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Fluoroquinolone resistance in *Mycobacterium tuberculosis*: an assessment of MGIT 960, MODS and nitrate reductase assay and fluoroquinolone cross-resistance

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***Objectives*: The aim of this study was to assess the sensitivity, specificity and time to results of mycobacterial growth indicator tube (MGIT) 960, microscopic observation drug susceptibility (MODS) assay and nitrate reductase assay (NRA) compared with the gold standard agar proportion method (PM), and to determine whether there is cross-resistance between older-generation fluoroquinolones and moxifloxacin.**

***Methods*: *Mycobacterium tuberculosis* isolates from culture-confirmed tuberculosis patients from 2002 to 2007 were tested for ofloxacin (2 mg/L) resistance by PM and MGIT 960. All isolates from 2005 and 2006 were also tested by MODS and NRA. Ofloxacin-resistant isolates by PM were further tested by all four methods using ciprofloxacin, levofloxacin and moxifloxacin. For each ofloxacin-resistant isolate, two ofloxacin-susceptible isolates were tested against all three fluoroquinolones using all four methods.**

***Results*: Of the 797 *M. tuberculosis* isolates, 19 (2.4%) were ofloxacin-resistant by PM. MGIT 960 had 100% sensitivity (95% CI, 83%–100%) and specificity (95% CI, 99.5%–100%). Of the 797 isolates, 239 were from 2005 to 2006 and 6 of these (2.5%) were resistant by PM. MODS had 100% sensitivity (95% CI, 61%–100%) and specificity (95% CI, 98%–100%). NRA had 100% sensitivity (95% CI, 61%–100%) and 98.7% specificity (95% CI, 96%–99.6%). The median time to results was shorter using MGIT 960 (8 days), MODS (6 days) or NRA (9 days) compared with PM (21 days) ($P<0.001$). All 19 ofloxacin-resistant isolates were resistant to ciprofloxacin, levofloxacin and moxifloxacin by PM.**

***Conclusions*: MGIT 960, MODS and NRA are sensitive and specific and more rapid than PM for identifying fluoroquinolone resistance in *M. tuberculosis*. Ofloxacin resistance was associated with cross-resistance to ciprofloxacin, levofloxacin and moxifloxacin.**

Keywords: tuberculosis, fluoroquinolones, susceptibility tests

Introduction

Timely drug susceptibility testing of *Mycobacterium tuberculosis* isolates is important for the successful treatment of tuberculosis. Delays in susceptibility testing may lead to prolonged use of ineffective drugs for persons with drug-resistant tuberculosis. Rapid susceptibility testing is increasingly important as the prevalence of drug-resistant tuberculosis increases in regions of the world.[1,2]

Fluoroquinolones are recommended for the treatment of multidrug-resistant tuberculosis (MDR-TB) (defined as

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resistance to at least isoniazid and rifampicin)[3] and are under investigation as first-line anti-tuberculosis therapy.[4,5] Newer fluoroquinolones, such as levofloxacin, gatifloxacin and moxifloxacin, have excellent *in vitro* and *in vivo* activities against *M. tuberculosis*[6,7] and are potentially more effective than ofloxacin.[8,9] The potential superior clinical activity of newer fluoroquinolones against drug-resistant tuberculosis led the WHO to recommend the use of levofloxacin or moxifloxacin for the treatment of extensively drug-resistant tuberculosis (XDR-TB) (defined as resistance to isoniazid, rifampicin, a fluoroquinolone and a second-line injectable drug) even when ofloxacin resistance is present.[10] There may be some clinical benefit from new generation agents for the treatment of *M. tuberculosis* isolates resistant to older generation fluoroquinolones.[11,12] However, the extent to which there is *in vitro* cross-resistance between older generation fluoroquinolones and new generation agents such as moxifloxacin is unclear.[13–15]

The gold standard for fluoroquinolone susceptibility testing in *M. tuberculosis* is the agar proportion method (PM). The primary disadvantage of this method is that it requires 3 weeks to obtain the results.[16] Rapid drug susceptibility tests for *M. tuberculosis* are available but have not been thoroughly investigated using fluoroquinolones. These include liquid media tests, such as mycobacterial growth indicator tube (MGIT) 960 (Becton–Dickinson) and microscopic observation drug susceptibility (MODS) assay, and the colorimetric test, nitrate reductase assay (NRA). We assessed the sensitivity, specificity and time to results for MGIT 960, MODS assay and NRA compared with PM.

