

Validation of the Use of Middlebrook 7H10 Agar, BACTEC MGIT 960, and BACTEC 460 12B Media for Testing the Susceptibility of *Mycobacterium tuberculosis* to Levofloxacin

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Levofloxacin, the active L-isomer of the quinolone ofloxacin, is now widely accepted for treatment of multidrug-resistant tuberculosis. Because the drug is now widely used, we sought to establish susceptibility test conditions for *Mycobacterium tuberculosis* against levofloxacin by the traditional reference method, agar proportion (AP), the commonly used BACTEC 460 radiometric system, and the newer BACTEC MGIT 960 method. To determine the stability of levofloxacin in the two newer test systems (BACTEC 460 and BACTEC MGIT 960), media containing subinhibitory levels of levofloxacin were prepared and stored at 4 and 37°C for 14 days. The stored media were inoculated with H37Rv, and the drug activity was compared to freshly prepared media. Results show that levofloxacin is stable over the course of testing. Next, optimum levofloxacin test concentrations were determined for AP, BACTEC 460, and BACTEC MGIT 960 methods. MICs were determined for 32 pan-susceptible isolates of *M. tuberculosis* obtained from presumably untreated patients and 14 quinolone-resistant isolates. The levofloxacin-resistant strains either were isolated from patients who remained culture-positive despite treatment with a quinolone agent (six strains) or contained known mutations in *gyrA* (eight strains). Levofloxacin MICs resulted in a bimodal pattern with values for resistant strains consistently higher than those for pan-susceptible strains. Results show that levofloxacin concentrations of 2 µg/ml (BACTEC 460 and BACTEC MGIT 960) and 1 µg/ml (AP) inhibited the growth of all pan-susceptible strains while permitting the growth of all levofloxacin-resistant strains. Confirmatory tests with a subset of pan-susceptible and levofloxacin-resistant isolates validated the selected test concentrations.

Increasing reports of multidrug-resistant tuberculosis (MDR-TB) and the emergence of TB in human immunodeficiency virus-infected persons have resulted in the need for new anti-TB agents (3). The fluoroquinolones, especially ofloxacin, have been shown to be highly useful as second-line anti-TB agents against drug-resistant strains. Studies have demonstrated that levofloxacin (LVX), the L-isomer of ofloxacin, is nearly twice as active against *Mycobacterium tuberculosis* in vitro as its parent compound ofloxacin (8, 9, 15). Because of its effectiveness, LVX has become widely used as a second-line anti-TB agent to treat patients with MDR-TB.

In response to the use of LVX for treatment of MDR-TB, the need to establish laboratory protocols for susceptibility testing of LVX has developed. To address this need, we investigated the conditions necessary to obtain valid LVX susceptibility results with the BACTEC 460 radiometric system (10) and the newer BACTEC MGIT 960 nonradiometric system (13), based on the previously published drug stability studies done by the agar proportion (AP) method (4).

Once susceptibility test conditions were determined, critical testing concentration levels for LVX for the three methods were established with fluoroquinolone-resistant and -susceptible strains of *M. tuberculosis*. Similarly, resistant and susceptible strains of *M. tuberculosis* were tested using each of the methods to validate the resulting choice of test concentration.

MATERIALS AND METHODS

Bacterial strains. Fourteen fluoroquinolone-resistant strains of *M. tuberculosis* were either selected from clinically resistant isolates submitted to the California Department of Health Services for susceptibility testing or obtained from a collection of well-characterized isolates provided by the Public Health Research Institute (PHRI) Tuberculosis Center, New York City, N.Y. (14). Six of the 16 *M. tuberculosis* isolates tested were strains identified by the California Department of Health Services as fluoroquinolone resistant by both BACTEC radiometric and AP methods and were from patients who were culture positive despite a clinical history of fluoroquinolone treatment for TB. The remaining eight resistant isolates were part of a collection of strains obtained from PHRI, which have a mutation in their *gyrA* gene region associated with fluoroquinolone resistance. The isolates from PHRI included four strains that are IS6110 type W and one strain that is type W1. Thirty-two pan-susceptible strains of *M. tuberculosis* were selected from isolates submitted to the California Department of Health Services for susceptibility testing between January 1998 and February 2000 from the Pacific Basin—Kwajalein, Marshall Islands (8); Palau (8); American Samoa (5); Saipan, Northern Mariana Islands (4); Yap, Federated States of Micronesia (FSM) (4); Majuro, Marshall Islands (1); Pohnpei, FSM (1); and Chuuk, FSM (1)—where treatment for *M. tuberculosis* or other infectious agents with fluoroquinolones is unlikely.

