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Microcolonies in fluoroquinolone agar proportion susceptibility testing of *Mycobacterium tuberculosis*: an indicator of drug resistance

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Abstract

Microcolony growth of *Mycobacterium tuberculosis* on agar proportion susceptibility testing is neither well-defined nor previously reported with fluoroquinolone susceptibility testing. We describe here *M. tuberculosis* microcolony growth with fluoroquinolones, and assess its clinical significance. We screened 79 *M. tuberculosis* isolates for ofloxacin resistance (2.0 µg/mL) by agar proportion; 19 ofloxacin-resistant and 38 ofloxacin-susceptible isolates were selected for more detailed susceptibility testing with ofloxacin, ciprofloxacin, levofloxacin (all at 2.0 µg/mL) and moxifloxacin (0.5 µg/mL). The 57 isolates were also tested at two concentrations both above and below the critical concentrations. Microcolonies were defined as colonies 0.2–0.4 mm in diameter; confirmed microcolonies were present on repeat testing. Of the 57 isolates tested in detail, 7 grew microcolonies, of which 2 (0.3% of all isolates tested) had confirmed microcolonies

on repeat testing (6 tests performed, and microcolonies were present on at least 4). Both *M. tuberculosis* isolates were ofloxacin-resistant on screening, and had ofloxacin minimum inhibitory concentration (MIC) >8 µg/mL. The five other isolates were ofloxacin-susceptible on screening, but had regular colony growth (i.e., resistance) at the drug concentration that initially resulted in microcolonies (ofloxacin 0.5 or 1.0 µg/mL). Microcolonies were observed infrequently with fluoroquinolone susceptibility testing, but when confirmed, they were associated with drug resistance.

Introduction

Drug susceptibility testing of *Mycobacterium tuberculosis* using the agar proportion method indicates drug resistance if the number of *M. tuberculosis* colonies growing in the presence of an anti-tuberculosis drug is $\geq 1\%$ of the number of colonies growing in the absence of drug [1]. Growth of *M. tuberculosis* colonies is visible without magnification, with a usual colony size of 2–4 mm. However, there have been reports of so-called “microcolonies” of *M. tuberculosis*, particularly in the presence of ethambutol [2]. The size of these colonies has not been reported nor standardized and their clinical significance is unknown. The Clinical and Laboratory Standards Institute (CLSI) suggests that microcolonies may represent complete antimicrobial resistance, partial resistance, or may be a result of drug degradation in the media followed by an overgrowth of susceptible organisms [1]. In the report of microcolonies in the presence of ethambutol, the microcolonies could not be confirmed as resistant by the BACTEC 460 TB method or by repeat testing with the agar proportion method; the authors therefore concluded that the microcolonies were not indicative of resistance [2].

We noted the presence of microcolonies while conducting a large study of fluoroquinolone resistance in *M. tuberculosis* [3, 4]. Because this has not previously been reported in fluoroquinolone susceptibility testing of *M. tuberculosis*, and because of the lack of a standard definition of *M. tuberculosis* microcolonies, we sought to describe the *M. tuberculosis* microcolonies we identified, and assess their clinical significance in fluoroquinolone susceptibility testing.

Materials and methods

Study population

A total of 797 *M. tuberculosis* isolates from culture-confirmed tuberculosis patients reporting to the Tennessee Department of Health from January 2002 through December 2007 were screened for resistance to ofloxacin as part of a large population-based study [3, 4]. All isolates were screened by agar proportion for resistance to 2.0 µg/mL ofloxacin. Nineteen isolates were ofloxacin-resistant and were selected for further testing with ofloxacin, ciprofloxacin, levofloxacin, and moxifloxacin [4]. These isolates were tested at a critical concentration of 2.0 µg/mL for ofloxacin, ciprofloxacin, and levofloxacin and 0.5 µg/mL for moxifloxacin [1]. They were also tested at two concentrations above and two concentrations below each critical concentration. For ofloxacin, ciprofloxacin, and levofloxacin, these additional concentrations were 0.5, 1.0, 4.0, and 8.0 µg/mL; for moxifloxacin, the additional concentrations were 0.125, 0.25, 1.0 and 2.0 µg/mL. The stability of all four fluoroquinolones was tested and confirmed.

For each ofloxacin-resistant isolate identified on screening, two ofloxacin-susceptible isolates were selected for the additional testing with the above four drugs at the five concentrations mentioned. The two susceptible isolates were from tuberculosis patients diagnosed in the same year as the patient with the resistant isolate; for each pair, one patient

had received outpatient fluoroquinolones prior to tuberculosis diagnosis and the other patient had not.

