Brazilian Journal of Microbiology 44, 1, 287-290 (2013)
ISSN 1678-4405

Short Communication

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

Katia Peñuelas-Urquides[1,2], Licet Villarreal-Treviño[2], Beatriz Silva-Ramírez[3], Liliana Rivadeneyra-Espinoza[4], Salvador Said-Fernández[5], Mario Bermúdez de León[1]

[1]Laboratorio de Biología Molecular, Centro de Investigación Biomédica del Noreste, Instituto Mexicano del Seguro Social, Monterrey, NL, México.
[2]Posgrado en Microbiología, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, NL, México.
[3]División de Genética, Centro de Investigación Biomédica del Noreste, Instituto Mexicano del Seguro Social, Monterrey, NL, México.
[4]División de Biología Celular y Molecular, Centro de Investigación Biomédica del Noreste, Instituto Mexicano del Seguro Social, Monterrey, NL, México.
[5]Departamento de Bioquímica y Medicina Molecular, Facultad de Medicina, Universidad Autónoma de Nuevo León, Monterrey, NL México.

Submitted: September 21, 2011; Approved: July 2, 2012.

Abstract

The quantification of colony forming units (cfu), turbidity, and optical density at 600 nm (OD_{600}) measurements were used to evaluate *Mycobacterium tuberculosis* growth. Turbidity and OD_{600} measurements displayed similar growth curves, while cfu quantification showed a continuous growth curve. We determined the cfu equivalents to McFarland and OD_{600} 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 (OD_{600}); though measuring *M. tuberculosis* concentrations using OD is controversial (Iona *et al.*, 2007; Taneja 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 OD_{600} 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% CO_2 atmosphere for 2 weeks. A portion of the mycobacterial colonies was transferred to Middlebrook 7H9 medium (Difco, Becton Dickinson, Le Pont de Claix, France) supplemented with 10% OADC (oleic acid, albumin, dextrose and catalase, Becton Dickinson and Company, Sparks, MD. USA.). The cultures were

Send correspondence to M.B. León. Laboratorio de Biología Molecular, Centro de Investigación Biomédica del Noreste, Instituto Mexicano del Seguro Social, Ave 2 de Abril 501, Col. Independencia, C.P. 64720, Monterrey, N.L., México. E-mail: mario.bermudez@imss.gob.mx.

incubated at 37 °C in 5% CO_2 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, OD_{600} 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 OD_{600} measurements. The turbidity of the aliquots was measured using a nephelometer (ATB 1550, BioMérieux, France). Prior to measuring the OD_{600} 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 Claix, 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 McFarlands growth curves showed a longer lag phase than observed at OD_{600}.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 OD_{600}, 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 OD_{600} 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 OD_{600} 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 OD_{600} 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). OD_{600} 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 10^6 cfu/mL or 0.39 OD_{600}, and an OD_{600} measurement of 1 is equivalent to either 3.13 x 10^7 cfu/mL or 3.66 McFarland units.

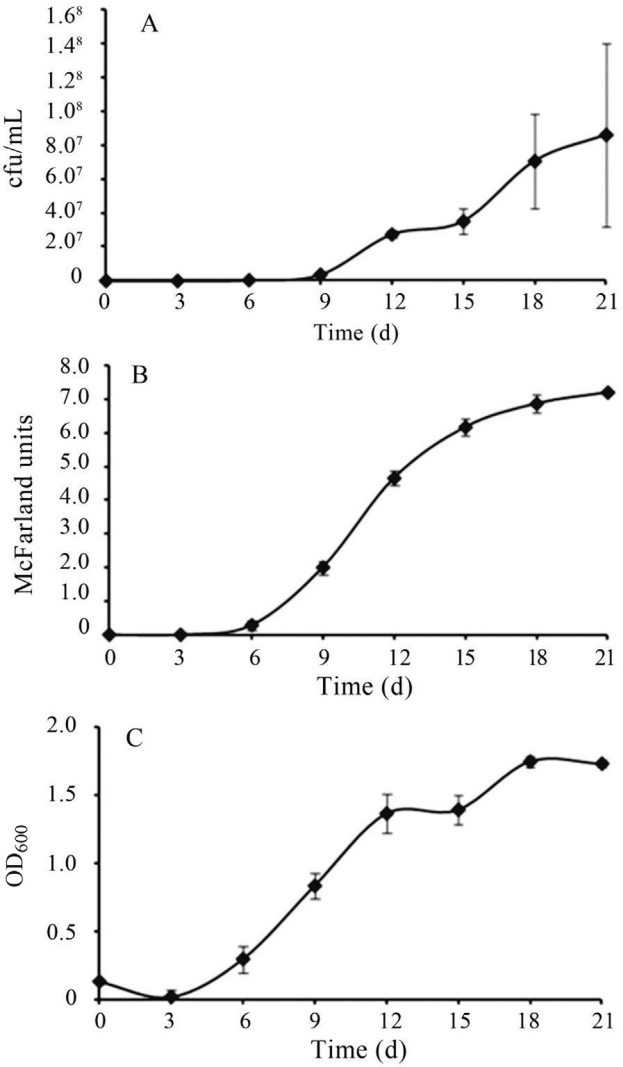

Figure 1 - Growth curves of *M. tuberculosis* over the course of 21 days using cfu/mL counting (A), turbidity measurements (B), and OD_{600} measurements (C). Each point corresponds to the average of three determinations ± standard deviation.