LVX. LVX stock solution (1,000 µg/ml) was prepared according to the manufacturer's recommendations and stored at –20°C until use.

LVX stability studies. For the mycobacterial growth indicator tube (MGIT) method, dilutions of LVX were prepared in sterile water, and then 0.1 ml of each drug dilution was added to sets of tubes containing 7.0 ml of MGIT broth supplemented with 0.8 ml of oleic acid-albumin-dextrose-catalase. Two subinhibitory concentrations of LVX, 0.5 and 0.25 µg/ml, were tested. Each tube was then inoculated based on the protocol of Bergmann and Woods (1) with 0.1 ml of a suspension of *M. tuberculosis* H37Rv equivalent to a McFarland turbidity standard of 0.5. One set of MGIT tubes containing drug dilutions was stored at 4°C for 14 days prior to inoculation with *M. tuberculosis* H37Rv. A second set of tubes containing drug dilutions was incubated for 14 days at 37°C prior to inoculation. A third set of tubes received added drug dilutions on the same day as inoculation. Five replicates were run per analysis. Stability comparisons were determined based on the number of days that were required to detect a positive

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result by the MGIT instrument. Results were compared for statistical significance using the one-way analysis of variance.

For the BACTEC 460 method, dilutions of LVX were prepared in sterile water such that when 0.1 ml was added to 12B medium, concentrations of 1.0 and 2.0 $\mu\text{g/ml}$ were obtained. These concentrations were selected to provide sufficient inhibition of growth of the test strain to yield usable endpoint values. To each drug-containing vial, 0.1 ml of a suspension of *M. tuberculosis* H37Rv adjusted to a McFarland turbidity standard of 1 was added. A drug-free control was inoculated with the same H37Rv suspension (McFarland turbidity standard of 1) diluted 1:100 as recommended by the manufacturer. To compare drug stability, one set of 12B vials (containing 1.0 and 2.0 μg of LVX/ml and a drug-free control) was stored at 4°C for 14 days prior to inoculation. A second set of vials was stored at 37°C for 14 days prior to inoculation. A third set of vials was prepared on the same day they were inoculated. Once inoculated, the 12B vials were analyzed with the BACTEC 460 instrument according to the manufacturer's recommendations for the standard nonweekend reading schedule. Five replicates were run per analysis. When the growth index (GI) of the control was ≥ 30 U, the GI values of the sets of drug-containing vials were compared using the one-way analysis of variance test.

Critical test concentration value determination using the BACTEC 460 radiometric method. Serial twofold dilutions of LVX were prepared with sterile water. For fluoroquinolone-resistant strains, LVX test concentrations were 4, 2, 1, 0.5, and 0.25 $\mu\text{g/ml}$. For susceptible strains, LVX test concentrations were 2, 1, 0.5, 0.25, and 0.125 $\mu\text{g/ml}$. The test concentrations selected were expected to be satisfactory, based on previously published MIC results of LVX for *M. tuberculosis* (5, 7, 12). BACTEC 460 radiometric MIC determinations were done by standard methods. The critical concentration for the BACTEC 460 radiometric method for LVX was the concentration that inhibited 100% of susceptible strains of *M. tuberculosis* while permitting growth of all LVX-resistant strains. Once critical concentration values were determined, the values were tested against a subset of susceptible and resistant strains for which MICs were near the critical values to validate the selected critical concentration, since those strains would be the most sensitive indicators of the validity of the selected critical test concentration. The results were then analyzed for statistical significance using chi-square analysis.