Methods

The study was conducted among newly diagnosed culture-confirmed tuberculosis patients reported to the Tennessee Department of Health from January 2002 to December 2007. From 2002 to 2006, *M. tuberculosis* isolates were included in the study if the patient had ever enrolled in TennCare (Medicaid).[17] TennCare is a managed healthcare programme that insures state residents who are eligible for federal Medicaid benefits and other low-income groups. The study was approved by the Institutional Review Boards of Vanderbilt University, Tennessee Department of Health, the Davidson County Metro Public Health Department and was also reviewed by the Bureau of TennCare.

All *M. tuberculosis* isolates from patients who met eligibility criteria underwent ofloxacin susceptibility testing by PM and MGIT 960. According to the Clinical and Laboratory Standards Institute guidelines, ofloxacin resistance was defined as at least 1% colony growth on agar at a critical concentration of 2 mg/L[16]; the same concentration was used for MGIT 960.[18,19] Isolates from 2005 and 2006 were also tested by MODS assay and NRA[20] using ofloxacin at 2 mg/L.

Isolates that were ofloxacin-resistant by PM underwent further fluoroquinolone susceptibility testing against ciprofloxacin (2 mg/L), levofloxacin (2 mg/L) and moxifloxacin (0.5 mg/L) by PM,[16] MGIT 960,[21] MODS assay and NRA.[20] For each ofloxacin-resistant isolate, two ofloxacin-susceptible isolates by PM from the same year as the ofloxacin-resistant isolate were also tested with these three drugs and four testing methods.

Standard powders of ofloxacin, ciprofloxacin and levofloxacin were obtained from Sigma Aldrich. Moxifloxacin powder was donated by Bayer Pharmaceuticals. Drug stock solutions were prepared according to the manufacturer's recommendations and working solutions were further prepared in sterile distilled water. Based on the potency of the fluoroquinolone, ~100 mg of each drug was dissolved in 10 mL of either sodium hydroxide or sterile distilled water resulting in a concentration of 10000 mg/L. The drug stock solution was filter sterilized through a cellulose membrane. Additional stock solutions of 1000 and 100 mg/L were prepared from the initial stock solution. An aliquot of 1 mL of all stock solutions was stored at −80°C for ≤3 months. Working solutions were prepared from any of the three stock solutions 24–48 h before use.

Laboratory methods

All clinical *M. tuberculosis* isolates were stored at the Tennessee State Mycobacteriology Laboratory at −70°C. *M. tuberculosis* isolates were thawed and subcultured onto Lowenstein–Jensen (LJ) medium. A 1.0 McFarland inoculum was prepared from colonies on the LJ slant and served as the standard inoculum for susceptibility testing for PM and NRA. A 0.5 McFarland inoculum was used for MGIT 960[22,23] and MODS assay.

PM

A 1:100 and a 1:10000 dilutions of the standard inoculum were prepared in sterile saline. For each dilution, 0.1 mL was inoculated onto both a drug-free quadrant of a 7H10 agar plate and a quadrant containing two 5 μg ofloxacin discs (final concentration 2 mg/L). Plates were incubated at 37°C in 5%–10% CO_2 and examined for growth once a week for 3 weeks. If sufficient growth was not present at 3 weeks, plates were re-incubated for another 3 weeks.[16]

The American Type Culture Collection (ATCC) #27294 drug-susceptible *M. tuberculosis* H37 Rv strain and a known fluoroquinolone-resistant *M. tuberculosis* strain from the Centers for Disease Control and Prevention (Division of TB Elimination, Mycobacteriology Laboratory Branch) were used as negative and positive controls for each test.

