) (GraphPad Software, CA, USA).

Results

Phase I

To establish breakpoints, multiple drug concentrations were evaluated (Table 1). For the fluoroquinolones, 58 results were available for analysis. Forty-seven strains were resistant and 11 susceptible by MGIT DST. Based on the ROC curves in Figure 1, we chose a concentration of 0.5 μg/ml for MOX and 1 μg/ml for OFX. At these concentrations, the sensitivity and specificity for MOX (Fig. 2A) was 91.5% (95%CI 84.3% to 98.7%) and 81.8% (95%CI 71.9% to 91.7%), and for OFX (Fig. 2B) 97.9% (95%CI 94.2% to 1.0) and 100%, respectively.

For the injectable drugs, 57 AMK and KAN results and 55 CAP results were available for analysis. Thirty strains were resistant and 27 susceptible to AMK; 38 resistant and 19 susceptible to KAN; and 29 resistant and 26 susceptible to CAP by MGIT DST. Based on the ROC curves (Fig. 1) we chose a concentration of 2 μg/ml for AMK, 5 μg/ml for KAN, and 2.5 μg/ml for CAP. At these concentrations, the sensitivity and specificity for AMK (Fig. 2C) was 93.3% (95%CI 86.8% to 1.0) and 100%; for KAN (Fig. 2D) 89.7% (95%CI 81.8% to 97.6%) and 100%; and for CAP (Fig. 2E) 93.1% (95%CI 86.5% to 1.0) and 96.2% (95%CI 91.2% to 1.0), respectively.

Phase II

Using the selected breakpoints from Phase I, the three GCDD laboratories performed a validation test of the second-line drugs in the MODS assay using isolates with known MGIT DST results. In total, 89 isolates were tested [53 isolates tested once; 3 isolates, 2 times; 3 isolates, 5 times and H37Rv, 15 times]. High concordance with both resistant and susceptible isolates was observed for MOX (95.5%), OFX (94.4%), AMK (96.6%), KAN (95.5%), and CAP (92.1%). For all drugs the discordance was always, with exception of one isolate, due to the MODS result being resistant and MGIT being susceptible, i.e., MODS/MGIT: R/S – MOX, 4; OFX, 5; AMK, 3; KAN, 4; CAP, 3; S/R – CAP, 1. Replicate MODS results showed perfect reproducibility.

Phase III

Using the same breakpoints a validation test was performed using smear-positive sputa. The smear grades 3+, 2+, and1+ were evenly distributed among the specimens, representing 90% of specimens tested with remaining 10% being scanty. Concordance between MODS and MGIT was 100% for all five drugs in two laboratories. In the third laboratory, 100% concordance was observed for AMK, KAN, and CAP, whereas it was 96% for MOX and OFX. The discordance observed with MOX and OFX was due to one discrepant result between the MODS and MGIT. In both instances, the discrepancy was one of the three

replicates tests with MODS being resistant and MGIT being susceptible. The reproducibility of the MODS assay was determined by comparing the results from tests conducted on three consecutive days. Of the 30 specimens tested in triplicate, 88/90 (98%) yielded reproducible results.

Phase IV

In the final phase of the study, a larger number of prospectively collected specimens were tested using the same breakpoints as in Phases II and III. Of the 156 sputum specimens tested, 23 (14.7%) were smear negative, seven (4.5%) were MGIT culture negative, and one (0.6%) was MGIT culture contaminated. 148 MGIT DST results were available for comparison to the MODS results.

Of the 156 MODS tests, 14 (9%) had no growth in growth control wells, five (3.2%) were contaminated, one grew non-tuberculous mycobacteria, and 136 (87.2%) were interpretable. The mean time to detection of MTB bacilli in the growth control wells (an interpretable MODS result) was 13.2 days (95%CI 11.9 – 13.3). MODS results were available on four samples that were either culture negative, culture contaminated or failed to provide a result on MGIT DST. MODS results were available for 10/23 (43.5%) smear-negative specimens.

One hundred and thirty-five samples with both MODS and MGIT DST results were analyzed further to determine MODS assay performance (Table 2). The MGIT results for AMK and KAN for one isolate were not available thus this isolate was removed from further analysis. Concordance between MODS and MGIT was high for all 5 drugs (Table 3). As observed in Phase II, with one exception all discrepant results were false-resistant MODS results.

Discussion

In this study we chose breakpoints for the fluoroquinolones and injectable drugs tested which demonstrated high sensitivity and specificity when compared to the MGIT DST results. A recent publication advocates the use of wild-type strains with minimum inhibitory concentration distributions, in combination with data on clinical outcomes, pharmacokinetics and pharmacodynamics to determine critical concentrations.[22] The isolates we used for determining the breakpoints were obtained from patients receiving combination therapy with fluoroquinolones and injectable second-line drugs. The inability to use wild-type isolates with known MICs and clinical outcomes in determining breakpoints is a limitation of this study but does not appear to have affected our ability to determine reliable breakpoints.