Critical test concentration value determination by the AP method. Middlebrook 7H10 agar quadrant plates were prepared by standard methods (6). For fluoroquinolone-resistant strains, the plates were configured such that quadrant I was the drug-free control, and quadrants II, III, and IV contained LVX at 4, 2, and 1 $\mu\text{g/ml}$, respectively. For fluoroquinolone-susceptible strains, quadrant I was the drug-free control, and quadrants II, III, and IV contained LVX at 1, 0.5, and 0.25 $\mu\text{g/ml}$, respectively. The test concentrations selected were expected to be satisfactory based on previously published MIC results of LVX for *M. tuberculosis* (5, 7, 13). Plates were inoculated by standard methods (6). Plates were inoculated according to previously published recommendations (6) with 0.1 ml of 10^{-2} and 10^{-4} dilutions of inoculum adjusted to the turbidity equivalent to a McFarland standard of 0.5. After 21 days of incubation, the colonies on the plates were counted with the aid of a Quebec colony counter and/or microscope. Susceptibility to LVX was determined by comparing the growth in the drug-containing quadrants to the growth in the drug-free control quadrant. Quadrants showing 1% growth or more compared to growth in the control quadrant were considered resistant to that concentration of LVX. The critical concentration for the AP method was the LVX concentration that inhibited 100% of susceptible strains of *M. tuberculosis* while permitting growth of all LVX-resistant strains (2). Once critical concentration values were determined, the values were tested against a subset of susceptible and resistant strains for which the MIC was near the critical values to validate the selected critical concentration.

Critical test concentration value determination for the nonradiometric BACTEC MGIT 960 method. Serial twofold dilutions of LVX were prepared in sterile water. For fluoroquinolone-resistant strains, LVX test concentrations were 4, 2, 1, 0.5, and 0.25 $\mu\text{g/ml}$. For susceptible strains, LVX test concentrations were 2, 1, 0.5, 0.25, and 0.125 $\mu\text{g/ml}$. MIC determinations were done with the BACTEC MGIT 960 instrument and were performed according to the method of Bergmann and Woods (1). Briefly, serial twofold dilutions of LVX were prepared in sterile water, and then 0.1 ml of each drug dilution was added to sets of tubes containing 7.0 ml of MGIT broth supplemented with 0.8 ml of oleic acid-albumin-dextrose-catalase. Each MGIT tube was then inoculated with 0.1 ml of a suspension of the *M. tuberculosis* isolate equivalent to a McFarland turbidity standard of 0.5. At the time of inoculation, a drop of the inoculum was streaked on a Middlebrook 7H10 plate and incubated at 37°C to check for purity. Once inoculated, the MGIT tubes were then entered into the instrument. The BACTEC MGIT 960 instrument monitors the tubes for oxygen utilization, which results in an increase in fluorescence as an indirect indicator of growth. The

TABLE 1. Drug stability study of BACTEC^a

LVX concn ($\mu\text{g/ml}$)	Treatment	Reading no.					
		1	2	3	4	5	Avg
2.0	Fresh	39	44	43	41	44	42.2
	4°C	41	41	45	35	46	41.6
	37°C	40	37	42	40	41	40.0
1.0	Fresh	90	81	75	78	103	85.4
	4°C	70	61	86	70	94	76.2
	37°C	131	63	46	57	58	71.0

^a Results of the drug stability studies in the BACTEC system for LVX at two concentrations in freshly prepared drug-containing medium, medium stored at 4°C for 14 days, and medium stored at 37°C for 14 days inoculated with *M. tuberculosis* strain H37Rv. GI readings in units at the time the drug-free control reached a GI of ≥ 30 (5 days) for the various treatments are shown.

criterion for drug resistance was based on the time to positivity of a drug-containing tube compared to the time to positivity of the drug-free control. A reading was considered resistant if the tube was positive within 2 days or less of the day the drug-free control tube became positive for that isolate. MGIT tubes showing drug resistance were smeared and stained with Ziehl-Neelsen stain to verify the presence of acid-fast bacilli. The critical concentration for the nonradiometric BACTEC MGIT 960 method for LVX was the concentration that inhibited 100% of susceptible strains of *M. tuberculosis* while permitting growth of LVX-resistant strains. Once critical concentration values were determined, the values were tested against a subset of susceptible and resistant strains for which MICs are near the critical values to validate the selected critical concentration.