MGIT 960

A 1:5 and a 1:500 dilution was prepared from the MGIT 960 standard inoculum. To each of two tubes, 0.8 mL of MGIT 960 oleic acid–albumin–dextrose complex (OADC) was added. To tube one, 0.5 mL of the 1:500 dilution of the standard inoculum was added without drug. To tube two, 0.5 mL of the 1:5 dilution of the standard inoculum was added along with 0.1 mL of fluoroquinolone (final concentrations of 2 mg/L for ofloxacin, ciprofloxacin and levofloxacin and 0.5 mg/L for moxifloxacin). The tubes were incubated in the MGIT 960 at 37°C.[24] Drug resistance was determined by a growth unit of at least 100 and was determined manually since the MGIT tube drug susceptibility testing set carrier was entered as an 'unknown' drug.

MODS assay

The MODS assay was performed according to Caviedes *et al.*[25] Briefly, a 0.5 McFarland suspension was prepared in Middlebrook 7H9 broth (7H9-S) supplemented with 2% glycerol and OADC. Sixty microlitres of each of the pre-mixed drug stock solutions was pipetted into the appropriate wells of a sterile 24-well microplate. To the drug-containing well(s), 540 μL of the 10^{-3} diluted bacterial suspension was added.

Controls were prepared by pipetting 60 μL of 7H9-S broth into a drug-free well containing 540 μL of diluted bacterial suspension. Another 600 μL of 7H9-S broth was pipetted into a drug-free and

bacterial-suspension-free well to serve as a control for cross-contamination. Plates were covered with lids, labelled and sealed with polyethylene tape. Each plate was incubated at 37°C in 5%–10% CO_2. Control wells were examined every 1–2 days under an inverted light microscope at ×40 magnification. The drug-containing wells were examined once growth was observed in the control wells. A drug-resistant isolate was defined as visualization of serpentine cording of *M. tuberculosis* by microscopic examination of the well in the presence of the drug, along with a positive acid-fast smear. A drug-susceptible isolate was defined as the absence of bacterial growth in the presence of the drug.

NRA

The NRA was performed according to a modified protocol of Angeby *et al.*[26] and Poojary *et al.*[27] A 1.0 McFarland and a 0.5 McFarland standard inoculum was prepared in Middlebrook 7H9 broth supplemented with 0.5% glycerol, 0.05% Tween 80, OADC and 850 mg/mL potassium nitrate (Sigma Aldrich). In separate tubes, a 1:5 and a 1:500 dilution of the 0.5 McFarland inoculum was prepared. Further steps of the NRA were performed according to Poojary *et al.*[27]

Quality control and fluoroquinolone stability testing

To check our susceptibility results, six *M. tuberculosis* isolates also underwent fluoroquinolone susceptibility testing at the Maryland State Laboratory. Maryland laboratory personnel were blinded to the fluoroquinolone susceptibility results from our laboratory. These six isolates were tested by PM using ofloxacin and ciprofloxacin at 2 mg/L and moxifloxacin at 1 mg/L.

The drug stability of each fluoroquinolone was tested by PM and MGIT 960. A standard inoculum of the ATCC reference strain #27294 was prepared for each test. Four sets of 7H10 agar plates and MGIT 960 tubes were used for each fluoroquinolone. One set of plates and MGIT 960 tubes was inoculated and incubated at 37°C (5%–10% CO_2 for PM). The remaining plates and tubes were incubated without any inoculum. Each week one set of plates and one set of tubes were removed from the incubator and inoculated. The agar plates were then reincubated for an additional 3 weeks. The MGIT 960 tubes were reincubated and removed when the growth control tube became positive.

Statistical analysis

Sensitivity and specificity of MGIT 960, MODS and NRA were evaluated using PM as the gold standard reference test. The Wilson method was used to estimate the standard error for proportions close to 1 when calculating the confidence intervals (CIs) for sensitivity and specificity.[28–30] The Friedman signed rank test for the global test assessing homogeneity among medians was used to compare the median time to test results of PM, MGIT 960, MODS and NRA. Pair-wise comparison was performed only when statistical significance was observed in the overall test. This avoided inflation of Type I error by multiple comparisons. The bootstrap resampling method provided 95% CI of the difference in median time between two methods, and the Wilcoxon signed rank test provided *P* values for pair-wise comparisons.[30] *P* values were not adjusted for multiple comparisons.