Susceptibility testing by agar proportion was performed according to standard protocols established by the CLSI [1]. The fluoroquinolone-susceptible control was a reference ATCC *M. tuberculosis* strain H37Rv (#27294). The fluoroquinolone-resistant control strain was provided by the Centers for Disease Control and Prevention (Division of Tuberculosis Elimination, Mycobacteriology Laboratory Branch).

For the expanded susceptibility testing of the 57 *M. tuberculosis* isolates, stock solutions of the powdered drugs were prepared in the recommended solvent and incorporated into 7H10 media during preparation. Ofloxacin, ciprofloxacin, and levofloxacin were purchased from Sigma Aldrich. Moxifloxacin was donated by Bayer Pharmaceuticals. All 7H10 agar plates were prepared on-site using the same amount of agar for each plate, and all reagents were either purchased or prepared on-site.

Laboratory methods

All clinical *M. tuberculosis* isolates were stored at the Tennessee State mycobacteriology laboratory at -70°C . *M. tuberculosis* isolates were thawed and sub-cultured onto was prepared in 7H9 broth from colonies on the LJ slant and served as the standard inoculum for susceptibility testing for agar proportion.

Drug susceptibility testing

Agar proportion method—A 10^{-2} and 10^{-4} dilution was made from the 1.0 McFarland standard inoculum. One drug-containing quadrant and one drug-free quadrant of a 7H10 agar plate were inoculated with 100 μL of each dilution; each quadrant contained 5 mL of agar. The plates were sealed and incubated at 37°C in 5–10% CO_2 and examined weekly for colony growth. At the end of 3 weeks each plate was read and the results were finalized and recorded. For drug susceptibility testing of microcolonies a standard incubation time of 3 weeks was used. If there was no growth on the control quadrants at the end of the 3-week incubation period the plates were incubated for an additional 3 weeks and the results finalized at 6 weeks.

Minimum inhibitory concentration (MIC) was determined using the agar proportion method by doubling the concentration of ofloxacin (in $\mu\text{g/mL}$) until growth of *M. tuberculosis* was inhibited. For *M. tuberculosis* isolates resistant at 2.0 $\mu\text{g/mL}$ ofloxacin, MIC testing started at that concentration. For isolates susceptible at 2.0 $\mu\text{g/mL}$, MIC testing started at 0.5 $\mu\text{g/mL}$. The MIC was determined once for each *M. tuberculosis* isolate.

Genotypic resistance testing—Genomic DNA extracted from *M. tuberculosis* colonies grown on Lowenstein–Jensen medium underwent polymerase chain reaction (PCR). Amplification of the quinolone resistance-determining region (QRDR) of *gyrA* and *gyrB* was performed in separate 50- μL reactions for each isolate. Purified DNA was cloned using the TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Resultant plasmid DNA was sequenced using an ABI Prism 3730xl DNA Analyzer. Sequences were analyzed using Polyphred software (University of Washington) and compared with the sequence of *M. tuberculosis* H37Rv (S. Cole, Pasteur Institute, France).

Study definitions

Microcolonies of *M. tuberculosis* were defined as colonies 0.2–0.4 mm in diameter (compared with 2–4 mm for regular colonies) (Fig. 1 and 2). Agar plates were inspected weekly for growth while tilted in a light source. Microcolonies, if present, were visible with

the naked eye; colony characteristics were visible with the use of a biological microscope at 10×, 20×, and 40×. If microcolonies were identified, the testing condition was repeated. The *M. tuberculosis* isolate and testing conditions were considered to result in confirmed microcolonies only if they were present in at least two tests.

Results

On screening of the 797 *M. tuberculosis* isolates for ofloxacin resistance at 2.0 µg/mL, no microcolony growth was observed. Of the 19 ofloxacin-resistant and 38 ofloxacin-susceptible *M. tuberculosis* isolates selected for additional susceptibility testing, 7 grew microcolonies at least once in the presence of fluoroquinolones (Table 1). Five of these isolates did not grow microcolonies on repeat testing. On initial testing, 4 of these 5 isolates grew microcolonies at ofloxacin 0.5 µg/mL; one of these isolates also grew microcolonies at ciprofloxacin 0.5 µg/mL. The final isolate grew microcolonies at ofloxacin 1.0 µg/mL. There were no microcolonies noted at the other concentrations of ciprofloxacin tested, or at any concentration of levofloxacin tested. Of note, on repeat testing, all 5 isolates grew regular colonies at the same concentration that had resulted in microcolonies on initial testing.

Two *M. tuberculosis* isolates (0.3% of all isolates tested) grew microcolonies on repeat testing (Table 1). Both isolates were resistant to ofloxacin on screening, and had ofloxacin MICs 8 µg/mL (Table 2). The MIC of the other 17 ofloxacin-resistant *M. tuberculosis* isolates ranged from 16 to 256 µg/mL (data not shown). Isolate # 3 grew microcolonies at ofloxacin 8.0 µg/mL and isolate # 7 grew microcolonies at moxifloxacin 0.5 µg/mL. Microcolony growth was noted 14–21 days after inoculation; all colonies present on the agar at these drug concentrations were microcolonies. Neither isolate had microcolony growth at any concentration of ciprofloxacin or levofloxacin.