Lot-to-lot variability testing. Each selected critical testing concentration of LVX for each of the three methods was tested in more than one lot of medium. Comparisons were made between expected and observed susceptible or resistant results.

RESULTS AND DISCUSSION

The stability of LVX was compared for the BACTEC 460 and the BACTEC MGIT 960 methods by determining the effective drug activity in media stored at 4 or 37°C for 14 days with the drug activity available in freshly prepared medium with concentrations of LVX that are subinhibitory against *M. tuberculosis* H37Rv. Results of the LVX stability study for the BACTEC 460 method are shown in Table 1. For the BACTEC 460 method, the GI values of the drug-containing media were compared at the completion of testing, which occurs when the GI of the drug-free control was ≥ 30 U (5 days). The findings showed no significant difference ($P = 0.51$) between the GI values of the LVX-containing medium that was freshly prepared and the GI values of the drug-containing medium that was stored at 4 or 37°C prior to testing. These results indicate that LVX is stable over the time course typically required for in vitro susceptibility testing of *M. tuberculosis* by the BACTEC 460 method. Had drug deterioration occurred, the GI values of the stored media would have been noticeably higher than those of the freshly prepared media, and a statistically significant difference would have been observed.

Table 2 shows the results of the LVX stability study for the BACTEC MGIT 960 method. Shown is the number of days required for the BACTEC MGIT 960 instrument to detect a positive growth signal for *M. tuberculosis* H37Rv in MGIT medium containing subinhibitory levels of freshly added LVX versus drug-containing MGIT medium that had been stored at either 4 or 37°C. No statistically significant difference was detected ($P = 0.20$) in the number of days to positivity between

TABLE 2. Drug stability study with BACTEC MGIT 960 system^a

LVX concn (µg/ml)	Treatment	No. of days to positivity for reading no.					
		1	2	3	4	5	Avg
0.5	Fresh	16.0	19.8	18.2	17.2	21.1	18.5
	4°C	24.5	23.4	16.2	25.9	23.5	22.7
	37°C	24.5	21.1	16.8	27.9	18.8	21.8
0.25	Fresh	5.3	5.4	5.8	4.6	6.1	5.4
	4°C	6.0	5.3	5.5	6.1	4.3	5.4
	37°C	4.7	4.9	5.2	4.8	4.8	4.9

^a Results of the drug stability studies in the BACTEC MGIT 960 system for LVX at two subinhibitory concentrations in freshly prepared drug-containing medium, medium stored at 4°C for 14 days, and medium stored at 37°C for 14 days inoculated with *M. tuberculosis* strain H37Rv. The number of days to positivity indicates the number of days to achieve detectable growth with the BACTEC MGIT 960 instrument. A control with no drug was also tested and became positive in 3.3 to 3.5 days.

the freshly prepared medium and the medium that had been stored at 4 or 37°C prior to inoculation. Had drug deterioration occurred, the number of days required for the BACTEC MGIT 960 instrument to detect growth in the stored media would have been less than the freshly prepared medium. Two concentrations of LVX were tested (0.5 and 0.25 µg/ml) in the stability study for the MGIT 960 method. The 0.5-µg/ml level was chosen as a subinhibitory concentration of LVX very near the MIC for *M. tuberculosis* H37Rv and the resulting time required for an indication of positive growth was quite prolonged (16 to 28 days); however, testing of LVX at this level

provided the most sensitive indicator of drug deterioration. The 0.25-µg/ml level of LVX best demonstrated the drug activity over a time course more in keeping with a typical BACTEC MGIT 960 susceptibility test with *M. tuberculosis* (4 to 7 days). A drug-free control was also tested and became positive in 3.3 to 3.5 days.

