BMC Infectious Diseases

BioMed Central

Research article

Open Access

# Quality assessment of an interferon-gamma release assay for tuberculosis infection in a resource-limited setting

Nguyen TL Hang[1], Naoki Ishizuka[2], Naoto Keicho*[2], Le T Hong[3], Do B Tam[3], Vu TX Thu[3], Ikumi Matsushita[2], Nobuyuki Harada[4], Kazue Higuchi[4], Shinsaku Sakurada[2] and Luu T Lien[3]

Address: [1]IMCJ-BMH Medical Collaboration Center, Bach Mai Hospital, Hanoi, Viet Nam, [2]International Medical Center of Japan, Research Institute, Tokyo, Japan, [3]Hanoi Tuberculosis and Lung Disease Hospital, Hanoi, Viet Nam and [4]Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, Tokyo, Japan

Email: Nguyen TL Hang - lehang0310@gmail.com; Naoki Ishizuka - naishi@ri.imcj.go.jp; Naoto Keicho* - nkeicho-tky@umin.ac.jp; Le T Hong - lethihong.hth@gmail.com; Do B Tam - dobangtam.hth@gmail.com; Vu TX Thu - vuthixuanthu.hth@gmail.com; Ikumi Matsushita - ikumi-tky@umin.ac.jp; Nobuyuki Harada - harada@jata.or.jp; Kazue Higuchi - higuchi@jata.or.jp; Shinsaku Sakurada - ssakura@mti.biglobe.ne.jp; Luu T Lien - luuthilien.hth@gmail.com

* Corresponding author

Published: 18 May 2009

*BMC Infectious Diseases* 2009, **9**:66 doi:10.1186/1471-2334-9-66

Received: 20 February 2009
Accepted: 18 May 2009

This article is available from: http://www.biomedcentral.com/1471-2334/9/66



## Abstract

**Background:** When a test for diagnosis of infectious diseases is introduced in a resource-limited setting, monitoring quality is a major concern. An optimized design of experiment and statistical models are required for this assessment.

**Methods:** Interferon-gamma release assay to detect tuberculosis (TB) infection from whole blood was tested in Hanoi, Viet Nam. Balanced incomplete block design (BIBD) was planned and fixed-effect models with heterogeneous error variance were used for analysis. In the first trial, the whole blood from 12 donors was incubated with nil, TB-specific antigens or mitogen. In 72 measurements, two laboratory members exchanged their roles in harvesting plasma and testing for interferon-gamma release using enzyme linked immunosorbent assay (ELISA) technique. After intervention including checkup of all steps and standard operation procedures, the second trial was implemented in a similar manner.

**Results:** The lack of precision in the first trial was clearly demonstrated. Large within-individual error was significantly affected by both harvester and ELISA operator, indicating that both of the steps had problems. After the intervention, overall within-individual error was significantly reduced ($P < 0.0001$) and error variance was no longer affected by laboratory personnel in charge, indicating that a marked improvement could be objectively observed.

**Conclusion:** BIBD and analysis of fixed-effect models with heterogeneous variance are suitable and useful for objective and individualized assessment of proficiency in a multistep diagnostic test for infectious diseases in a resource-constrained laboratory. The action plan based on our findings would be worth considering when monitoring for internal quality control is difficult on site.

## Background

Assuring quality is essential for clinical laboratories in the field of infectious diseases. Beneficiaries are not only patients obtaining a diagnosis on site but also future patients receiving benefits of clinical research supported by qualified laboratories. Quality assurance in modern laboratories is realized by total quality management including external quality assurance (EQA) and internal quality control (IQC) [1-3].

In most resource-constrained countries, however, regulations on quality assurance have not been laid down by the authorities and accuracy and precision of clinical measurements have not been monitored systematically [4]. Under such disadvantageous circumstances, when important but rather complicated testing for infectious diseases is undertaken, we cannot easily be confident that the skill has been transferred and maintained properly until the procedure becomes familiar and stably performed in accordance with a desirable quality control system [5]. During this vulnerable period, how to assess proficiency of the testing effectively and objectively, and how to assure and improve the quality are open issues to be addressed.

Currently, immunoassay is commonly used to make a serological diagnosis of infectious diseases involving human immunodeficiency virus, a variety of hepatitis virus and other sexually transmitted or blood-borne pathogens [6,7], which are serious problems in the developing world. Enzyme linked immunosorbent assay (ELISA) is often used to make diagnosis of these diseases in the clinical laboratories. Because of the complexity of the method, however, quality control of these assay systems is challenging [8]. In this context, trend of point of care (POC) tests that facilitate immediate and on-site diagnosis as well as early treatment of infectious diseases has been emphasized [7]. However, their usage in resource-constrained countries is still hampered by high cost and difficulties in testing for high throughput screening and thus laboratory-based immunoassays would be irreplaceable in many fields.

Recently, a two-step immunoassay to detect tuberculosis (TB) infection has also been developed and used extensively [9]. It consists of whole blood stimulation with TB-specific antigens followed by measurement of interferon-gamma using ELISA. Our objective in the present study is to demonstrate that the quality of laboratory tests can be assessed objectively even in a resource-constrained laboratory if the optimum design of experiments and appropriate statistical models are chosen. As a result of this attempt, we experienced marked improvement of the quality of this multi-step immunoassay made by more than one laboratory staff member in a hospital of Viet Nam. We proposed a general plan to evaluate skills of laboratory staff members efficiently and quantitatively to perform qualified immuno-diagnostic testing especially for infectious diseases until such time as they establish a total quality management system by themselves.

