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A New and Rapid Testing Method for Drug Susceptibility of *Mycobacterium leprae* Using RT-PCR

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Abstract Due to the uncultivable nature of *Mycobacterium leprae in vitro*, the fast, easy, and accurate measurement of the antimicrobial drug susceptibility of this microbe has been difficult. Conventional methods for such testing are subjective, cumbersome, and expensive in some cases. Here, the utility of a reverse transcriptase-PCR (RT-PCR)-based assay for testing was examined and compared with a Buddmeyer-type radiorespirometric assay. The susceptibility of *M. leprae* to rifampin was determined by probing the presence of *M. leprae*-specific 18 kDa gene mRNA in *M. leprae*-infected IC-21 macrophage cells after drug treatment. The results showed that, as the rifampin concentration was increased, the 360-bp cDNA products generated by the RT-PCR-based assay decreased in a dose-dependent manner as in the drug susceptibility observed in the Buddmeyer-type assay. The drug susceptibility testing of *M. leprae* by the RT-PCR based assay was found to be not only faster but also nearly 10^4-fold more sensitive than the Buddmeyer-type assay. Moreover, it was also found that, unlike the RT-PCR based assay, the same testing by a DNA-PCR resulted in no differences in the 360-bp signal, regardless of the rifampin concentrations used. Accordingly, these results demonstrate that the drug susceptibility of *M. leprae* can be determined effectively by an RT-PCR-based assay, thereby providing a new, fast, and sensitive testing method.

Key words: *Mycobacterium leprae*, leprosy, antibiotic drug susceptibility, reverse transcriptase-PCR

Mycobacterium leprae, an obligatory intracellular parasite, is the etiological agent of leprosy. Although the annual incidence of leprosy has declined substantially since the introduction of multidrug therapy, it remains to be one of the major health threats in leprosy endemic countries and continues to be a world health problem because of its contagious nature [1]. Moreover, there have been an increasing number of reports concerning the emergence of antibiotic resistant *M. leprae* [2, 3], which necessitates the search for new anti-leprotic drugs.

A prerequisite for the development of new anti-leprotic drugs is an easy and efficient method for measuring the drug susceptibility of *M. leprae*. However, this is not easy with *M. leprae* due to its uncultivable nature *in vitro*. Conventional methods, such as measuring the CFU (colony-forming-unit) after drug exposure, and more recently developed methods for other mycobacteria, which exploit an exogenously introduced reporter gene like luciferase [4, 5], are all inapplicable to *M. leprae*. Until now, an *in vivo* nude mouse footpad system is mostly used to test the anti-leprotic drug susceptibility of *M. leprae* [6, 7]. However, this method takes at least 10 months to determine a drug's effect and is, therefore, too cumbersome, and also difficult to quantitate.

Another method currently used is the Buddmeyer-type assay system which measures the radiolabeled $^{14}CO_2$ produced from [1-^{14}C]-palmitic acid by the respiration of live *M. leprae* [8]. Although this method is relatively quick and more quantitative than others, it still requires two to four weeks to measure a drug's effect and is very expensive in the case of the BACTEC 460 system [9]. In addition, there is also a possibility of fortuitous contamination by other microorganisms during the long incubation period required for the assay, which can give rise to unavoidable false positives as the assay measures only the amount of bacterial respiration. Accordingly, another easy, fast, and sensitive means of measuring the drug susceptibility of *M. leprae* needs to be developed.

In this study, the effectiveness of a reverse transcriptase polymerase chain reaction (RT-PCR)-based assay in assessing the drug susceptibility of *M. leprae* was examined and then compared with the Buddmeyer assay system.

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MATERIALS AND METHODS

Infection of *M. leprae* and Antibiotic Treatment

To measure the antibiotic drug susceptibility of *M. leprae*, the mouse macrophage cell line IC-21 (American Tissue & Cell Collection) was infected with *M. leprae* (Thai-53 strain) as follows. 1×10^6 IC-21 cells in 1 ml of RPMI containing 10% FBS (Hyclone Lab Inc., Logan, U.S.A.) were plated in 6-well culture plates and infected with *M. leprae* at a 1:30 ratio for 8 h. After infection, the cells were harvested using 2 ml of RPMI containing 10 mM EDTA, washed twice with DPBS (Dulbecco's phosphate buffered saline; Sigma Co., St. Louis, U.S.A.), and centrifuged at 200 ×g for 3 min at 4°C to remove any unphagocytosed *M. leprae*. The resulting *M. leprae*-infected IC-21 cells were resuspended in a complete medium, replated in 6-well plates in equal numbers, and incubated in a 5% CO_2 incubator (Forma Scientific, Marietta, U.S.A.) for 1 h at 37°C. The culture medium was exchanged with 2 ml of a fresh complete medium containing predetermined concentrations of rifampin and the cells were further incubated in the CO_2 incubator for 16 h at 37°C. At the end of the incubation, the cells were harvested again as above and subjected to either a radiorespiratory assay or an RT-PCR-based assay as described below. The isolation of *M. leprae* from granulomas in the footpads of nude mice (B & K Universal Ltd., U.K.) and AFB staining to count the bacteria, have been described previously [10, 11].