LVX MICs were determined for 32 pan-susceptible strains, 6 clinically resistant strains, and 8 resistant strains with known *gyrA* mutations to establish a testing level of LVX that would result in all susceptible strains testing susceptible and all resistant strains testing resistant for the three methods. The results of the LVX MIC determinations are shown in Fig. 1. LVX MICs resulted in a bimodal pattern with values for resistant strains consistently higher than those for the pan-susceptible strains. MICs for all of the quinolone-resistant strains were ≥2 µg/ml. MICs for all of the pan-susceptible strains were ≤1 µg/ml. These results are in agreement with LVX MIC data for *M. tuberculosis* published by other authors (5, 7, 11, 12). Based on our MIC results, a critical testing concentration of LVX of 2 µg/ml was selected for the BACTEC MGIT 960 and BACTEC 460 methods, and a concentration of 1 µg/ml was chosen as the critical testing level for the AP method.

The critical testing levels that were selected of 2 µg/ml for BACTEC 460 and BACTEC MGIT 960 and 1 µg/ml for the AP method were challenged with a subset of pan-susceptible and resistant *M. tuberculosis* strains to verify that findings correlated with expected results. The results are shown in Table 3. There was a statistically significant difference by chi-square

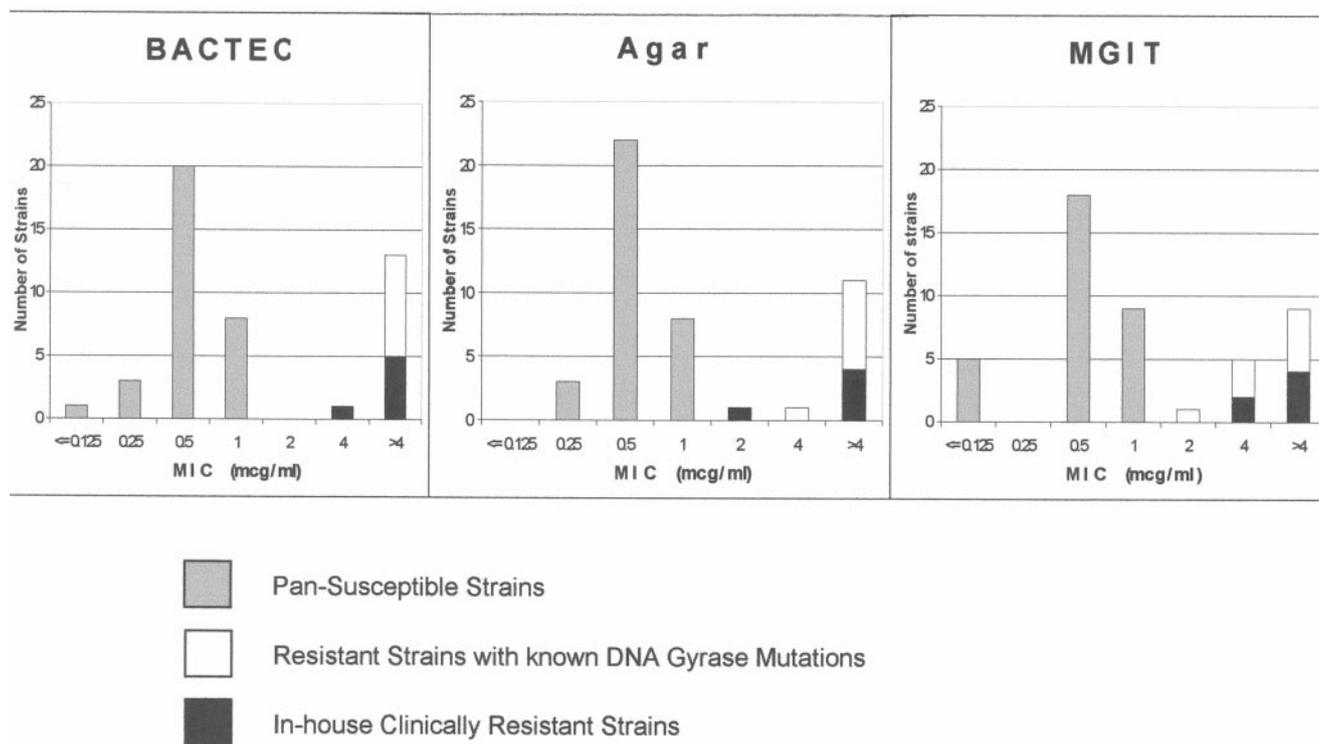


FIG. 1. LVX MIC results for susceptible and resistant clinical isolates of *M. tuberculosis*. LVX MIC results for BACTEC 460, BACTEC MGIT 960, and AP methods are shown for 32 pan-susceptible clinical isolates of *M. tuberculosis* and 14 *M. tuberculosis* isolates that either were clinically resistant to LVX or had known DNA gyrase resistance mutations.

TABLE 3. Results of critical concentration testing of three methods^a

Method	LVX test concn (μg/ml)	No. of isolates with susceptible results/No. susceptible	No. of isolates with resistant results/No. resistant
BACTEC 460	2	8/9 ^b	14/14
BACTEC MGIT 960	2	9/9	14/14
AP	1	23/24 ^c	13/14 ^d

^a Results of LVX critical concentration testing for the BACTEC 460, BACTEC MGIT 960 and AP methods. The critical test concentrations of LVX were chosen for each method based on results of testing clinical isolates of *M. tuberculosis* with known LVX susceptibilities. The selected critical test concentrations were then tested against a subset of LVX-susceptible *M. tuberculosis* isolates as well as isolates that were either clinically resistant to LVX or had known *gyrA* mutations associated with quinolone resistance.