The MODS assay was tested in three phases – one with clinical isolates and two with sputum specimens. In all three phases the MODS results for the second-line drugs were highly concordant with the MGIT DST results. Furthermore, reproducibility among repetitive tests was 98%. The number of indeterminate results due to contamination was very low when testing sputum sediments. In one laboratory there were 14 MODS test failures due to no growth in control wells in the second phase of sputum testing. The failures did not correlate with low MTB loads in the sputum and occurred at the beginning of the

testing phase. This suggests the “no growth” control wells were a result of technical issues that were resolved once the test was performed on a routine basis. Surprisingly, 43.5% of the smear negative specimens yielded results for all five drugs.

The number of discrepant results between MODS and MGIT was relatively low; however, discrepancies were observed with each of the drugs when testing clinical isolates and sputum sediments. All discrepancies, with two exceptions, were false-resistant MODS results that suggest that the MODS breakpoints for some of the drugs may be too low and/or the minimum inhibitory concentrations of the isolates with discrepant results are close to the critical concentrations. The diagnostic accuracy of this assay is being evaluated with a large number of prospectively collected specimens in the GCDD Clinical Observation Phase. These data will either substantiate or refute this trend.

There are only two published reports describing critical concentrations for MODS second-line drug testing. In a comparative study of agar proportion, MGIT DST, Nitrate Reduction Assay and MODS assay (indirect testing) the breakpoint for OFX was determined to be 2 μg/ml and 0.5 μg/ml for MOX.[22] The MOX concentration coincides with that determined in our study; however, the OFX concentration is 2-fold higher (1 μg/ml). A more recent study used ROC curves and Kaplan Meier analysis to determine the critical concentrations for KAN and CAP in the MODS assay with culture-positive sputa (direct testing).[14] The cutoff point for KAN was defined as 5 μg/ml which is the same as in our study whereas the breakpoint for CAP was 4-fold higher than the concentration we chose to use. Thus these studies suggest that higher cutoff concentrations for OFX and CAP may more clearly differentiate isolates that are drug susceptible and resistant.

The median time to obtain an interpretable MODS result was 13.2 days which is longer than reported in some studies[23,24]; however, shorter than the mean time of 23 days required to perform MGIT 960 culture and MGIT DST[25]. Reasons for the longer MODS completion time could be the relative inexperience of the laboratories in reading and interpreting MODS assay data and/or the slow growth rate of some drug resistant isolates.

The strengths of our study are we tested MTB isolates and sputa from three distinct geographic locations where the frequency of resistance for these five drugs differs and the exposure to these drugs and treatment regimens vary, and we demonstrated accurate and reproducible results when MODS assays were prepared and tested in three separate laboratories. In conclusion, the second-line MODS assay yielded comparable results to MGIT second-line DST, and is thus a promising alternative. Further studies with sputum specimens are needed to confirm the accuracy of the individual drug breakpoints and the reliability of the MODS second-line DST as a direct test.

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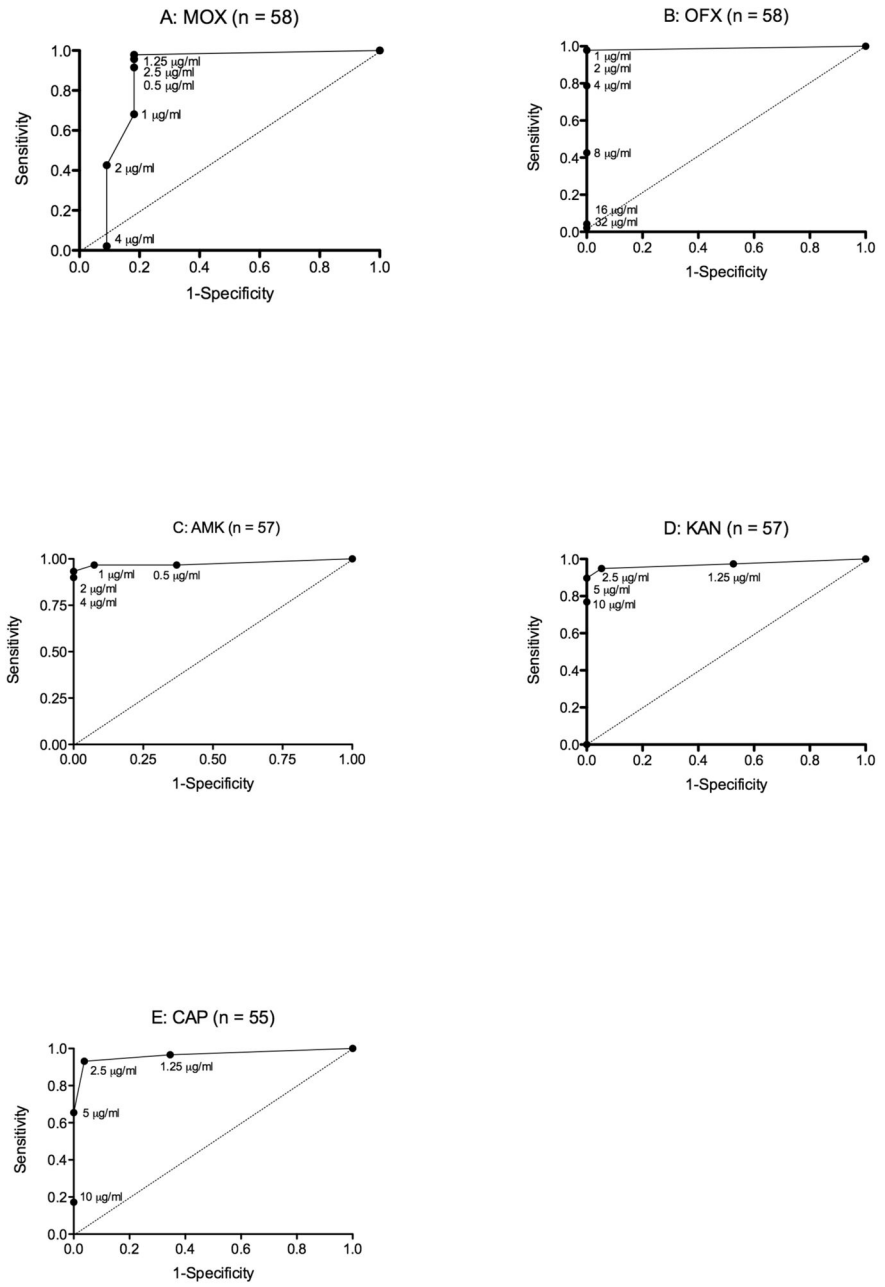