## Methods

### *Interferon-gamma release assay (IGRA) for diagnosis of TB infection*

IGRA is a general method to measure interferon-gamma induced by *Mycobacterium tuberculosis*-specific antigens (TB-Antigen) for detecting TB infection. In the ELISA-based IGRA (QuantiFERON-TB Gold In-Tube™, Cellestis, Victoria, Australia), one milliliter of the whole blood was collected into the Nil tube for negative control, Mitogen for positive control, and TB-Antigen separately. The blood in the tubes was mixed and placed in the incubator for 18 hours at 37°C (Cool incubator NC-25B, Funakoshi, Tokyo, Japan). Approximately 200 μl of plasma were harvested from each tube after centrifugation (Kubota 2010, Kubota, Tokyo, Japan).

Interferon-gamma concentrations in the plasma were measured by ELISA, using microtiter plate washer and reader (Wellwash Plus Microplate Washer and Multiscan JX Microplate Reader, Thermo Electron Corporation, Vantaa, Finland) with the analysis software provided by the manufacturer (QuantiFERON-TB Gold Analysis Software, ver. 2.50, Cellestis). In this study, interferon-gamma concentrations obtained from this calculation were directly used for further analysis.

### *Study setting*

Two trials were carried out in Hanoi TB and Lung Disease Hospital, Viet Nam. Between the first and second trial, statistical analysis was made and an intervention was planned to ensure counterchecking and correct questionable manipulations. Each trial consisted of two runs. In each run, three milliliters of blood were collected from volunteers after informed consent had been obtained. Study protocols using IGRA were approved by ethical committees of the Ministry of Health, Viet Nam and International Medical Center of Japan respectively.

Two laboratory members, A and B, performed either plasma harvest or ELISA operation or both: Harvest included labeling and placing plasma storage tubes properly and transferring plasma from centrifuged blood collection tubes to these tubes by pipetting. ELISA was a process including preparing reagents and transferring plasma samples into the microtiter plate. ELISA ended with calculation of interferon-gamma concentration. Because their roles were changed occasionally due to the limited manpower of the laboratory, their performance in both Harvest and ELISA was the subject to be analyzed.

***Balanced incomplete block design (BIBD)***

A single specimen obtained from routine blood collection was not sufficient to assess two staff members' performance. Because additional blood sampling was not easily accepted in many countries including Viet Nam, BIBD was attempted to obtain analytical information from small volume of plasma samples in this study: Of four possible combinations of harvester and ELISA operator, two combinations were cyclically chosen, using the limited amount of specimen. Allocation of observed combinations by BIBD in this study was described in Table 1. In each trial, there were two levels of Harvest (two different Harvesters), two levels of ELISA (two different ELISA operators) and 12 levels of Specimen (12 different blood donors).

***Outliers***

To identify outliers, Mahalanobis distance D was calculated, which took the distance from the mean and correlation into account [10]. When D > 2.0, the value of that observed pair was regarded as outlier.

***A fixed-effect model and three-way analysis of variance (ANOVA)***

To assess effects of factors of interest and error variance, we used a fixed-effect model:

$$y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \varepsilon_{ijk}$$

of which,

$y_{ijk}$: Interferon-gamma concentration in the plasma

$\mu$: Grand mean of all measurements

$\alpha_i$: Harvest with i levels: i = 1, 2 (= A and B)

$\beta_j$: ELISA with j levels: j = 1, 2 (= A and B)

$\gamma_k$: Blood specimen with k levels: k = 1, 2,..., 12

$\varepsilon_{ijk}$: Within-individual error; following normal distribution with mean = 0 and variance = $\sigma^2$: N (0, $\sigma^2$)

In this clinical setting, effects of interaction terms were not considered in the above model, because harvesting plasma and performing ELISA are independent steps and it is unlikely that the exchanging of staff roles in itself could increase the chances of error.

***Analysis of heterogeneous error variance affected by a given factor***

To determine whether individuals of Harvest or ELISA affect within-individual error, we assessed a fixed-effect model with heterogeneous variance of error in the following way:

$$y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \varepsilon_{ij}$$

where error follows the normal distribution $N(0, \sigma_{ij}^2)$.

Error variance affected by Harvesters was evaluated in the following formula:

$$y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \varepsilon_{i.}$$

Similarly, the following formula was used for error variance affected by ELISA operators:

$$y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \varepsilon_{.j}$$

***Coefficient of variation (CV) before and after intervention***

Error variance $\varepsilon_{ijk}$ that included sources of Harvest and ELISA was calculated in a simple one-way ANOVA model adjusted by specimen. Based on the following formula, CVs of the two trials were assessed:

$$y = \mu + \gamma_k + \varepsilon_{ijk}$$

**Table 1: Allocation of observed combinations of Harvester and ELISA operator.**

| Sample | Specimen* | Harvest | ELISA | Data | Sample | Specimen | Harvest | ELISA | Data |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | A | A | Observed | 7 | 4 | A | A | Observed |
| 2 | 1 | A | B | Observed | | 4 | A | B | Not observed |
| | 1 | B | A | Not observed | 8 | 4 | B | A | Observed |
| | 1 | B | B | Not observed | | 4 | B | B | Not observed |
| | 2 | A | A | Not observed | | 5 | A | A | Not observed |
| | 2 | A | B | Not observed | 9 | 5 | A | B | Observed |
| 3 | 2 | B | A | Observed | | 5 | B | A | Not observed |
| 4 | 2 | B | B | Observed | 10 | 5 | B | B | Observed |
| 5 | 3 | A | A | Observed | | 6 | A | A | Not observed |
| | 3 | A | B | Not observed | 11 | 6 | A | B | Observed |
| | 3 | B | A | Not observed | 12 | 6 | B | A | Observed |
| 6 | 3 | B | B | Observed | | 6 | B | B | Not observed |

*To each specimen, two measurements were assigned. This layout was repeated twice by using different sets of specimens in each trial.

$$CV(\%) = \frac{\text{Root mean square error}}{\text{Mean}} \times 100$$

CV should not be larger than 20% in any types of immunoassay [8].