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

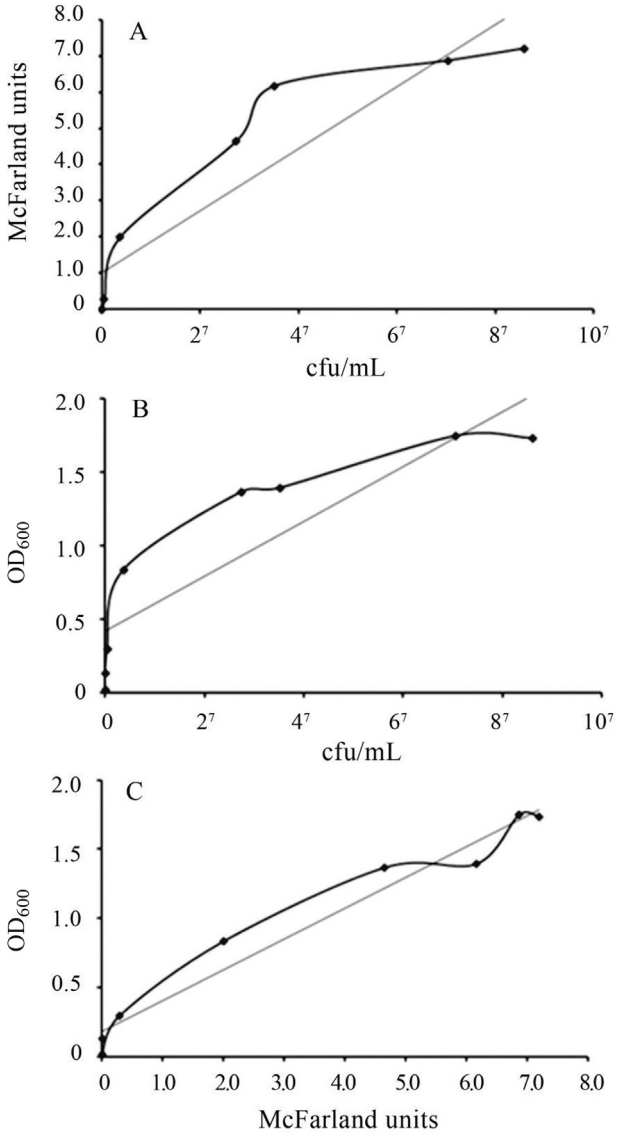

Figure 2 - Correlation of *M. tuberculosis* growth curves evaluated by different methods: (A) cfu/mL counting and turbidity measurement, (B) cfu/mL counting and OD_{600} measurement, and (C) turbidity and OD_{600} measurements.

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. 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. 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. 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. 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. tuberculosis* (Will *et al.*, 1951); *M. tuberculosis* has a higher sedimentable mass than *E. coli,* as well as a suspension of *E. coli* scatters the light more intensely than *M. 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. 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. 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. tuberculosis* growth in liquid cultures, whereas the growth curve is more consistent using McFarland method, particularly between 9-15 days.

Acknowledgments

This work was supported by a grant from CONACyT (99792-M)

References

Bergmann JS, Fish G, Woods GL (2000) Evaluation of the BBL MGIT (Mycobacterial growth indicator tube) AST SIRE system for antimycobacterial susceptibility testing of Mycobacterium tuberculosis to 4 primary antituberculous drugs. Arch Pathol Lab Med 124:82-86.

Bollela VR, Sato DN, Fonseca BA (1999) McFarland nephelometer as a simple method to estimate the sensitivity of the polymerase chain reaction using Mycobacterium tuberculosis as a research tool. Braz J Med Biol Res 32:1073-1076.

Damato JJ, Collins MT, Rothlauf MV, McClatchy JK (1983) Detection of mycobacteria by radiometric and standard plate procedures. J Clin Microbiol 17:1066-1073.

Davey HM, Kell DB, Weichart DH, Kaprelyants AS (2004) Estimation of microbial viability using flow cytometry. Curr Protoc Cytom, Chapter 11, Unit 11 13.

Elbir H, Abdel-Muhsin AM, Babiker A (2008) A one-step DNA PCR-based method for the detection of Mycobacterium tuberculosis complex grown on Lowenstein-Jensen media. Am J Trop Med Hyg 78:316-317.

