Measuring of *Mycobacterium tuberculosis* growth. A correlation of the optical measurements with colony forming units

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Abstract

The quantification of colony forming units (cfu), turbidity, and optical density at 600 nm (OD₆₀₀) measurements were used to evaluate *Mycobacterium tuberculosis* growth. Turbidity and OD₆₀₀ measurements displayed similar growth curves, while cfu quantification showed a continuous growth curve. We determined the cfu equivalents to McFarland and OD₆₀₀ units.

Key words: bacterial growth, colony forming units, McFarland, *Mycobacterium tuberculosis*, optical density.

The McFarland standards are a series of references of different densities used to estimate the concentration of cells in microbial cultures (Versalovic *et al.*, 2011). The turbidity of these standards is compared to a defined number of *Escherichia coli* microorganisms per mL (Perilla *et al.*, 2003), and frequently has been used to estimate the density of *Mycobacterium tuberculosis* cultures (Bergmann *et al.*, 2000; Bollela *et al.*, 1999; Elbir *et al.*, 2008; Leonard *et al.*, 2008; Syre *et al.*, 2003). It is commonly assumed that *E. coli* and *M. tuberculosis* cultures with equivalent concentrations have equivalent turbidities (Bollela *et al.*, 1999; Elbir *et al.*, 2008). This assumption has not been validated and could be contributing to erroneous quantifications. Another method for quantifying the concentration of microbial cultures includes measuring the optical density at 600 nm (OD₆₀₀); though measuring *M. tuberculosis* concentrations using OD is controversial (Iona *et al.*, 2007; Taneya and Tyagi, 2007). The method considered the most reliable for measuring the concentration of viable microorganisms in culture is the quantification of colony-forming units (cfu) per unit volume of culture (Davey *et al.*, 2004). However, this approach is laborious, and for *M. tuberculosis*, requires lengthy time periods to obtain results (Damato *et al.*, 1983; von Groll *et al.*, 2010). The aim of this study was to define the number of viable *M. tuberculosis* cells equivalent to McFarland and OD₆₀₀ measurements in liquid cultures and to compare the reliability of these methods in estimating *M. tuberculosis* concentrations in suspension.

*M. tuberculosis* cultures (strain H37Rv, ATCC 27294) were inoculated in Lowenstein-Jensen slants and incubated at 37 °C in 5% CO₂ atmosphere for 2 weeks. A portion of the mycobacterial colonies was transferred to Middlebrook 7H9 medium (Difco, Becton Dickinson, Le Pont de Clai, France) supplemented with 10% OADC (oleic acid, albumin, dextrose and catalase, Becton Dickinson and Company, Sparks, MD. USA.). The cultures were
incubated at 37 °C in 5% CO₂ atmosphere until they reached a turbidity equivalent to the McFarland standard No. 1. A 100 μL aliquot was inoculated in 10 mL of Middlebrook 7H9 medium (Becton Dickinson) supplemented with 10% OADC (Becton Dickinson and Company). To avoid clump formation the cultures were incubated in constant shaking at 300 rpm.

The turbidity, OD₆₀₀ and cfu per milliliter (cfu/mL) of 1 mL aliquots of mycobacteria cultures were measured every three days for 21 days. Data obtained with cfu/mL counting were compared with McFarlands and OD₆₀₀ measurements. The turbidity of the aliquots was measured using a nephelometer (ATB 1550, BioMérieux, France). Prior to measuring the OD₆₀₀ using a spectrophotometer (DU 800 Beckman Coulter, CA, USA), cultures were inactivated with 10% (v/v) formaldehyde (Sigma-Aldrich, Steinheim, Germany) and adjusted to the appropriate dilution. Before formaldehyde addition, cultures were serially diluted 10-fold (from 10⁻² to 10⁻⁸) to quantify cfu/mL. 100 μL aliquots from these dilutions were inoculated on Middlebrook 7H10 agar media (Difco, Becton Dickinson, Le Pont de Clai, France) and incubated as described above until colonies were visible. The *M. tuberculosis* colonies were counted and adjusted according to the dilution factor. The generation time was calculated using cfu/mL counting by the following equation: \( \log(N) = \log(N_0) + Kt \), where \( N \) = final number of microorganisms, \( N_0 \) = inoculum, \( K \) = slope, and \( t \) = incubation time.