Cost estimate analysis

The cost of each method to perform ofloxacin susceptibility testing for one isolate was compared. Common items among all four methods included pipettes, pipette tips, ofloxacin and glass beads. Unique items for PM included agar, glycerol, OADC, 7H9 broth, Petri dishes, tape and tubes. For MGIT, the items included 7H9 broth, glass tubes with and without saline, and Steriflip. For MODS, the items included an incubator, inverted microscope, tubes with OADC and 7H9 broth, tubes, tape, 24-well plate and wooden applicator. For NRA, the items were tubes with 7H9 broth, Tween, OADC and potassium nitrate, tubes with and without saline, wooden applicator and Greiss reagent. One time cost of a CO_2 incubator for PM, a MGIT 960 machine for MGIT, an incubator for MODS and an incubator for NRA was not included in the cost estimate for testing one isolate. Personnel costs were also not included.

Results

There were 797 *M. tuberculosis* isolates available for fluoroquinolone susceptibility testing during the study period. There were 19 (2.4%) ofloxacin-resistant and 778 (97.6%) ofloxacin-susceptible isolates by PM. The same 19 ofloxacin-resistant and 778 ofloxacin-susceptible isolates were also identified by MGIT 960. Therefore, MGIT 960 had 100% sensitivity and specificity (Table 1).

Of the 797 isolates, 239 isolates were from 2005 to 2006 and also underwent ofloxacin susceptibility testing by MODS and NRA. Of these 239 isolates, 6 (2.5%) were ofloxacin-resistant by PM. The same six isolates were resistant using MODS and NRA. The MODS assay had a sensitivity of 100% and specificity of 100%. NRA identified three additional ofloxacin-resistant isolates that were ofloxacin-susceptible by PM. Therefore, NRA had a sensitivity of 100% and specificity of 98.7%.

The median time to susceptibility results for PM and MGIT 960 was determined for all 797 isolates and median time to susceptibility results for MODS and NRA was determined for the 239 isolates. Positive results were available in a median of 21 days for PM [inter-quartile range (IQR), 21–21]. Final results were available in a median of 8 days for MGIT 960 (IQR, 7.4–8.6) ($P<0.001$), 6 days for MODS assay (IQR, 5–7) ($P<0.001$) and 9 days for NRA (IQR, 7–11) ($P<0.001$) (Table 1); all *P* values are for the comparison with PM. The global test for homogeneity of median time to obtain test results was statistically significant ($P<0.001$).

In pair-wise comparisons, the time to susceptibility results for each test was significantly different from the other tests. Comparing the same 239 susceptibility results, the time to detection of resistance occurred a median of 2.5 days sooner with MODS than with MGIT 960 (95% CI, 2.2–2.6) ($P<0.001$) and 3 days sooner with the MODS assay than with NRA (95% CI, −3 to −2) ($P<0.001$).

A total of 57 isolates (19 ofloxacin-resistant and 38 ofloxacin-susceptible isolates) were tested for ciprofloxacin, levofloxacin and moxifloxacin susceptibility by PM. The 19 ofloxacin-resistant isolates were resistant to ciprofloxacin, levofloxacin and moxifloxacin by PM. Results were similar when using MGIT 960, MODS and NRA (Table 2). Of the 38 ofloxacin-susceptible isolates, 38 (100%) were ciprofloxacin- and levofloxacin-susceptible and 37 (97%) were moxifloxacin-susceptible by PM.

Ofloxacin had good sensitivity and specificity for identifying fluoroquinolone resistance among newer fluoroquinolones.