### *Assessment of heterogeneous variance between the two trials*

To analyze overall within-individual error between the two trials statistically, we used a fixed-effect model with heterogeneous variance of error, under the assumption that α and β were fixed throughout the trials. The effect of each blood specimen γ was expected to be different between the two trials.

$$y_{ijk1} = \mu + \alpha_i + \beta_j + \gamma_k + \varepsilon_{ijk1} \text{(the first trial)}$$
$$\| \quad \| \quad \backslash\!\backslash \quad \backslash\!\backslash$$
$$y_{ijk2} = \mu + \alpha_i + \beta_j + \gamma_k + \varepsilon_{ijk2} \text{(the second trial)}$$

of which, $\varepsilon_{ijk1}$ and $\varepsilon_{ijk2}$ were within-individual errors of the first and the second trial respectively. On the above assumption, $\varepsilon_{ijk1}$ and $\varepsilon_{ijk2}$ would be heterogeneous error between the trials.

Calculation of Mahalanobis distance, three-way ANOVA and estimation of heterogeneous variance were performed by SAS version 9.1 (SAS Institute Cary, NC, USA). Differences in error variance of two trials and error variance affected by a given factor were considered to be significant when *P*-value was less than 0.05.

## Results

### *Evaluation of outliers and three-way ANOVA in the first trial*

Out of 72 measurements obtained from the first trial, seven outliers were identified: One was in Nil condition, three in TB-Antigen and three in Mitogen (Mahalanobis D = 2.64 to 4.69).

To assess effects of individuals for Harvest and ELISA and character of errors involved in the first trial, we first performed three-way ANOVA using a fixed-effect model, in which three factors, Harvest, ELISA and individual blood specimens may have possible effects on the interferon-gamma concentration respectively. This model decomposes the total variance into between-individual error (or bias) and within-individual error (or imprecision). Herein, "between-individual error" indicates deviation in interferon-gamma values caused by the difference between Harvesters or ELISA operators, and "within-individual error" represents fluctuation of interferon-gamma values measured by a single Harvester or ELISA operator.

As shown in Table 2, mean square error indicating magnitude of within-individual error was large in all conditions of the first trial, which was indicated by remarkably large CV (> 20%) for Nil, TB-Antigen and Mitogen. Furthermore, in the condition of Mitogen, the mean-square value directing the effect of ELISA, or "between-individual error", was significantly large ($P = 0.017$). In the other two conditions, the effects of ELISA and Harvest were also considerably large but did not reach significant levels, as compared with the corresponding mean square errors. These findings indicate that their performance is unstable. Problems specific to ELISA and Harvest should be considered, although not statistically significant in all conditions.

### *Analysis of heterogeneous error variance in the first trial*

We then analyzed which factor affected within-individual error. Because two laboratory members were involved in each step of this experiment, we assumed that within-individual error, i.e. error variance, could be different depending on the personnel in each step. Thus, we chose a fixed-effect model with heterogeneous variance of error affected by Harvest and ELISA (Table 3).

In Nil condition, difference in error variance was statistically significant between Harvesters A and B ($P = 0.0040$), when error variance caused by ELISA operator was not considered. Difference of error variance caused by ELISA operators A and B was also significant ($P = 0.024$), when error variance caused by Harvester was not taken into account. These findings imply that under the model, the error variance was affected significantly by different Harvesters or ELISA operators, respectively.

### *Intervention*

By means of the above-mentioned statistical analysis of the first trial, we identified several points to be improved: a) there was a considerable number of outliers. Within-individual error was large and between-individual error

**Table 2: Three-way analysis of variance in the first trial.**

| | Nil | TB-Antigen | Mitogen |
|---|---|---|---|
| Mean (IU/ml) | 0.7821 | 5.4013 | 15.3638 |
| Harvest | | | |
| Mean Square | 0.0000 | 1.5252 | 51.2656 |
| F value | 0.0000 | 0.2200 | 2.5600 |
| *P* value | 0.9984 | 0.6482 | 0.1404 |
| ELISA | | | |
| Mean Square | 1.8838 | 12.3026 | 161.3535 |
| F value | 1.2600 | 1.7800 | 8.0700 |
| *P* value | 0.2847 | 0.2112 | 0.0175 |
| Error | | | |
| Mean Square | 1.4741 | 6.8935 | 19.9916 |
| Root Mean Square | 1.2142 | 2.6255 | 4.4712 |
| Coefficient of Variation (%) | 155.2476 | 48.6099 | 29.1022 |

**Table 3: Error variance affected by Harvester (left) and error variance affected by ELISA operator (right) in the first trial.**

| εi. | Harvester | P value | εj | ELISA operator | P value |
|---|---|---|---|---|---|
| Nil | A:1.9150<br>B:0.0160 | 0.0040 | Nil | A:0.0036<br>B:3.2723 | 0.0244 |
| TB-Antigen | A:2.9897<br>B:9.7114 | 0.2546 | TB-Antigen | A: 0.1270<br>B:15.2216 | 0.0830 |
| Mitogen | A:33.5782<br>B: 5.6792 | 0.2780 | Mitogen | A:41.1154<br>B: 3.0221 | 0.3584 |

was also comparably large, and b) within-individual error was affected by both Harvesters and ELISA operators at least when Nil was measured.

