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New Drug Susceptibility Test for *Mycobacterium tuberculosis* Using the Hybridization Protection Assay

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We developed a novel method for early detection of drug-resistant strains of *Mycobacterium tuberculosis* by using the hybridization protection assay (HPA). The number of viable bacteria during the incubation period correlated well with the number of relative light units measured by the HPA. In addition, the relative light unit values of susceptible strains on the first, third and fifth days of incubation were significantly different from those of resistant strains for both isoniazid and rifampin. Our results suggest that after isolation of the organism from clinical specimens, drug-resistant strains of *M. tuberculosis* are accurately detected by the HPA even after 1 day of incubation with the drug.

The conventional indirect drug susceptibility tests for *Mycobacterium tuberculosis* are time-consuming and usually require 2 to 4 weeks. Recently, newer methods have been developed. These include tests with fluorescent dyes, such as rhodamine 123 and fluorescein diacetate (3); radiometric techniques, such as the BACTEC system (12, 16–18); and the bioluminescence assay of ATP (14). Furthermore, other methods applying molecular biological techniques, such as those involving the Gen-Probe DNA hybridization system (8), luciferase (4, 6) and amplification of rRNA (21), have been reported in the last few years. The majority of these methods, however, are still not commercially available, and some can be used only in a limited number of laboratories because they involve radioactive material. We describe here a new, simple, and fast *M. tuberculosis* drug susceptibility test with a commercially available hybridization protection assay (HPA).

A standard strain of *M. tuberculosis*, H37Ra, and 17 clinically isolated strains of *M. tuberculosis* were used in the present study. The clinical isolates were divided into four groups according to the MICs of isoniazid (INH) and rifampin (RIF) (Daiichi Seiyaku Co. Ltd., Tokyo, Japan). These groups included INH-susceptible strains for which the MICs were <1 μg/ml, INH-resistant strains for which the MICs were ≥ 1 μg/ml, RIF-susceptible strains for which the MICs were <1 μg ml, and RIF-resistant strains for which the MICs were ≥ 1 μg/ml. The Ogawa egg medium was used to culture strains of *M. tuberculosis*. Middlebrook 7H9 broth (Difco Laboratories, Detroit, Mich.), supplemented with 100 ml of Middlebrook OADC enrichment (Difco) and 0.05% Tween 80 per liter, was used for in vitro drug susceptibility tests by both microdilution and the novel method with the HPA. Middlebrook 7H10 agar (Difco), supplemented with 100 ml of Middlebrook OADC enrichment and 0.5% glycerol per liter, was used to measure the number of viable bacteria in representative strains of INH and RIF-susceptible and -resistant bacteria.

The MICs of INH and RIF for *M. tuberculosis* were determined by the broth microdilution method (9, 10, 22). Briefly, 50 μl of serial twofold dilutions of each antituberculous agent solution, ranging from 0.125 to 1,024 μg/ml, was dispensed into 96-well plates (Corning 2580; Iwaki Glass Co., Chiba, Japan). One to three colonies grown on Ogawa egg agar were inoculated into 10 ml of Middlebrook 7H9 broth and incubated at 37°C for 1 week. The inocula were adjusted to the density of McFarland standard no. 0.5, and 50 μl of the suspension was added to each well. The final concentrations of the antituberculous agent ranged from 0.063 to 512 μg/ml. The plates were covered with lids and incubated at 37°C. The MICs were determined with a reading stand equipped with a mirror after 7 to 21 days of incubation, when successful mycobacterial growths were observed in the drug-free wells. The MIC was defined as the lowest concentration of the antituberculous agent at which the organisms showed no visible growth.

The appropriate density of mycobacterial suspensions for the HPA was determined in a series of preliminary experiments. Serial 10-fold dilutions of H37Ra suspension adjusted to McFarland standard no. 0.5, without antituberculous agents, were prepared and incubated. The viable bacterial count and number of relative light units (RLU) of each suspension were measured according to the HPA protocol described below at 0-, 1-, 3-, and 5-day intervals. To determine the number of viable bacteria, 500 μl of each sample was drawn and serial 10-fold dilutions (10 to 10^5) were prepared in Middlebrook 7H9 broth. Next, 100 μl of each sample was inoculated on Middlebrook 7H10 agar. Two to 4 weeks later, the number of colonies was counted and the concentration of viable mycobacteria was calculated.