Table 1. Sensitivity, specificity and time to results for MGIT 960, MODS assay and NRA compared with the PM

	PM	MGIT 960 (95% CI)	MODS assay (95% CI)	NRA (95% CI)
Total number of *M. tuberculosis* isolates	797	797	239	239
Total number of fluoroquinolone-resistant isolates	19	19	6	9
Sensitivity		100% (83%–100%)	100% (61%–100%)	100% (61%–100%)
Specificity		100% (99.5%–100%)	100% (98%–100%)	98.7% (96%–99.6%)
Median time (days) to results (IQR)	21	8 (7.4–8.6)	6 (5–7)	9 (7–11)

CI, confidence interval; IQR, inter-quartile range.

Table 2. Fluoroquinolone cross-resistance among 19 ofloxacin-resistant *M. tuberculosis* isolates using four different drug susceptibility methods

Fluoroquinolone (critical concentration)	PM	MGIT 960	MODS assay	NRA
Ofloxacin resistant (2 mg/L)	19	19	19	19
Ciprofloxacin resistant (2 mg/L)	19	18	19	18
Levofloxacin resistant (2 mg/L)	19	18	19	17
Moxifloxacin resistant (0.5 mg/L)	19	16	19	19

The values shown are the number of fluoroquinolone-resistant isolates detected according to the laboratory method used and fluoroquinolone tested.

Table 3. Cost for ofloxacin susceptibility testing[a]

	PM[b]	MGIT 960[c]	MODS assay[d]	NRA[e]
One time equipment cost	CO_2 incubator US$14000	MGIT machine US$47500	incubator US$8000, inverted microscope US$4000	incubator US$8000
Cost per isolate for ofloxacin susceptibility testing	US$2.86	US$13.92	US$1.38	US$3.92

[a]All methods required the use of transfer pipettes, wooden applicator, glass beads and flat glass tube with cap. Equipment cost is not included in the cost per isolate estimate.
[b]Includes cost of 7H9 broth, OADC, pipette tips, 7H10 agar, glycerol, Petri dish, tape and ofloxacin disc.
[c]Includes cost of 7H9 broth, OADC, pipette tips, tubes, MGIT tubes, Steriflip and ofloxacin.
[d]Includes cost of 7H9 broth, OADC, pipette tips, tubes, tape, 24-well plate and ofloxacin.
[e]Includes cost of 7H9 broth with OADC, Tween, KNO_3, tubes, Greiss reagent and ofloxacin.

Ofloxacin had 100% sensitivity (95% CI, 83%–100%) and specificity (95% CI, 91%–100%) in detecting ciprofloxacin and levofloxacin resistance. Ofloxacin also had 95% (95% CI, 76%–99.7%) sensitivity and 100% (95% CI, 91%–100%) specificity in detecting moxifloxacin resistance.

Three ofloxacin-resistant and three ofloxacin-susceptible *M. tuberculosis* isolates were also tested by the Maryland State Laboratory. Susceptibility results were in 100% agreement with our results. Based on colony growth and resistance percentages, we found fluoroquinolones to be stable for 6 weeks at 37°C in 5%–10% CO_2 for PM. Fluoroquinolones were also stable in the MGIT 960 liquid media for up to 6 weeks.

The cost estimate for ofloxacin susceptibility testing for one *M. tuberculosis* isolate varied between the four methods: PM cost US$2.86, MGIT cost US$13.92, MODS cost US$1.38 and NRA cost US$3.92 (Table 3).

Discussion

To our knowledge, this is the largest population-based study to examine MGIT 960, MODS and NRA for fluoroquinolone susceptibility testing in *M. tuberculosis*. Using ofloxacin, all three methods were highly sensitive and specific compared with the gold standard PM. Results for all three methods were also available significantly faster than with PM.