Based on these results, an intervention was introduced: 1) reviewing all procedures of Harvest and ELISA, 2) reconsidering and strengthening standard operation procedures, 3) checking working condition of machines, and 4) developing a checklist for countercheck. First, we attempted to find out which procedure of harvesting and ELISA operation would be unstable and all questionable manipulations were listed up. Essential laboratory skills, such as mixing the solution by pipetting, were reviewed. Secondly, standard operation procedures were rechecked and corrected seeing that the laboratory personnel were handling three blood collection tubes and three other plasma storage tubes from each blood donor at a time, they should take every care to identify the tubes during Harvest and ELISA and to confirm the right position of corresponding tubes. Thorough instruction for handling ELISA plates and tubes with manipulation of the pipette was given to avoid carry-over error or contamination. After intensive discussions, more attention was paid to basic laboratory practice and reduction of preventable mistakes. Thirdly, performance of the ELISA plate washer and reader and the quality of distilled water were also checked. Technical requirements from the manufacturer, such as temperature for reagent reservation, time of incubation, were strictly followed. Finally, a checklist for the countercheck of each step was developed for practical use.

### *General assessment by CV before and after intervention*

To assess the overall improvement after intervention, CV was compared between the two trials. Because variation due to Harvest and ELISA was of interest, CV adjusted by the effect of specimens was calculated and used. The CV had decreased remarkably in each condition of the second trial, as compared with that of the first trial, indicating the overall improvement of test performance after intervention (Table 4).

### *Evaluation of outliers and three-way ANOVA in the second trial*

In the second trial, only one outlier was seen in Nil condition (Mahalanobis D = 2.59); the number of outliers was lower than that of the first trial.

We then proceeded to analyze the change of parameters that had possibly contributed to overall improvement of test performance. As shown in Table 5, both mean square error and mean-square values showing effects of Harvest and of ELISA were markedly lower in the second trial. The former implies the decrease in within-individual error and the latter shows the reduction of between-individual error. The latter change was also clearly shown when differences of least square means between Harvesters and between ELISA operators in each condition of the second trial were compared with those in the first trial (Figure 1).

### *Analysis of heterogeneous error variance affected by harvester and ELISA operator in the second trial*

In contrast to the first trial, there were no significant differences of error variance affected by Harvesters or ELISA operators (Table 6). This finding showed that the heterogeneous error variance indicating personnel-dependent unstableness was small enough in each step of the second trial.

### *Assessment of heterogeneous variance between the two trials*

We further evaluated the decrease in overall within-individual error statistically. For this purpose, we used a fixed-effect model with heterogeneous variance between the two trials. Under the assumption that influence of Harvest and ELISA was not changed between the two trials, estimated overall error variances of the two trials were com-

**Table 4: CV adjusted by specimen in the two trials.**

| | CV (%) | |
|---|---|---|
| Condition | 1st trial | 2nd trial |
| Nil | 150.5036 | 2.1661 |
| TB-Antigen | 48.6219 | 2.3967 |
| Mitogen | 38.1630 | 9.8776 |

**Table 5: Three-way analysis of variance in the second trial.**

| | Nil | TB-Antigen | Mitogen |
|---|---|---|---|
| Mean (IU/ml) | 0.2308 | 0.3071 | 11.0017 |
| Harvest | | | |
| Mean Square | 0.0000 | 0.0000 | 3.4225 |
| F value | 1.0000 | 0.1000 | 3.2100 |
| *P* value | 0.3409 | 0.7572 | 0.1036 |
| ELISA | | | |
| Mean Square | 0.0000 | 0.0000 | 0.0770 |
| F value | 1.0000 | 0.4000 | 0.0700 |
| *P* value | 0.3409 | 0.5393 | 0.7937 |
| Error | | | |
| Mean Square | 0.0000 | 0.0001 | 1.1707 |
| Root Mean Square | 0.0050 | 0.0079 | 1.0330 |
| Coefficient of Variation (%) | 2.1661 | 2.5615 | 9.3898 |

pared. As shown in Table 7, values indicating the overall within-individual error in all conditions had significantly decreased in the second trial ($P < 0.0001$).

## Discussion

We have demonstrated that a study design BIBD and statistical analysis using fixed-effect models with heterogeneous variance of error are useful for objective and quantitative assessment of laboratory testing for the first time. A series of experiments in our study clearly showed that proficiency of the personnel was improved by an appropriate intervention between the first and second trials of a two-step ELISA-based immunoassay for tuberculosis newly introduced to a resource-constrained laboratory.

Design of clinical experiments including block designs can be used to estimate effect of factors and their possible interaction [10]. In block designs including BIBD, introduction of blocks usually provides extra precision for comparison of other factors, while difference between blocks is of no intrinsic interest [10]. In our proficiency testing, variation of individual specimens was not the point of interest, but analysis of the other two factors, Harvest and ELISA was of importance. Roles of laboratory members are occasionally changed because of limited manpower. In such a case, our analysis is indispensable for assessment of their individual skills in each step of the testing, since this kind of approach has not been evaluated by the conventional IQC methods [11].