The HPA susceptibility test was performed as follows. The strains grown on Ogawa egg medium were inoculated into Middlebrook 7H9 broth and cultured at 37°C for 5 to 7 days. Inocula were prepared by 10-fold dilution of a bacterial suspension equivalent to a McFarland no. 0.5 standard. The inoculum was mixed with an antituberculous drug solution to prepare final concentrations of 0.1 and 1.0 μg/ml for INH and 1.0 and 10 μg/ml for RIF. The resulting bacterial suspension, with or without the test drug, was cultured on an agitating plate at 37°C for up to 5 days. At 0-, 1-, 3-, and 5-day intervals, each sample was removed and subjected to the HPA protocol with an acridinium ester (AE)-labeled DNA probe (AccuProbe; Gen-Probe, Inc., San Diego, Calif.) (5, 11). Briefly, 50 μl of each sample was sonicated in a tube containing glass beads and lysing agents in a sonication bath (Bransonic ultrasonic

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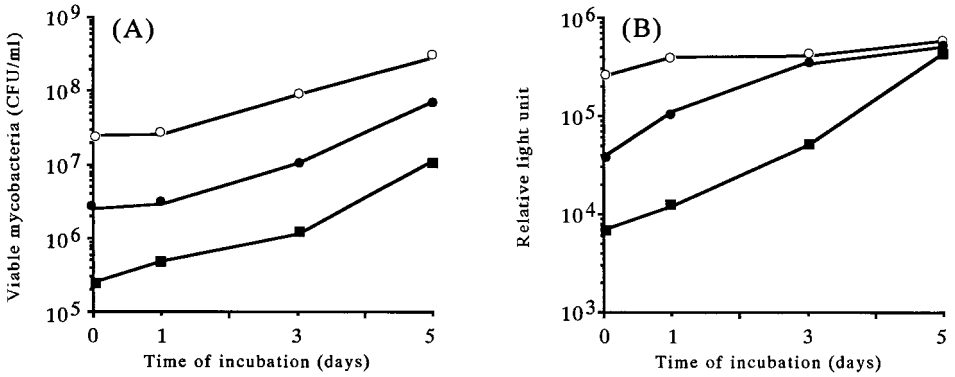

FIG. 1. Changes in the numbers of viable mycobacteria (A) and RLU (B) at various initial culture densities. ○, McFarland standard no. 0.5; ●, 10-fold dilution; ■, 100-fold dilution.

cleaner, model B2200; Emerson, Tokyo, Japan) for 20 min, followed by heating of the mixture at 95°C for 10 min. One hundred microliters of each lysate was then incubated for 15 min at 60°C in a water bath with the reconstituted lyophilized DNA probe. In the next step, 300 μl of a selection reagent was added, and the mixture was incubated at 60°C for 5 min to allow for the differentiation of nonhybridized and hybridized acridinium ester-labeled DNA probe. After incubation at room temperature for 5 min, the assay results were read on a luminometer (Leader 50; Gen-Probe) and expressed as RLU. Each suspension was assayed in duplicate for hybridization, and the results were expressed as the average of the two readings. At the same time, the concentration of viable mycobacteria in each suspension of representative INH- and RIF-susceptible and -resistant strains was calculated as described above. The RLU ratio was calculated by dividing the number of RLU on day 1, 3, or 5 by the number of RLU on day 0 and represented the change in RLU value in each strain tested. The RLU ratios between groups with and without antituberculous agents were compared by Student's *t* test, and statistical significance was set at $P < 0.01$.

The following seven strains of *M. tuberculosis* were selected as INH-susceptible strains (INH MIC [micrograms per milliliter] in parentheses): U106 (0.063), U107 (0.063), U111 (0.125), U112 (0.063), U115 (0.063), U118 (0.063), and U125 (0.063). In addition, the following five strains were also used as RIF-susceptible strains (RIF MIC [micrograms per milliliter] in parentheses): U106 (0.063), U107 (0.063), U111 (0.063), U112 (0.063), and U115 (0.063). On the other hand, seven strains (INH MIC [micrograms per milliliter] in parentheses), T103 (256), T104 (512), U108 (1), U113 (64), U122 (8), N129 (512), and K136 (128), were selected as INH-resistant strains, while five strains (RIF MIC [micrograms per milliliter] in parentheses), T103 (>512), N129 (512), N130 (4), K134 (512), and K137 (>512), were used as RIF-resistant strains.

Examination of the density of mycobacteria demonstrated a good correlation between the number of viable bacteria and the number of RLU in 10- and 100-fold culture dilutions adjusted to the density of McFarland standard no. 0.5 (Fig. 1). When the initial density was that of McFarland standard no. 0.5, the RLU values were persistently about 4×10^5, although the bacterial count continued to increase during the incubation period. Consequently, since 10-fold culture dilutions at the density of McFarland standard no. 0.5 showed an adequate initial RLU value which was slightly over the cutoff value of 3×10^4 and the presence of a gradual increase in RLU during the 5-day incubation, this inoculum was used as the initial density of bacterial suspension in the subsequent susceptibility tests.