Figure 1.

ROC curves for (A) MOX, (B) OFX, (C) AMK, (D) KAN, (E) CAP. Drug concentrations that reach furthest to the top left corner represent those with highest sensitivity and specificity, and these were selected as the candidate breakpoints for the MODS second-line DST assay

Table 1

Drug concentrations used in the development of the MODS 2nd line DST

Drug	MGIT DST (μg/ml)	Phase I (μg/ml)	Phase II, III, IV (μg/ml)
Isoniazid (INH)	0.1	Not applicable	0.4
Rifampicin (RIF)	1.0	Not applicable	1.0
Moxifloxacin (MOX)	0.25	0.125, 0.25, 0.5, 1.0, 2.0, 4.0	0.5
Ofloxacin (OFX)	2.0	1.0, 2.0, 4.0, 8.0, 16.0, 32.0	1.0
Amikacin (AMK)	1.0	0.5, 1.0, 2.0, 4.0	2.0
Kanamycin (KAN)	2.5	1.25, 2.5, 5.0, 10.0	5.0
Capreomycin (CAP)	2.5	1.25, 2.5, 5.0, 10.0	2.5

Table 2

Distribution of MGIT DST patterns of isolates from MGIT cultures of sputum sediments in Phase IV

MGIT DST patterns	(n = 135)
Pan susceptible	50
INH mono-resistant	4
RIF mono-resistant	2
OFX & MOX resistant only	1
INH, RIF, & OFX resistant	2
MDR (INH & RIF resistant)	41
Pre-XDR (MDR + MOX or OFX resistant)	24
Pre-XDR (MDR + KAN or AMK or CAP resistant)	2
XDR (MDR + MOX or OFX + KAN or AMK or CAP)	9

Table 3

Concordance of the MODS 2nd line DST as compared to MGIT DST in sputum sediments in Phase IV (n = 135)

A: MOX (Concordance = 97%)

MGIT DST = 0.25 μg/ml	MODS = 0.5 μg/ml	MGIT DST Susceptible	MGIT DST Resistant
MODS Susceptible		94	0
MODS Resistant		7	34

B: OFX (Concordance = 97%)

MGIT DST = 2 μg/ml	MODS = 1 μg/ml	MGIT DST Susceptible	MGIT DST Resistant
MODS Susceptible		95	0
MODS Resistant		4	36

C: AMK (Concordance = 99.3%)

MGIT DST = 1 μg/ml	MODS = 2μg/ml	MGIT DST Susceptible	MGIT DST Resistant
MODS Susceptible		123	0
MODS Resistant		1	10

D: KAN (Concordance = 99.3%)

MGIT DST = 2.5 μg/ml	MODS = 5μg/ml	MGIT DST Susceptible	MGIT DST Resistant
MODS Susceptible		123	0
MODS Resistant		1	10

E: CAP (Concordance = 94.8%)

MGIT DST = 2.5 μg/ml	MODS = 2.5μg/ml	MGIT DST Susceptible	MGIT DST Resistant
MODS Susceptible		119	1
MODS Resistant		6	9