Gomez-Flores R, Gupta S, Tamez-Guerra R, Mehta RT (1995) Determination of MICs for Mycobacterium avium-M. intracellulare complex in liquid medium by a colorimetric method. J Clin Microbiol 33:1842-1846.

Iona E, Giannoni F, Pardini M, Brunori L, Orefici G, Fattorini L (2007) Metronidazole plus rifampin sterilizes long-term dormant Mycobacterium tuberculosis. Antimicrob Agents Chemother 51:1537-1540.

Jaiswal R, Panda D (2009) Differential assembly properties of Escherichia coli FtsZ and Mycobacterium tuberculosis FtsZ: an analysis using divalent calcium. J Biochem 146:733-742.

Kitchen LW, Weston CM, Day SP (1998) Diethylcarbamazine-related antimicrobial activity in Mycobacterium tuberculosis-infected blood. J Antimicrob Chemother 42:241-243.

Lambrecht RS, Carriere JF, Collins MT (1988) A model for analyzing growth kinetics of a slowly growing Mycobacterium sp. Appl Environ Microbiol 54:910-916.

Leonard B, Coronel J, Siedner M, Grandjean L, Caviedes L, Navarro P, Gilman RH, Moore DA (2008) Inter- and intra-assay reproducibility of microplate Alamar blue assay results for isoniazid, rifampicin, ethambutol, streptomycin, ciprofloxacin, and capreomycin drug susceptibility testing of Mycobacterium tuberculosis. J Clin Microbiol 46:3526-3529.

Martin-Casabona N, Xairo Mimo D, Gonzalez T, Rossello J, Arcalis L (1997) Rapid method for testing susceptibility of Mycobacterium tuberculosis by using DNA probes. J Clin Microbiol 35:2521-2525.

Meyers PR, Bourn WR, Steyn LM, van Helden PD, Beyers AD, Brown GD (1998) Novel method for rapid measurement of growth of mycobacteria in detergent-free media. J Clin Microbiol 36:2752-2754.

Molina-Salinas GM, Ramos-Guerra MC, Vargas-Villarreal J, Mata-Cardenas BD, Becerril-Montes P, Said-Fernandez S (2006) Bactericidal activity of organic extracts from Flourensia cernua DC against strains of Mycobacterium tuberculosis. Arch Med Res 37:45-49.

Perilla MJ, Ajello MS, Bopp C, Elliott J, Facklam R, Popovic T, Wells J (2003) Manual for the Laboratory Identification and Antimicrobial Susceptibility Testing of Bacterial Pathogens of Public Health Importance in the Developing World. Centre for Disease Control and Prevention: National Centre for Infectious Diseases, Atlanta, Georgia, USA.

Raut U, Narang P, Mendiratta DK, Narang R, Deotale V (2008) Evaluation of rapid MTT tube method for detection of drug susceptibility of Mycobacterium tuberculosis to rifampicin and isoniazid. Indian J Med Microbiol 26:222-227.

Sanchotene KO, von Groll A, Ramos D, Scholante AB, Honscha G, Valença M, Scaini CJ, da Silva PEA (2008) Comparative evaluation of the Nitrate Reductase Assay and the Resazurin Microtitre Assay for drug susceptibility testing of Mycobacterium tuberculosis against first line anti-tuberculosis drugs. Braz J Microbiol 39:16-20.

Syre H, Phyu S, Sandven P, Bjorvatn B, Grewal HM (2003) Rapid colorimetric method for testing susceptibility of Mycobacterium tuberculosis to isoniazid and rifampin in liquid cultures. J Clin Microbiol 41:5173-5177.

Taneja NK, Tyagi JS (2007) Resazurin reduction assays for screening of anti-tubercular compounds against dormant and actively growing Mycobacterium tuberculosis, Mycobacterium bovis BCG and Mycobacterium smegmatis. J Antimicrob Chemother 60:288-293.

Versalovic J, Carrol KC, Funke G, Jorgensen JH, Landry ML, Warnock DW (2011) *Manual of Clinical Microbiology*. 10th ed.; American Society for Microbiology Press, Washington, DC.

von Groll A, Martin A, Portaels F, Almeida da Silva PE, Palomino JC (2010) Growth kinetics of Mycobacterium tuberculosis measured by quantitative resazurin reduction assay: a tool for fitness studies. Braz J Microbiol 41:300-303.

Will DW, Bishop F, Bogen E, Djang AH, Carpenter CM (1951) Comparative morphology of acid-fast bacilli. Dis Chest 19:387-410.

Zobell CE, Cobet AB (1962) Growth, reproduction, and death rates of Escherichia coli at increased hydrostatic pressures. J Bacteriol 84:1228-1236.