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McFarland nephelometer as a simple method to estimate the sensitivity of the polymerase chain reaction using *Mycobacterium tuberculosis* as a research tool

V.R. Bollela[1], D.N. Sato[2] and B.A.L. Fonseca[1]

[1]Departamento de Clínica Médica, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil
[2]Instituto Adolfo Lutz de Ribeirão Preto, Ribeirão Preto, SP, Brasil

Abstract

Polymerase chain reaction (PCR) has been widely investigated for the diagnosis of tuberculosis. However, before this technique is applied on clinical samples, it needs to be well standardized. We describe the use of McFarland nephelometer, a very simple approach to determine microorganism concentration in solution, for PCR standardization and DNA quantitation, using *Mycobacterium tuberculosis* as a model. Tuberculosis is an extremely important disease for the public health system in developing countries and, with the advent of AIDS, it has also become an important public health problem in developed countries. Using *Mycobacterium tuberculosis* as a research model, we were able to detect 3 *M. tuberculosis* genomes using the McFarland nephelometer to assess micobacterial concentration. We have shown here that McFarland nephelometer is an easy and reliable procedure to determine PCR sensitivity at lower costs.

Correspondence
B.A.L. Fonseca
Departamento de Clínica Médica
FMRP, USP
Av. dos Bandeirantes, 3900
14049-900 Ribeirão Preto, SP
Brasil
Fax: +55-16-633-6695

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Tuberculosis (TB) is a worldwide disease that has never lost its importance in developing countries, and after the 80's it has also become a problem in developed countries. There are almost 20 million cases of active tuberculosis in the world with almost 5,000 deaths every day (1). The laboratory diagnosis of TB is currently based on the demonstration of *Mycobacterium tuberculosis* in acid-fast stained (Ziehl-Neelsen) or fluorochrome-stained smears, and on culture growth on solid or liquid media. Staining is a rapid screening test but with a low sensitivity, detecting acid-fast bacilli only when there are more than 10^4 mycobacteria per ml. Even though culture on solid media is the gold standard diagnostic test for tuberculosis, it is laborious and slow for clinical use, requiring at least 4 weeks to detect the *M. tuberculosis* (2).

PCR (3) is a remarkably specific and sensitive method of DNA amplification capable of amplifying as little as 1 copy of a given DNA. While this technique has been

widely used for the diagnosis of viral infections, bacterial infections still rely on culture methods. Assuming that PCR may play a role in the diagnosis of bacterial infections, we devised a method for assaying concentration of microorganisms detected by PCR using *M. tuberculosis* as a tool.

Tuberculosis diagnosis by PCR has been possible since the identification of singular DNA sequences present in the genome of organisms of the *M. tuberculosis* complex (4). A PCR assay can be run in a few hours with high rates of specificity and for this reason it has become an excellent option for rapid diagnosis of pulmonary tuberculosis. Proper standardization is the first step for the use of PCR in the clinical diagnosis of tuberculosis.

In this study, we describe the use of the McFarland nephelometer procedure to determine the concentration of tubercle bacilli in solution and its use for PCR standardization and quantitation of *M. tuberculosis* in solution. Even though several quantitation methods have been described for PCR, including methods for the *M. tuberculosis* genome, they are not always feasible in developing countries. We have used a simple procedure to estimate *M. tuberculosis* concentration, which should be useful to standardize PCR protocols. The McFarland nephelometer approach offers the advantage of being less expensive when compared to ordinary methods of DNA quantitation by PCR.

The McFarland nephelometer was described in 1907 by J. McFarland as an instrument for estimating the number of bacteria in suspensions used for calculating the bacterial opsonic index and for vaccine preparation. The McFarland nephelometer No. 1 standard procedure was performed as described by McFarland (5). In a large test tube, 0.1 ml of a 1% solution of anhydrous barium chloride was mixed with 9.9 ml of a cold solution of 1% chemically pure sulfuric acid. The tube was sealed and kept in the refrigerator until a fine white precipitate of barium sulfate became visible after vigorous shaking. At that time the tube had a density corresponding to approximately 3 x 10^8 mycobacteria/ml of suspension. We prepared a solution of the *M. tuberculosis* reference strain (HRa37) corresponding to McFarland No. 1 standard. By sequentially diluting the sample ten-fold, we ended up with concentrations of *M. tuberculosis* from 10^8 to 10^{-1} bacilli/ml.

DNA extraction was performed by boiling 1 ml of each HRa37 *M. tuberculosis* dilution for 10 min and centrifuging the samples for 10 min at 13,500 rpm. The resulting supernatant was used for PCR.