Measurement of Antibiotic Susceptibility Using a Radiorespirometric Assay

The assay was performed according to the method described previously [8]. Briefly, 0.3 ml of 0.1 N NaOH was added to the above harvested IC-21 cells to lyse the cells. After a 5 min incubation at room temperature, the whole suspension containing the liberated *M. leprae* was placed in 6-ml screw-capped vials containing 4 ml of 7H9 broth (pH 5.6, Difco) with 1 μCi of [1-^{14}C]-palmitic acid (specific activity 5.7 mCi mmol^{-1}, Amersham Pharmacia Biotech., Buckinghamshire, England) and the vials were capped loosely. The vials were then placed in scintillation vials, covered inside with a piece of Whatman filter paper precharged with NaOH, and incubated at 33°C for 7 days. The precharging was performed by dipping the filter in an Aquasol-2 cocktail solution (Packard, Meriden, U.S.A.) and air drying, followed by wetting with 200 l of 2 N NaOH. The filters were counted every 24 h in a Beckman LS6500 liquid scintillation counter (Beckman, Fullerton, U.S.A.).

Measurement of Antibiotic Susceptibility Using the RT-PCR Assay

IC-21 cells, harvested after rifampin treatment as above, were resuspended in 1 ml of DPBS. Fifty μl of the cell suspension was set aside for a DNA-PCR analysis. The remaining cell suspension was recentrifuged at 250 ×g for 3 min at 4°C and resuspended in 100 μl of DEPC-treated ddH_2O. To isolate the *M. leprae* RNA, 900 μl of the TRI reagent (Sigma Co., St. Louis, U.S.A.) was added to the cell suspension and the reaction was proceeded according to the manufacturer's protocol. The resulting RNA precipitate was washed with 75% cold ethanol and resuspended in 20 μl of DEPC-treated ddH_2O.

To synthesize cDNA, 10 μl of the isolated RNA was first mixed with 250 ng of random primers (Gibco-BRL, Gaithersburg, U.S.A.) and 1 pmol of 18 kDa gene-specific primer #2 (see the legend in Fig. 2). Thereafter, it was heated for 2 min at 75°C and immediately placed in ice. The first-strand cDNA was synthesized by adding 100 units of MMLV Reverse Transcriptase (Promega, Madison, U.S.A.) and 10 units of an RNase inhibitor (Promega, Madison, U.S.A.) in a total 20 μl of a reaction buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM of each of dNTPs) and incubated at 42°C for 60 min. After the reaction, the mixture was heated for 5 min at 95°C to inactivate the enzyme and 2 μl of the resulting reaction mixture was subjected to a DNA-PCR. To eliminate any contaminating DNA, 2 units of RNase-free DNase I (Boehringer Mannheim, Indianapolis, U.S.A.) instead of the reverse transcriptase were added prior to the first-strand cDNA synthesis. The mixture was then incubated for 60 min at 37°C followed by heat-inactivation of the enzyme for 5 min at 75°C.

The isolation of *M. leprae* DNA was performed by placing the *M. leprae* cell suspension in liquid nitrogen for 1 min followed by 1 min in boiling water for a total of five times. After incubating for 10 min at 95°C, 2 μl of the resulting solution was subjected to a DNA-PCR as described previously [13, 14].

RESULTS

Determination of Antibiotic Susceptibility of *M. leprae* Using the Buddmeyer Method

In order to evaluate the usefulness and effectiveness of an RT-PCR based assay as a new assay for determining the drug susceptibility of *M. leprae*, the susceptibility of *M. leprae* to rifampin, a major anti-leprotic drug, was first measured by a Buddmeyer-type assay for comparison. Using this assay, it was found that the amounts of cumulative $^{14}CO_2$ generated after the drug treatment decreased inversely as the rifampin concentration was increased, and reached a base level at around 80 μg ml^{-1} of rifampin (Fig. 1), thereby indicating a dose-dependent susceptibility of *M. leprae* to rifampin. The IC_{50} level by this assay was determined to be 10 μg ml^{-1} of rifampin.