Previous studies in clinical fields other than laboratory medicine showed the advantage of BIBD over the sample size [12-14]. In the present study, this design enabled us to evaluate essential components of the blood testing procedure systematically without collecting an extra specimen from each donor. If all combinations of Harvesters and ELISA operators were to be tested at the same time, a twice-larger volume of blood should be collected from each volunteer, however, obtaining consent of this often causes difficulties in a country where blood sampling is not easily accepted. We have shown furthermore that this design is suitable for clinical settings in which many different specimens are to be handled at the same time.

In the first trial before intervention, we found that within-individual error was large and between-individual error tended to be so. However, a number of outliers also affected both within- and between-individual errors. The cause of outliers was probably due to mixing up of speci-

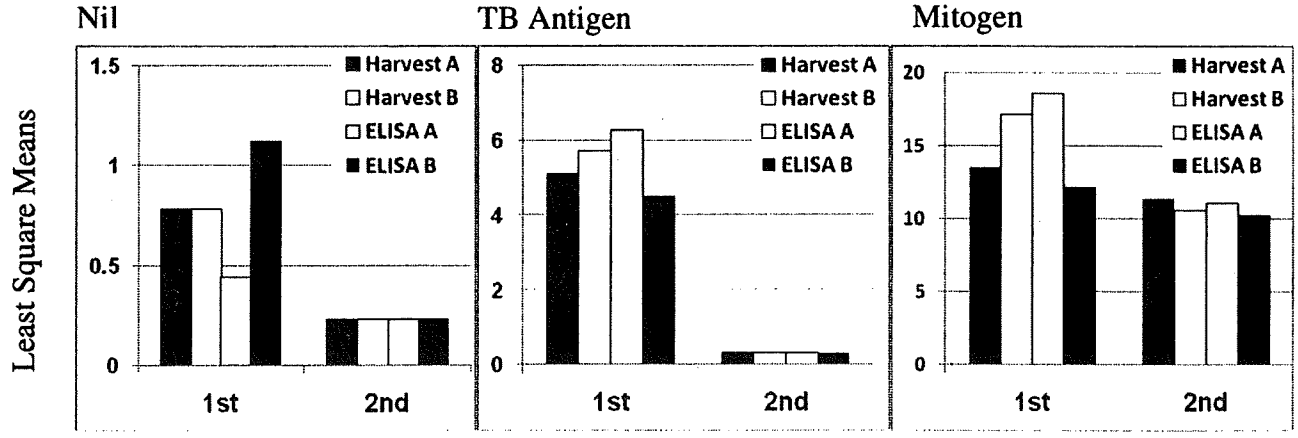


**Figure 1**
**Least square means of measurements in the first and the second trials.** Differences in least square means between Harvesters and between ELISA operators in the conditions of Nil, TB-Antigen and Mitogen in the second trial were compared with those in the first trial.

**Table 6: Error variance affected by Harvester (left) and error variance affected by ELISA operator (right) in the second trial.**

| $\varepsilon i.$ | Harvester | *P* value | $\varepsilon .j$ | ELISA operator | *P* value |
|---|---|---|---|---|---|
| Nil | NE* (A > B) | | Nil | A:0.000014<br>B:0.000035 | 0.4832 |
| TB-Antigen | A:0.0001<br>B:0.00002 | 0.2391 | TB-Antigen | NE (A > B) | |
| Mitogen | A:2.1184<br>B: 0.1788 | 0.3291 | Mitogen | A:1.9065<br>B: 0.2747 | 0.33347 |

*NE = not estimable by this calculation.

men tubes or contamination of samples resulting from unfamiliar handling of multiple samples, although this was not easily determined [15,16]. Using a fixed-effect model with the heterogeneity of error variance, we further illustrated that within-individual error was affected by Harvesters and ELISA operators. The results indicated that there were problems with both steps of Harvest and ELISA, and with both laboratory members, and this represented a strong motivation to improve the skills of the laboratory personnel in both steps of Harvest and ELISA.

After timely intervention including checkup of all steps and standard operation procedures, marked improvement was observed in all parameters including CV, a general parameter for precision of measurements [8]. In case of IGRA in this study, CV should be kept less than 10% [17,18] and in the second trial, this criterion was met satisfactorily.

We propose as a consequence the following action plan to improve diagnostic capacity in resource-constrained settings. This could be generalized not only for complicated immunoassay for infectious disease but also for other kinds of clinical tests:

- Set the target CV derived from simple one-way ANOVA model of specimen (for example, 10%). This value should be defined before the commencement of study.

- Design experiment to evaluate between- and within-individual error.

- Conduct experiment.

- Analyze data with ANOVA model with and without heterogeneous error variance.

- If CV exceeds the target, review the operating procedures.

- Conduct experiment a second time.

- Consequently analyze data to ascertain any improvement.

- Return to step 5 until CV becomes less than the target.

**Table 7: Difference in estimated overall within-individual error between the two trials.**

| | Estimated overall error variance ($\varepsilon_{ijk}$) | |
|---|---|---|
| Condition | 1st trial | 2nd trial* |
| Nil | 1.3866 | 0.000025 |
| TB-Antigen | 6.9003 | 0.000062 |
| Mitogen | 35.3152 | 1.0814 |

*$P < 0.0001$

In-house quality control for effective transfer of skills is a topic of interest in our proposal and this should be carried out easily, at a low cost, whilst assuring objective and quantitative assessment in a clinical laboratory where resources such as reagents, manpower and feasibility of sample collection are limited. Our plan meets the above requirement. Measurements could be sent via the internet and analyzed in a statistical way by a joint-research facility inside or outside the country and an immediate feedback should be sent in an appropriate manner. Such continuous efforts to share information are important to maintain quality levels over a long distance [19].