These two isolates were each re-tested five additional times using the same drug and drug concentrations at which microcolonies were initially noted. Isolate # 3 grew microcolonies 5 out of 6 times at ofloxacin 8.0 µg/mL. All colonies present were microcolonies, and the number of microcolonies present in these five experiments ranged from 2 to 40. There were 50–200 colonies (all of regular size) in the control quadrant without drug. Isolate # 7 grew microcolonies 4 out of 6 times with moxifloxacin 0.5 µg/mL (Table 1). The number of microcolonies on agar ranged from 50 to 200, similar to the number of regular-sized colonies in control quadrants without drug.

The microcolonies grown by these two ofloxacin-resistant *M. tuberculosis* isolates were subsequently restreaked to a drug-free 7H10 agar plate and allowed to incubate for 3 weeks at 37°C in 5–10% CO₂. The resultant colonies had normal size and morphology. Kinyoun stains of the colonies were positive for acid-fast organisms.

Isolate # 3 had the Ala90Val mutation in the QRDR of *gyrA*. There were no other mutations identified in the QRDR of *gyrA* or *gyrB* among the seven isolates tested (Table 2).

Two out of 19 (10.5%) fluoroquinolone-resistant *M. tuberculosis* strains grew reproducible microcolonies compared with 0 out of 38 (0%) fluoroquinolone-susceptible *M. tuberculosis* strains (Fisher's exact test $P=0.11$).

Discussion

We report here 7 *M. tuberculosis* isolates in which microcolonies were noted on fluoroquinolone susceptibility testing, 2 of which grew microcolonies on repeat testing. The microcolonies were distinctly smaller than regular *M. tuberculosis* colonies (0.2–0.4 mm vs 2–4 mm in diameter; roughly one-tenth the size), yet were visible without magnification.

Growth was present 21 days after inoculation of the agar. Additional studies are needed to develop and standardize a definition of *M. tuberculosis* microcolonies. Such studies might include genotypic resistance testing in which DNA from microcolonies (rather than regular colonies, as was done in this study) are cloned and sequenced for fluoroquinolone resistance mutations in *gyrA* and *gyrB*. In addition, microcolonies could be sub-cultured onto Lowenstein–Jensen agar for subsequent agar proportion drug susceptibility testing to see if normal colony growth occurs in the presence of fluoroquinolones.

The frequency of confirmed microcolonies in this study was low, seen in only 2 out of 797 (0.3%) *M. tuberculosis* isolates tested. Both of these isolates were resistant to ofloxacin 2 µg/mL on screening, and both had ofloxacin MICs 8 µg/mL. One isolate had the Ala90Val mutation in the QRDR of *gyrA*, which has been associated with fluoroquinolone resistance in *M. tuberculosis* [5, 6]. Thus, the presence of confirmed microcolonies did not add significant clinical information regarding fluoroquinolone resistance in these two isolates. However, the identification of microcolonies on testing of these isolates provides some evidence that microcolonies are associated with drug resistance.

All five of the *M. tuberculosis* isolates in which microcolonies could not be replicated grew regular colonies on repeat testing, consistent with drug resistance. Of note, 2 of these patients had received outpatient fluoroquinolone exposure prior to tuberculosis diagnosis. Isolate # 4 was the most interesting, in that regular colony growth was noted at ofloxacin 0.5 µg/mL, microcolonies were noted on initial testing at 1.0 µg/mL (regular colonies on repeat testing), and no growth was noted at 2.0 µg/mL. This patient had received a 28-day course of fluoroquinolones as an outpatient prior to diagnosis of tuberculosis. Increased duration of fluoroquinolone exposure prior to diagnosis has been associated with an increased risk of fluoroquinolone resistance in this same study population [3]. These findings provide additional support for the hypothesis that microcolonies represent some level of drug resistance, and that when microcolonies are identified in fluoroquinolone susceptibility testing performed in a clinical microbiology laboratory, they should be evaluated further.

We required microcolonies to be reproducible to ensure that it was a true phenomenon. In isolates in which microcolonies were not present on repeat testing, it is unclear whether this was an unstable effect, or whether such isolates simply did not have this phenotype. If there were phenotypic flexibility, it could be due to the isolates having gyrase variants that result in a level of resistance that allows them to remain viable at the precise antibiotic concentration in the selective medium. We did not identify resistance mutations in the QRDR of *gyrA* or *gyrB* in the five isolates in which microcolonies could not be reproduced. However, this does not rule out the possibility of other polymorphisms that could contribute to this phenotypic flexibility.