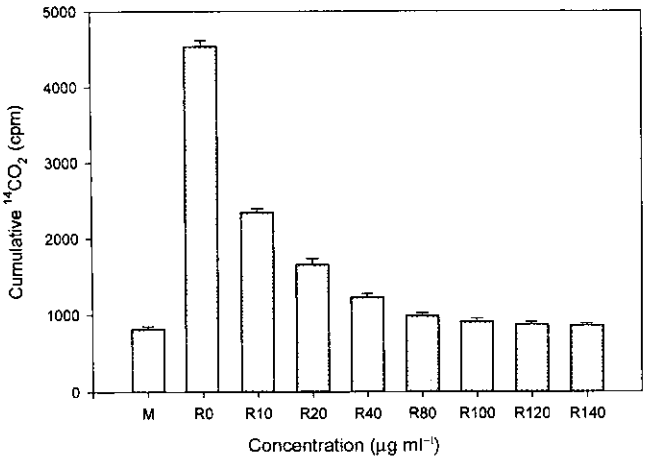

Fig. 1. Rifampin susceptibility of *M. leprae* determined by the Buddmeyer assay.

The cumulative amounts of radiolabeled $^{14}CO_2$ generated after rifampin treatments on mouse macrophage IC-21 cells infected with *M. leprae*, as a function of the drug susceptibility of *M. leprae* to rifampin, were measured using a Buddmeyer-type assay, as described in "Materials and Methods". The final rifampin concentrations used were as indicated. M, mouse macrophage IC-21 cells without *M. leprae* infection.

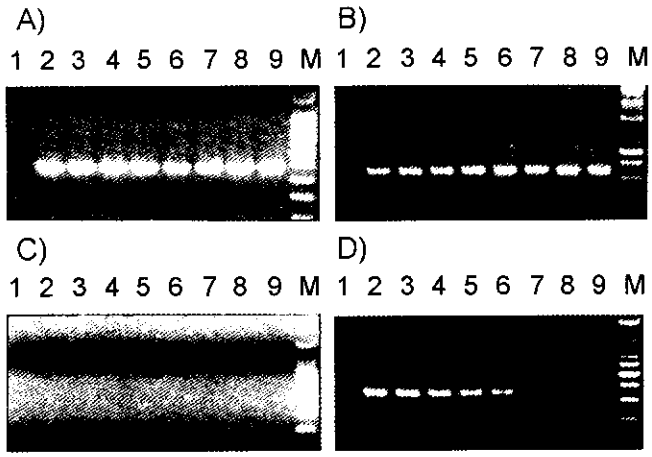

Fig. 2. Rifampin susceptibility of *M. leprae* determined by the RT-PCR-based assay.

The RT-PCR amplified DNA products were resolved using 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The primers used for the cDNA synthesis and amplification were primer #1; 5'-ATT CGT CGT CGA GTT CGA CCT TCCT-3' and primer #2; 5'-CTT AGC TTG TTG CGC AAA CAA CAGT-3' [12]. The DNA-PCR was run for 40 cycles consisting of a 30 sec denaturation at 94°C, 30 sec annealing at 60°C, 90 sec extension at 72°C, and final 10 min extension at 72°C at the end of the cycle. Lanes: lane 1, mouse macrophage IC-21 cells without *M. leprae* infection; lanes 2-9 except in A, 0, 10, 20, 40, 80, 100, 120, and 140 μg/ml of rifampin treated, respectively; M, molecular weight DNA marker of 100 bp. A, DNA-PCR analysis of each sample after *M. leprae* infection without rifampin treatment; B, DNA-PCR analysis after *M. leprae* infection and rifampin treatment; C, DNA-PCR examination after DNase I treatment prior to RT-PCR analysis; D, RT-PCR analysis after *M. leprae* infection and rifampin treatment.

Determination of Antibiotic Susceptibility of *M. leprae* Using the RT-PCR-Based Method

Next, it was examined whether monitoring the *M. leprae*-specific 18 kDa mRNA by RT-PCR, which is a *M. leprae*'s unique gene product with no other known function than that it is related to a family of small heat shock proteins, present in IC-21 cells after an antibiotic treatment, could be a measure of the drug susceptibility of *M. leprae*. The 360 bp cDNA products (signal) generated by the RT-PCR using primers specific for 18 kDa mRNA decreased in a dose-dependent manner as the rifampin concentration was increased (Fig. 2D), as in the Buddmeyer assay, thereby demonstrating the effectiveness of the assay in measuring the drug susceptibility of *M. leprae*. In addition, no other band was observed in the agarose gel, thus exhibiting the specificity of the assay. Furthermore, the RT-PCR-based assay was found to be more sensitive than the Buddmeyer method, since the signal in the RT-PCR assay was easily detected even at 100 μg ml^{-1} of rifampin, whereas it was difficult to differentiate signals beyond 80 μg ml^{-1} of rifampin in the Buddmeyer assay from the background level (compare Figs. 1 & 2D). Based on the detection limit of 18 kDa mRNA with the RT-PCR using isolated *M. leprae* bacilli (data not shown), the signal corresponding to 100 μg ml^{-1} of rifampin accounted for approximately 5× 10^2-10^3 bacilli. This represents about a 10^3 to 10^4-fold higher sensitivity in the *M. leprae* detection limit compared to the Buddmeyer-type assay.