In this age of evidence-based medicine and development of new diagnostic technologies, quality of laboratory tests is essential. There is an urgent need for validation and standardization of the new assays before they are adopted into clinical diagnostics [20]. Until such time as an effective quality control system is established, our approach is valuable to assure the quality of laboratory tests for timely diagnosis and treatment of infectious diseases. Another favorable design or analytical method might be suggested by others in the future studies, seeing that no standard way of quality monitoring has been proposed so far. We expect that the successful experience gathered in the present study will provide useful information for further comparison and discussion.

Our study has some limitations. It was obvious that outliers influenced statistical analysis in the first trial and exact causes of error in each condition were not clearly specified by the present analysis itself [15]. Through repeated experiments, the causes of error might be clearer, although all errors in our study decreased dramatically after a single intervention. We should also emphasize in conclusion, that a number of procedures should be combined to establish a total quality assurance system.

## Conclusion

In a setting where a modern quality control system has not been entirely established, a laboratory test could be assessed quantitatively and such objective assessment is helpful for quality improvement of the test, if an appropriate design of experiment and statistical method are chosen. The design of experiment BIBD and analytical models for ANOVA were useful for objective assessment of individual skills in each stage of a multi-step immunoassay for tuberculosis in a laboratory with limited resources. A proposed plan to assess the level of proficiency might be useful for skill improvement of clinical testing especially for infectious diseases when monitoring is difficult to assure the sustainability of the technology transferred.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

NTLH participated in supervising the on-site implementation of the study, drafting the paper or substantially revising it. NI was responsible for making conception, design and overall supervision of the study, analysis and interpretation of data, drafting the paper or substantially revising it. NK participated in making conception and design of the study, analysis and interpretation of data, drafting the paper or substantially revising it. LTH carried out the immunoassays. DBT participated in on-site implementation. VTXT carried out the immunoassays. IM participated in technical transfer and supervision. NH was responsible for technical transfer and supervision. KH was responsible for technical transfer and supervision. SS participated in conception and design of the study. LTL participated in conception, design and supervision of the study. All authors read and approved the manuscript.

## Acknowledgements

The authors thank Dr. Hiroshi Ohara (IMCJ-BMH Medical Collaboration Center) and Dr. Pham Huu Thuong (Hanoi Tuberculosis and Lung Disease Hospital) for supporting management. The authors also thank Kazuko Tanabe D.V.M. for her critical reading of this manuscript. This work was supported by grants from the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases, MEXT and International Cooperation Research Grant, the Ministry of Health, Labor and Welfare, Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## References

1. Westgard JO: **Basic Planning for Quality.** Madison: Westgard QC Inc; 2000.
2. Kazmierczak SC: **Laboratory quality control: using patient data to assess analytical performance.** *Clin Chem Lab Med* 2003, **41**:617-27.
3. Westgard JO, Barry PL, Hunt MR, Groth T: **A multi-rule Shewhart chart for quality control in clinical chemistry.** *Clin Chem* 1981, **27**:493-501.
4. Heuck CC, Deom A: **Healthcare in the developing world: Need for appropriate laboratory technology.** *Clin Chem* 1991, **37**:490-6.
5. Smith PG, Morrow RH: **Field trials of health interventions in developing countries: A toolbox.** 2nd edition. Oxford: Macmillan Press Limited; 1996.
6. Peruski AH, Peruski LF Jr: **Immunological methods for detection and identification of infectious disease and biological warfare agents.** *Clin Diagn Lab Immunol* 2003, **10**:506-13.
7. Peeling RW, Mabey D, Herring A, Hook EW 3rd: **Why do we need quality-assured diagnostic tests for sexually transmitted infections.** *Nat Rev Microbiol* 2006, **4**:S7-19.
8. Lock RJ: **My approach to internal quality control in a clinical immunology laboratory.** *J Clin Pathol* 2006, **59**:681-684.
9. Pai M, Riley LW, Colford JM Jr: **Interferon-gamma assays in the immunodiagnosis of tuberculosis: a systematic review.** *Lancet Infect Dis* 2004, **4**:761-76.
10. Armitage P, Berry G, Matthews JNS: **Statistical method in medical research.** 4th edition. Malden: Blackwell science Ltd; 2002.
11. Williams GW, Schork MA: **Basic statistics for quality control in the clinical laboratory.** *Crit Rev Clin Lab Sci* 1982, **17**:171-99.
12. Fleiss JL: **The design and analysis of clinical experiments.** New York: John Wiley & Sons, Inc; 1999.
13. Endres HG, Hucke C, Holland-Letz T, Trampisch HJ: **A new efficient trial design for assessing reliability of ankle-brachial index measures by three different observer groups.** *BMC Cardiovasc Disord* 2006, **6**:33.
14. Varma AAO, Chilton NW, Kutscher AH: **Studies in the Design and Analysis of Dental Experiments: 13. Balanced Incomplete Block Design.** *J Dent Res* 1970, **49**:1166-1171.
15. Marks V: **False-positive immunoassay results: a multicenter survey of erroneous immunoassay results from assays of 74 analytes in 10 donors from 66 laboratories in seven countries.** *Clin Chem* 2002, **48**:2008-16.
16. Ricós C, García-Victoria M, de la Fuente B: **Quality indicators and specifications for the extra-analytical phases in clinical laboratory management.** *Clin Chem Lab Med* 2004, **42**:578-82.
17. Cellestis Inc: *QuantiFERON Precision and reproducibility report* [http://www.cellestis.com/IRM/content/gold/Precisionproductivity.pdf]. Accessed 18 October 2008.
18. Menzies D, Pai M, Comstock G: **Meta-analysis: new tests for the diagnosis of latent tuberculosis infection: areas of uncertainty and recommendations for research.** *Ann Intern Med* 2007, **146**:340-54.
19. Coloma J, Harris E: **Sustainable transfer of biotechnology to developing countries: fighting poverty by bringing scientific tools to developing-country partners.** *Ann N Y Acad Sci* 2008, **1136**:358-68.
20. Lalvani A, Meroni PL, Millington KA, Modolo ML, Plebani M, Tincani A, Villalta D, Doria A, Ghirardello A: **Recent advances in diagnostic technology: applications in autoimmune and infectious diseases.** *Clin Exp Rheumatol* 2008, **26(1 Suppl 48)**:S62-6.