Though the samples measured by each method in the study were processed from the same *M. tuberculosis* cultures, under comparable conditions, cfu/mL and McFarland's growth curves showed a longer lag phase than observed at OD₆₀₀. The logarithmic growth phase lasted nine days in all cases, conserving the starting delay. The stationary phase was observed by day 15 in growth curves using the McFarland method and OD₆₀₀, whereas the cfu/mL curve did not have a stationary phase within the 21 days of incubation (Figure 1). The discrepancy observed between the McFarland and OD₆₀₀ curves could be due to a higher threshold of detection for the nephelometer (BioMérieux) as compared to the spectrophotometer (Beckman Coulter). When the multidrug resistant clinical isolate CIBIN:UMF:15:99 (Molina-Salinas et al., 2006) of *M. tuberculosis* was used to evaluate the equivalents of McFarland and OD₆₀₀ measurements, we did not observe a correlation with the growth curve of H₃₇Rv reference strain (data not shown).

In quantifying cfu/mL by colony counting, colonies were not observed until the 9th day of incubation. During log phase, the generation time was calculated in 24.91 h. Figure 2 shows the correlation of the growth curves generated from the different methods. The correlation between cfu/mL quantification and OD₆₀₀ measurement was the lowest (\( R^2 = 0.8913 \), Figure 2A), followed by the correlation between cfu/mL quantification and turbidity measurement (\( R^2 = 0.9252 \), Figure 2B). OD₆₀₀ and turbidity measurements were highly correlated (\( R^2 = 0.9823 \), Figure 2C). We determined in *M. tuberculosis* H₃₇Rv ATCC 27294 that 1 McFarland unit is equivalent to either 1.97 x 10⁶ cfu/mL or 0.39 OD₆₀₀, and an OD₆₀₀ measurement of 1 is equivalent to either 3.13 x 10⁷ cfu/mL or 3.66 McFarland units.

Quantification of *M. tuberculosis* in liquid cultures is difficult as this microorganism is prone to clump formation (Lambrecht et al., 1988). Results from this study show that turbidity and optical density measurements yield similar growth curves, with lag, log and stationary phases clearly defined (Figure 1). Though these methods are easy to perform and the equipment is readily available, these methods do not distinguish between live and dead microorganisms. Thus, the measurements do not reflect the concentration of actively growing cells. In addition, turbidity measurement
by nephelometry uses versatile equipment that can be maintained in biosafety rooms, but the real mycobacterial number equivalent to the McFarland standards is not known with certainty (Kitchen et al., 1998; Martin-Casabona et al., 1997; Raut et al., 2008).

In this study, we compare turbidity and OD$_{600}$ measurements to cfu/mL quantification. Our results demonstrate there is a relationship between turbidity and OD$_{600}$ lectures and cfu/mL quantification. These measurements are not quite as precise, since $M. \text{tuberculosis}$ tends to form clumps. Nevertheless, we have established equivalents of McFarland and OD$_{600}$ units for a defined number of viable cells in $M. \text{tuberculosis}$ H37Rv ATCC 27294 cultures. The lack of correlation of McFarland and OD$_{600}$ equivalents found between a multidrug-resistant clinical isolate and H37Rv strain of $M. \text{tuberculosis}$ could be explained by its high rate of clumping and the slower growing. On the other hand, McFarland standards for *Escherichia coli* cultures (Perilla et al., 2003) are not comparable with those of $M. \text{tuberculosis}$ because they have different physical properties such as cell size, sedimentation, and light scattering. For example, the bacterial size for *E. coli* is 1-2 µm length (Zobell and Cobet, 1962) vs. 3.5-4 µm of $M. \text{tuberculosis}$ (Will et al., 1951); $M. \text{tuberculosis}$ has a higher sedimentable mass than *E. coli*, as well as a suspension of *E. coli* scatters the light more intensely than $M. \text{tuberculosis}$ (Jaiswal and Panda, 2009). These data support the idea of an erroneous quantification when McFarland equivalents in *E. coli* are used to evaluate the growth of *M. \text{tuberculosis}*, which lead to a misinterpretation of results in liquid cultures. Other methods such as respiration rate (Gomez-Flores et al., 1995), resazurin reduction (Sanchotene et al., 2008; von Groll et al., 2010), as well as protein and ATP measurements (Meyers et al., 1998) have measured the growth kinetics of *M. \text{tuberculosis*}, but all of these methods require further incubation time and analysis resulting in a delay of additional assays. In conclusion, OD$_{600}$ measuring is the most sensible method for the evaluation of $M. \text{tuberculosis}$ growth in liquid cultures, whereas the growth curve is more consistent using McFarland method, particularly between 9-15 days.

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