It is unclear what microcolonies represent physiologically for the *M. tuberculosis* strain and humans. In a novel in vitro assay in which human skin fibroblasts were infected with attenuated (H37Ra) and virulent (H37Rv, Erdman, and clinical) strains of *M. tuberculosis*, microcolonies were visible to the naked eye after 7–10 days. The microcolonies of the virulent strains were larger than those of the avirulent strain, and the former exhibited a characteristic elongated appearance and extensive cording [7]. The authors suggest that these differences in microcolony characteristics might be associated with the differences in cell-to-cell spread of avirulent and virulent *M. tuberculosis* strains. These investigators also noted that the anti-tuberculosis agent streptomycin had different effects on microcolony formation, with avirulent strains having the smallest microcolonies and greatest decrease in the number of colonies in the presence of the drug [7]. The above suggests the possible importance of characterizing the physical features of microcolonies to assess their clinical significance.

Although the agar proportion method is the gold standard for *M. tuberculosis* drug susceptibility testing [1], there is no standardized methodology for fluoroquinolone susceptibility testing using agar. However, there are guidelines for fluoroquinolone drug susceptibility testing, and they were followed in this study [8].

This study is important in that it reports for the first time microcolonies identified on fluoroquinolone susceptibility testing in *M. tuberculosis*. The occurrence of microcolonies was infrequent. However, when confirmed, microcolonies were associated with drug resistance.

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Fig. 1.
Regular *M. tuberculosis* colonies after incubation for 3 weeks on 7H10 agar. The plastic bar is 6 mm wide and 1 mm high. Photograph magnification, $\times 1.4$

Fig. 2.
Microcolonies of *M. tuberculosis* after incubation for 3 weeks on 7H10 agar. The plastic bar is 6 mm wide and 1 mm high. Photograph magnification, $\times 1.4$

Table 1

M. tuberculosis colony growth according to fluoroquinolone and drug concentration tested by the agar proportion method. Of the seven *M. tuberculosis* isolates that had microcolony growth, 5 did not grow microcolonies on repeat testing; all 5 had normal colony growth at those same drug concentrations

Patient isolate	Drug concentration (µg/mL)	Number of times tested/ number times microcolony growth occurred
Ofloxacin		
1	1	0 0 0 2/1
2	1	0 0 0 2/1
3	2	2 2 2 6/5
4	2	1 0 0 2/1
5	1	1 0 0 2/1
6	1	0 0 0 2/1
7	2	2 2 2 1/0
Ciprofloxacin		
1	2	0 0 0 1/0
2	1	0 0 0 2/1
3	2	2 2 2 1/0
4	2	0 0 0 1/0
5	2	0 0 0 1/0
6	0	0 0 0 1/0
7	2	2 2 2 1/0
Levofloxacin		
1	0	0 0 0 1/0
2	0	0 0 0 1/0
3	2	2 2 0 1/0
4	0	0 0 0 1/0
5	0	0 0 0 1/0
6	0	0 0 0 1/0
7	2	2 2 2 1/0
Moxifloxacin		
	0.125	0.25 0.5 1.0 2.0

Patient isolate	Drug concentration (µg/mL)		Number of times tested/ number times microcolony growth occurred	
1	0	0	0	1 / 0
2	2	2	0	0 1 / 0
3	2	2	2	0 1 / 0
4	2	2	2	0 1 / 0
5	2	2	0	0 1 / 0
6	0	0	0	0 1 / 0
7	2	2	1	0 6 / 4

Code for colony growth (*italics*): 0=no growth; 1=microcolony growth; 2=regular colony growth

Table 2

Characteristics of the seven *M. tuberculosis* isolates that grew microcolonies on at least one occasion. Ofloxacin susceptibility 2 µg/mL, the minimum inhibitory concentration (MIC), genotypic resistance mutations identified, and outpatient fluoroquinolone exposure prior to tuberculosis diagnosis. No resistance mutations were identified in the QRDR of *gyrB*

Patient isolate	Microcolony on repeat testing	Ofloxacin susceptibility	Ofloxacin MIC (µg/mL)	Mutations in QRDR of <i>gyrA</i>	Days of outpatient fluoroquinolone exposure prior to TB diagnosis
1	No	S	1		0
2	No	S	1		12 ^a
3	Yes	R	8	Ala90Val	23 ^b
4	No	S	2		28 ^c
5	No	S	2		0
6	No	S	1		0
7	Yes	R	128		24 ^d

QRDR: quinolone-resistance determining region; S: susceptible; R: resistant

^a 12 days of levofloxacin

^b 23 days of levofloxacin

^c 28 days of ciprofloxacin

^d 7 days of ciprofloxacin, 17 days of levofloxacin