## Pre-publication history

The pre-publication history for this paper can be accessed here:

http://www.biomedcentral.com/1471-2334/9/66/prepub

J Infect Chemother
DOI 10.1007/s10156-010-0057-5

NOTE

# Comparison of rifabutin susceptibility and *rpoB* mutations in multi-drug-resistant *Mycobacterium tuberculosis* strains by DNA sequencing and the line probe assay

**Shiomi Yoshida · Katsuhiro Suzuki · Tomotada Iwamoto · Kazunari Tsuyuguchi · Motohisa Tomita · Masaji Okada · Mitsunori Sakatani**

Received: 4 November 2009 / Accepted: 1 March 2010


**Abstract** We compared rifabutin susceptibility and *rpoB* mutations in 98 multi-drug-resistant strains of *Mycobacterium tuberculosis* (MDR-TB) by DNA sequencing and with a line probe assay using the commercially available INNO-LiPA Rif. TB kit (the LiPA). Our results indicated that rifabutin continues to remain active against MDR-TB strains harboring certain genetic alterations and also that the LiPA might be useful in identifying MDR-TB strains susceptible to rifabutin.

**Keywords** Tuberculosis · Drug resistance · Rifabutin · *rpoB* · Line probe assay

The recent global expansion of multi-drug-resistant *Mycobacterium tuberculosis* (MDR-TB) poses a serious threat to human health. Numerous previous studies have shown that the majority of rifampicin-resistant isolates of *M. tuberculosis* are also isoniazid resistant [1]. The detection of rifampicin resistance therefore has the potential benefit of simultaneously detecting MDR-TB [1, 2]. One of the commercial kits used to determine drug resistance is the INNO-LiPA Rif. TB kit (the LiPA; Innogenetics, Ghent, Belgium). This assay is an excellent tool for detecting mutations in hot-spot regions of *rpoB*, a gene that encodes a subunit of RNA polymerase. Such mutations occur in up to 95% of rifampicin-resistant strains [2].

Rifabutin is a semisynthetic spiropiperidyl derivative of rifampicin, which is more active than rifampicin itself against *M. tuberculosis* in immunocompromised patients [3]. Rifabutin is also useful as an alternative to rifampicin when serious side effects occur during tuberculosis treatment [4]. Moreover, the minimum inhibitory concentration (MIC) of rifabutin in rifampicin-resistant strains of *M. tuberculosis* carrying *rpoB* mutations varies depending on the specific site of the mutation in the *rpoB* gene [5–10]. Rifabutin might therefore be active against some MDR-TB strains. However, rifabutin susceptibility testing using the time-consuming proportional method on Middlebrook 7H10 medium or by 7H9 microdilution could postpone the effective treatment of patients infected with MDR-TB.

This study aimed to determine the MICs of rifampicin and rifabutin for MDR-TB isolates with known *rpoB* sequences and also to assess results of the LiPA, thereby helping to establish whether this test enables detection of rifabutin susceptibility in MDR-TB strains.

A total of 128 *M. tuberculosis* strains retrieved from a culture collection of the Kinki-chuo Chest Medical Center were tested by the mycobacterial growth-indicator tube–aspartate aminotransferase (MGIT-AST) method (Becton–Dickinson and Company, Fukushima, Japan), and WelPack method (Nihon BCG Inc, Tokyo, Japan) that was established by the egg-based Ogawa medium in commercial susceptibility test systems. Ninety-eight of these strains

S. Yoshida (✉) · K. Suzuki · K. Tsuyuguchi · M. Okada
Clinical Research Center, National Hospital Organization, Kinki-chuo Chest Medical Center, 1180 Nagasone-cho, Sakai 591-8555, Japan
e-mail: dustin@kch.hosp.go.jp

M. Tomita
Department of Clinical Microbiology, National Hospital Organization, Kinki-chuo Chest Medical Center, Sakai, Japan

T. Iwamoto
Department of Microbiology, Kobe Institute of Health, 4-6 Minatojima-nakamachi, Chuo-ku, Kobe 650-0046, Japan

M. Sakatani
Department of Respiratory Medicine, National Hospital Organization, Kinki-chuo Chest Medical Center, 1180 Nagasone-cho, Kita-ku, Sakai 591-8555, Japan

Published online: 31 March 2010

were considered to be resistant to rifampicin as determined by these media. Thirty pan-drug-sensitive (DS) strains were collected between 1 and 31 August 2008, and 98 MDR strains were collected between 1 January 2001 and 31 December 2008. All patients from whom the strains were derived were negative for both human immunodeficiency virus (HIV)-1 and HIV-2. With the exception of one MDR patient, these patients represent all of the DS- and MDR-TB patients treated in this hospital during the strain collection periods.