^b One isolate failed to grow in 12B medium.

^c One 7H10 plate was unevaluable due to contamination.

^d One isolate tested borderline resistant (1 to 10%). One isolate failed to grow on 7H10 medium.

analysis between the resistant and susceptible results ($P \leq 0.001$) for the resistant and susceptible isolates, respectively. For the BACTEC 460 method, all of the resistant isolates tested resistant with the critical concentration of 2 μg/ml while all of the susceptible strains but one tested susceptible. The remaining susceptible isolate was nonviable. For the BACTEC MGIT 960 method, 100% of the resistant strains tested resistant and all of the susceptible *M. tuberculosis* strains tested susceptible when the critical concentration of 2 μg/ml was used. For the AP method, 13 of 14 resistant isolates tested resistant at a critical concentration of 1 μg/ml. One resistant isolate was borderline resistant (1 to 10%), but this isolate would be considered resistant for reporting purposes because the colony count in the LVX-containing quadrant was >1% of that of the drug-free control quadrant. Additionally, one other resistant strain failed to grow on the Middlebrook 7H10 medium used for testing. All but one of the susceptible *M. tuberculosis* strains tested susceptible at the 1-μg/ml concentration by AP. This one susceptible strain could not be evaluated, due to overgrowth of the medium by a fungal contaminant.

The selected critical concentrations for all three test methods were repeated with more than one lot of medium. No differences in susceptible or resistant results were seen, due to lot-to-lot variability of media by any of the methods.

LVX is stable over the course of testing for each of the methods examined. For the BACTEC 460 and BACTEC MGIT 960 methods, a critical testing concentration of 2 μg of LVX/ml appears to be a valid testing concentration, because that drug level reliably tested pan-susceptible strains as susceptible and clinically resistant strains and strains with known *gyrA* mutations as resistant. Results also show that a critical testing concentration of 1 μg/ml appears to be a valid testing level for the AP method. Conveniently, this testing level of LVX can easily be obtained in agar by using one commercially available 5-μg LVX disk per 5-ml quadrant of Middlebrook medium, which simplifies the testing process. The findings sug-

gest that results are unaffected by lot-to-lot variability of media by any of the three methods.

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