The PCR was based on the amplification of the insertion sequence *IS6110* with primers described by Eisenach et al. (6). The primers were TB1 5'-CCTGCGAGCGTAG GCGTCGG-3' and TB2 5'-CTCGTCC AGCGCCGCTTCGG-3' (Gibco-BRL, Gaithersburg, MD, USA) and amplified a 123-bp fragment of the repetitive *IS6110* sequence. The DNA amplification protocol used the *GeneAmp*® PCR reagent kit with native *Taq* DNA polymerase (Perkin Elmer Corporation, Branchburg, NJ, USA). Five microliters of the extracted DNA solution was added to 45 µl of PCR reaction mixture containing Ampli*Taq* DNA polymerase (1.25 U), deoxynucleotides (200 µM each), PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.15 mM $MgCl_2$, 0.01% gelatin), and 20 pmol of DNA primers. Thermal cycling was performed on a Perkin-Elmer DNA thermal cycler 480, with an initial cycle of 5 min at 94°C followed by 35 cycles consisting of 1 min at 94°C, 2 min at 65°C and 1 min at 72°C. The procedure was stopped after a 10-min final extension step at 72°C to allow polymerization of incomplete strands.

One-tenth of the amplification reaction mixture was analyzed electrophoretically on 3% agarose gel. The gel was then stained

with 1 µg/ml ethidium bromide solution and visualized under UV-light in order to check for DNA bands of appropriate size. The genome of *M. tuberculosis* was correctly amplified as demonstrated by the 123-bp fragment visible on ethidium bromide-stained gel (Figure 1). Our TB PCR was capable of detecting up to 3 copies of *M. tuberculosis* (Figure 1).

The TB resurgence on the world scenario in recent years has led researchers to spend significant energy to develop more rapid diagnostic tests for mycobacterial diseases. The recently developed nucleic acid amplification methods might provide a very sensitive, specific and rapid test for the detection of *M. tuberculosis* (7-9). PCR has been used for rapid diagnosis of pulmonary tuberculosis using different primers and protocols of amplification (7,10,11). Since standardization of this technique requires a sensitivity evaluation step, we set up an experiment to evaluate the ability of the McFarland technique as a quantification tool. With this method, we were able to detect 3 copies of the *M. tuberculosis* genome although we did not further dilute this sample to reach the concentration of 1 bacillus/ml. Eisenach et al. (6), using serial 10-fold dilutions of *M. tuberculosis* DNA, demonstrated that this PCR is able to detect samples containing up to 1 fg of input DNA, which is equivalent to one copy of the *M. tuberculosis* chromosome (~3000 kb).

The McFarland nephelometer was chosen because it allows the use of a solution with a known concentration of bacilli. It is a very helpful and extremely simple procedure compared with growing and counting colony-forming units and is suitable for standardization of PCR in developing countries.

In developing countries such as Brazil, tuberculosis is still a great public health problem and many efforts have been made to control *M. tuberculosis* dissemination. An adequate control of tuberculosis involves the treatment of known cases as well as isolation of those being treated. Molecular biology techniques such as PCR can be of great value in tuberculosis control since they provide a rapid diagnosis, detecting very few bacilli, and allow for early institution of tuberculosis treatment. Even though these methodologies might be expensive for developing countries, the cost-benefit of this test must be considered. It is less expensive than the prolonged permanence of a patient on the hospital wards, as tuberculosis patients often do, many times only waiting for confirmation of the diagnosis. Also, in order to cut the costs, we must look for simple and efficient ways to standardize these methods and the McFarland nephelometer is a tool that can be easily used to analyze PCR sensitivity for mycobacteria or other bacteria, especially the slow growers. Thus, the results described here suggest that PCR can be used to detect mycobacteremia or for the early detection of *M. tuberculosis* growth on liquid medium. Also, it could be used in those situations where the mycobacterial culture is contaminated with rapidly growing microorganisms. A solution resembling that used in the McFarland nephelometer could be prepared and typing of *M. tuberculosis* could be easily performed. In conclusion, the McFarland nephelometer is an easy and reliable procedure to assess PCR sensitivity and may allow developing countries to access modern technology at a lower cost.

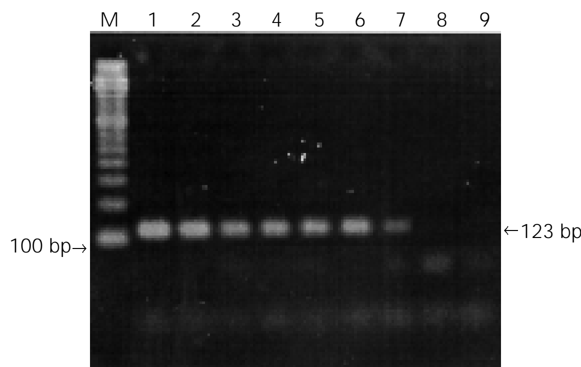

Figure 1 - Electrophoretic analysis of M. tuberculosis amplicons on 3% agarose gel showing their decreasing concentrations, as defined by the McFarland nephelometer. M: 100-bp marker, 1 to 8: 3×10^6 to 3×10^{-1} HRa37 M. tuberculosis and 9: negative control.

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