It should be noted that, unlike the RT-PCR assay, the DNA-PCR analysis after the same drug treatment showed no differences in the signal regardless of the rifampin concentrations tested (Fig. 2B), indicating that a DNA-PCR can not distinguish effectively between viable and dead *M. leprae* under these conditions. To ensure that the RT-PCR-based assay is both accurate and reliable for determining the drug susceptibility of *M. leprae*, the followings were also examined. First, to determine whether the IC-21 cells were infected equally by *M. leprae*, a DNA-PCR was performed on each sample after infection without rifampin treatment. As shown in Fig. 2A, equal intensities of the 360 bp DNA band were observed in all samples, which indicates that the signals observed after the RT-PCR assay were not due to unequal infection levels of *M. leprae*. Secondly, a DNA-PCR examination of all samples after the DNase treatment, prior to the RT-PCR, did not generate any signals (Fig. 2C), indicating that the signals obtained by the RT-PCR were not from any contaminating chromosomal DNA but from the RNA template present in the cells after the drug treatment. We have also determined that rifampin treatment on IC-21 cells did not affect its viability as the drug is known to be specific to bacterial RNA polymerase (data not shown). Accordingly, it would appear that an RT-PCR-based assay can measure the drug susceptibility of *M. leprae* effectively.

DISCUSSION

One of the difficulties in the treatment of leprosy stems from the uncultivable nature of *M. leprae in vitro*. Because of this characteristic, development of an easy and efficient method for measuring the drug susceptibility of *M. leprae* continues to be a challenging task in leprosy research. There have been a number of antibiotic drug testing systems developed for *M. leprae*. These include the mouse footpad technique [6, 7], Buddmeyer-type detection systems including the BACTEC 460 system [8, 9], mass spectrometric analysis [15], and ATP bioluminescence [16]. Most of these methods, however, require expensive equipment and are not easy to perform on a routine basis.

In this study, an easy, fast, and sensitive assay was developed for the determination of the anti-leprotic drug susceptibility of *M. leprae* using an RT-PCR based method. Using primers targeted for *M. leprae*-specific 18 kDa gene mRNA, it was found that the cDNA amplification of the mRNA decreased linearly in proportion to the amount of rifampin, similar to the drug susceptibility of *M. leprae* observed in the Buddmeyer-type assay. It was also demonstrated that the differences observed in the 360 bp cDNA band after the drug treatment were not due to unequal infection or contamination by nondegraded *M. leprae* chromosomal DNA. Accordingly, these results indicate that an RT-PCR-based assay can effectively measure the drug susceptibility of *M. leprae* and provide direct evidence for a previous suggestion that *M. leprae* RNA can serve as a practical indicator for determining the effect of anti-leprotic drugs [17]. The advantages of the RT-PCR-based assay over the Buddmeyer-type assay are that, besides higher sensitivity and specificity, it can be performed within a maximum of 2 days, is easy to conduct, and does not require any radioactivity.

Because of the superior sensitivity of DNA-PCR, a number of DNA-PCR-based methods have been developed for the fast and sensitive detection of *M. leprae* in clinical specimens for the diagnosis of leprosy [18-20]. DNA-PCR-based methods have also been applied for the assessment of chemotherapy in leprosy patients [10, 21, 22]. However, whether the identification of *M. leprae* DNA by DNA-PCR-based methods fully represents the existence of live organisms has long been questioned, as it has been shown that mycobacterium DNA can be detected from ancient skeletons using a DNA-PCR method [23, 24]. Here, it was found that, unlike the RT-PCR-based assay, no difference in the 360 bp signal was observed in a DNA-PCR analysis, regardless of the rifampin concentrations used. Accordingly, these results demonstrate clearly that DNA-PCR-based methods do not necessarily discern the life status of *M. leprae* accurately, thereby raising caution against using DNA-PCR for assessing the efficacy of antibiotics on *M. leprae*.

Recently, an RT-PCR-based method combined with a chemiluminescence detection system exhibited an improved sensitivity in the detection of *M. leprae* in tissue biopsy specimens [25]. This, along with the present results, would therefore seem to support that an RT-PCR-based assay would be effective in monitoring the efficacy of chemotherapy in leprosy patients and useful in the screening of new anti-leprotic drugs.

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