A statistically significant difference in the average RLU ratios between cultures of INH-susceptible strains incubated with INH and those incubated without INH was observed as early as the first day of incubation, although the RLU ratio in the presence of 0.1 or 1.0 μg of INH per ml remained constant throughout the incubation period (Fig. 2A). On the other hand, there was no difference in the RLU ratios between cultures of INH-resistant strains incubated with INH and those incubated without INH (Fig. 2B). Furthermore, the change in RLU ratio in one strain, U108, for which the MIC was 1.0 μg/ml, was similar to that in the INH-susceptible strains when incubated with 1.0 μg of INH per ml, although no difference between samples with INH (0.1 μg/ml) and those without INH was observed (Fig. 2C). In addition, we also examined the RLU ratio and the change in the number of viable bacteria for each representative INH-susceptible strain (U106) and INH-resistant strains (T104 and U108) in the presence and absence of INH. A remarkable reduction in the viable bacterial count was observed in strain U106 after 1-, 3-, and 5-day incubations with INH (Fig. 2D), although there was no difference between samples with INH and those without INH in strain T104 during the incubation period (Fig. 2E), indicating that the changes in viable bacterial counts correlated well with those of the RLU ratio. In the case of U108, a good correlation between the RLU ratio and the viable bacterial count was also observed (Fig. 2F). As with INH, a statistically significant difference in the RLU ratio during a 5-day incubation period was observed in RIF-susceptible strains between cultures prepared with RIF and those prepared without RIF, but no difference in RIF-resistant strains was observed (Fig. 3A and B). The change in RLU ratio in another strain, N130, for which the MIC was 4.0 μg/ml, was similar to that in the RIF-susceptible strains when incubated with 10 μg of RIF per ml, while the RLU ratio with 1.0 μg/ml was intermediate between samples with RIF (10 μg/ml) and those without RIF (Fig. 3C). Changes in the number of viable bacteria for each representative RIF-susceptible strain (U107) and the RIF-resistant strains (T104 and N130) correlated well with those of the RLU ratio, as was the case with INH (Fig. 3D, E, and F).

In recent years, new methods designed to detect mycobacteria by molecular biological techniques have been developed and used clinically (1, 7, 13, 15, 20). Furthermore, the genetic characteristics of drug resistance of mycobacteria have been identified, and the presence of specific genes related to resistance to a few drugs has already been confirmed (2, 19, 23).

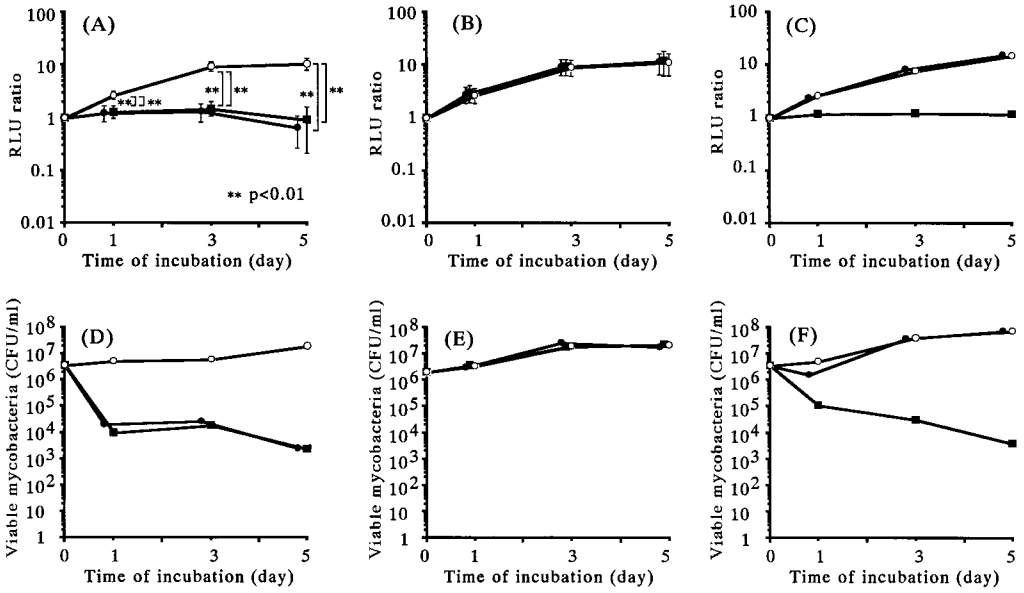

FIG. 2. Changes in RLU ratio (A, B, and C) and numbers of viable mycobacteria (D, E, and F) in the presence (● [0.1 μg/ml]; ■ [1.0 μg/ml]) or absence (○) of INH. (A) Seven susceptible strains. (B) Six resistant strains. (C) U108. (D) U106. (E) T104. (F) U108.

However, the application of these techniques to drug susceptibility tests for *M. tuberculosis* is still inadequate and is difficult to perform in clinical laboratories. In this study, we described a novel method for the early detection of drug-resistant strains of *M. tuberculosis* by using the HPA. This DNA probe kit is already available commercially and has been widely used for the identification of mycobacteria in many laboratories.