In this study, we noted that ofloxacin-resistant isolates were consistently cross-resistant to newer fluoroquinolones. The 19 ofloxacin-resistant isolates were resistant to ciprofloxacin, levofloxacin and moxifloxacin by PM. This finding is important since current practice for the treatment of XDR-TB is to replace ofloxacin or ciprofloxacin with newer fluoroquinolones if resistance to the former is present. In a study of 48 XDR-TB patients in Peru with ciprofloxacin resistance who received levofloxacin

or moxifloxacin as part of their treatment regimen, 29 (60.4%) were cured, similar to cure rates among patients with MDR-TB.[12] *In vitro* susceptibility results to levofloxacin or moxifloxacin were not reported. In a retrospective study of 99 MDR-TB patients who were treated with ofloxacin or levofloxacin, 59 patients received ofloxacin and 40 received levofloxacin. Of the 40 patients receiving levofloxacin, 14 had ofloxacin-resistant isolates, of whom 11 were cured with a levofloxacin-containing regimen.[11] The isolates were not tested for levofloxacin susceptibility. Although in our study ofloxacin resistance correlated with *in vitro* resistance to other fluoroquinolones, the two studies mentioned here suggest that there may be some clinical response to fluoroquinolone-containing regimens even in the presence of fluoroquinolone resistance. Studies defining the MIC and MBC of individual fluoroquinolones may explain the effectiveness of new generation fluoroquinolones when ofloxacin or ciprofloxacin resistance is present. However, it should be noted that all patients in those studies received combination anti-tuberculosis therapy, so the beneficial clinical effect could have been due to medications other than the fluoroquinolone.

The use of liquid culture and drug susceptibility testing, such as MGIT 960, is recommended for the early detection of drug-resistant *M. tuberculosis*.[16] MGIT 960 is also fully automated and requires minimal training of personnel. However, MGIT 960 and its supplies are expensive and therefore less appealing for developing countries. In late 2007, Becton–Dickinson, the manufacturer of MGIT 960, in partnership with the non-profit organization Foundation for Innovative New Diagnostics (FIND) decreased the cost of MGIT 960 and its reagents for 39 high-burden low-income countries.[31] With this partnership, faster TB drug susceptibility results may become a possibility for many low- and middle-income countries.

MODS assay is an excellent method for fluoroquinolone susceptibility testing for several reasons. It is fast, sensitive and specific, and personnel can be trained to use MODS in ~2 weeks.[32] NRA has good sensitivity and specificity compared with PM and results were available in under 2 weeks. Both MODS and NRA are relatively inexpensive. The greatest expense for the MODS assay is an inverted microscope and incubator. Unlike MGIT 960 or MODS, NRA does not rely on expensive equipment other than an incubator. The price per isolate for NRA testing was higher than expected because the protocol we used required 11 tubes per isolate. Other NRA protocols are available that use fewer tubes and therefore cost ~$2 per isolate.[33,34] Finally, both MODS and NRA had a wide 95% CI from 61% to 100%, which is likely due to the smaller sample size used for MODS and NRA susceptibility testing compared with the PM and MGIT.

The prevalence of fluoroquinolone resistance was low in this population, so these tests should be evaluated in settings with higher prevalence of fluoroquinolone-resistant *M. tuberculosis*. This would better define the sensitivity of each method and would also be a practical assessment of each method in an environment where rapid tests are needed most. It may be reasonable in areas with high rates of MDR-TB to perform fluoroquinolone susceptibility testing along with first-line drug susceptibility testing. Our data suggest that there is cross-resistance between fluoroquinolones and therefore ofloxacin, the current class drug, could be used for susceptibility testing. All four methods were performed by laboratory personnel experienced in fluoroquinolone susceptibility testing of *M. tuberculosis*. Therefore, our results do not take into account the training required to learn MODS and NRA. However, most people can be trained to perform these methods in under 2 weeks. To verify our susceptibility results, we randomly selected isolates to be tested by PM at another laboratory experienced in fluoroquinolone susceptibility testing. Our findings were reproduced by the external laboratory.

In this study, MGIT 960, MODS assay and NRA were highly sensitive and specific, and faster for fluoroquinolone susceptibility testing than the PM. Identifying fluoroquinolone resistance in *M. tuberculosis* is increasingly important as fluoroquinolones undergo evaluation as first-line tuberculosis therapy and remain a crucial drug for treatment of drug-resistant tuberculosis. Advancing and standardizing the use of these methods for fluoroquinolone susceptibility testing in *M. tuberculosis* are necessary and will be an important tool in the treatment of tuberculosis.

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Transparency declarations

None to declare.

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