Our results demonstrated the presence of an excellent relationship between the number of viable bacteria and RLU, suggesting that the increase in RLU exactly reflected increases in the number of viable bacteria. The RLU, however, reached a plateau, so that no further increases in its value occurred at bacterial densities greater than that of McFarland standard no. 0.5, corresponding to 10^7 CFU/ml. This phenomenon is probably due to a limited amount of DNA probe coating the probe tube and its saturation with mycobacterial RNA. As for the lower limit, RLU values higher than 3×10^4 are considered positive for the identification of *M. tuberculosis* according to the manufacturer. Therefore, in the present susceptibility test, the appropriate initial density of mycobacteria was considered a 10-fold culture dilution at the density of McFarland standard no. 0.5 because the initial RLU value was slightly higher than 3×10^4 and reached the upper limit within a 5-day incubation period.

When INH or RIF was added, an excellent correlation between the number of viable bacteria and the RLU ratio was

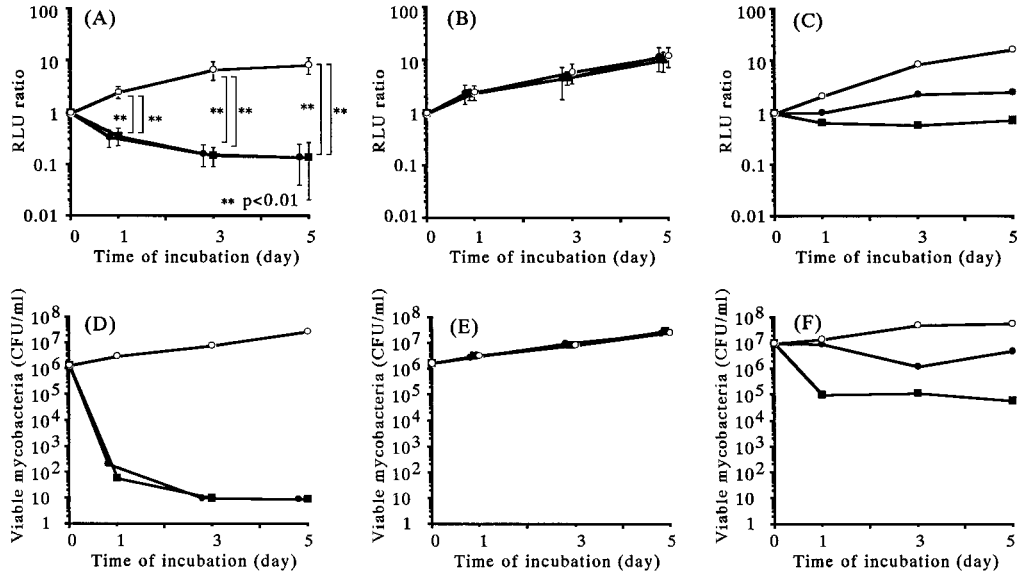

FIG. 3. Changes in RLU ratio (A, B, and C) and numbers of viable mycobacteria (D, E, and F) in the presence (● [1.0 μg/ml]; ■ [10 μg/ml]) or absence (○) of RIF. (A) Five susceptible strains. (B) Four resistant strains. (C) N130. (D) U107. (E) T104. (F) N130.

also noted. Consequently, since differences between RLU ratios of INH- or RIF-susceptible and -resistant strains and the ratios under drug-free conditions were observed during 1- to 5-day incubations, the characteristics of the tested resistant strains of *M. tuberculosis* were considered to be confirmed as early as the first day of incubation with the drug after culture and isolation of the organism. In particular, when RIF-susceptible strains were treated with RIF, both the number of viable bacteria and the RLU value decreased remarkably. This phenomenon may reflect the effect of RIF on the RNA polymerase and a strong inhibition of the synthesis of rRNA, which is a target nucleoside in HPA. In addition, changes in the RLU ratio of two strains with MICs close to the breakpoints for INH or RIF were influenced as expected according to the concentrations of the drugs added.

Since the HPA method is safe, simple, and fast, this assay may be used in the average laboratory processing routine clinical specimens. The test is also inexpensive, costing about $60 for three samples (a control sample on day 0 and two incubated samples with and without a test drug on any day up to day 5) of one *M. tuberculosis* strain. Although this method may be more expensive than the radiometric or conventional agar method, obtaining early test results is useful in certain clinical conditions such as in immunocompromised patients infected with multidrug-resistant *M. tuberculosis*. Furthermore, the HPA method is likely to overcome the problem of mixed cultures (*M. tuberculosis* and *Mycobacterium avium* complex) frequently seen in specimens from immunocompromised patients.

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