The MICs for these strains were determined by the validation protocol, performing the commercial and the in-house-prepared microdilution method in parallel for a series of these strains. We elected to use the BrothMIC MTB-1 (Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo, Japan) and a similar system for slowly growing mycobacteria by using 7H9 broth [11]. BrothMIC MTB-1 susceptibility test system with a shorter incubation period has been previously demonstrated to determine MICs that correlate with those obtained from the standardized agar proportion method. According to the manufacturer's instructions, the proposed breakpoints for rifampicin are $\leq$0.06 μg/ml (susceptible), 0.125–2 μg/ml (intermediate), and $\geq$4 μg/ml (resistant). For the microdilution method using 7H9 broth, 100 μl of serial twofold dilutions of rifampicin or rifabutin were dispensed into each well. The final concentrations of the test drugs ranged from 0.015 to 256 μg/ml. All microdilution plates were incubated at 37°C in plastic bags to increase carbon dioxide ($CO_2$) and were read after 7, 14, and 21 days by looking for macroscopic growth with an indirect light source. MICs were the lowest dilutions exhibiting no growth. Quality control testing using *M. tuberculosis* H37Rv was performed once each testing. Each microdilution plate included basal medium without antimicrobial agents to assess viability of the test organisms. Each microdilution testing was performed in duplicate on different days.

The MDR-TB strains were analyzed for the presence of mutations in the rifampicin-resistance-determining region (RRDR). A set of primers described by Kim et al. [12], MF (5′-CGACCACTTCGGCAACCG) and MR (5′-TCGATC GGGCACATCCGG), were used to amplify a 342-bp fragment of the *rpoB* gene containing the 81-bp RRDR. The polymerase chain reaction (PCR) product was sequenced using an automated DNA sequencer (ABI Genetic Analyzer 310, Applied Biosystems, Foster City, CA, USA) with MF and MR primers. The LiPA we employed was used in accordance with the manufacturer's instructions. This kit comprises the *M. tuberculosis* complex-specific probe, five overlapping sensitive probes (wild-type S: 19–23 bases long), and four resistance probes (R-type) from a region of the *rpoB* gene encoding amino acids 509–534. The lack of reactivities of an amplified fragment with the wild-type S probes (probes S1 through S5) was used to detect mutations that lead to rifampicin resistance. Furthermore, R-type probes were specifically designed to hybridize to the sequences of the four most frequently observed mutations: R2 (Asp-516-Val), R4a (His-526-Tyr), R4b (His-526-Asp), and R5 (Ser-531-Leu). When all the wild-type S probes gave a positive signal and all the R-type probes reacted negatively (wild-type profile), the *M. tuberculosis* isolate was considered susceptible to rifampicin. When at least one negative signal was obtained with the wild-type S probes, the isolate was considered rifampicin resistant (ΔS profiles). When the resistance to rifampicin was due to one of the four most frequently observed mutations described above, a positive reaction was obtained with one of the four R-type probes and was always accompanied by a negative reaction with the corresponding wild-type S probe (R profiles). We used *M. tuberculosis* strain H37RV as a positive control.

The ranges of the MICs in DS-TB strains were $\leq$0.03 μg/ml for rifampicin and $\leq$0.015 μg/ml for rifabutin. The corresponding ranges of the MICs in MDR-TB strains were 0.5 to $\geq$256 μg/ml and $\leq$0.015 to $\geq$256 μg/ml, respectively. Whereas rifabutin MICs for 78 of the 98 MDR-TB strains ranged between 0.5 and $\geq$256 μg/ml, which were threefold lower than or equal to those of rifampicin, the other 20 MDR-TB strains had rifabutin MICs ranging between $\leq$0.015 and $\leq$0.25 μg/ml, which were 4- to 15-fold lower than those of rifampicin. As shown in Table 1, our study revealed 20 mutations in the *rpoB* gene. Single-point mutation at codon 513, 525, 526, 531, 533, or 572, which was detected in 72 MDR-TB strains, influenced susceptibility to rifabutin. We also demonstrated that novel mutations such as two strains with double-point mutations (Asp516Ala and Leu533Pro, or Ser512Ile and His526Pro), one strain with an insertion (at codon 525), and one strain with an His526Ser mutation showed rifabutin resistance. In contrast, 20 (20.4%) of the MDR-TB strains that had single-point mutation at codon 511, 516, or 522 and double-point mutation (Asp516Gln and Ser522Leu) were susceptible to rifabutin (MIC, <0.5 μg/ml). The observations that some rifampicin-resistant strains remained susceptible to rifabutin suggest that *rpoB* mutation position and type of amino acid change influence rifabutin susceptibility.

In this study, four MDR-TB strains with a wild-type profile by the LiPA exhibited rifabutin resistance as well. Moreover, 72 strains exhibiting R4a, R4b, R5, ΔS4, ΔS5, ΔS1 + ΔS4, or ΔS2 + ΔS4 + R5 profiles were also resistant to rifabutin. Conversely, 19 strains that exhibited R2 (one of the four most frequently observed mutations), ΔS3, or ΔS2 + ΔS3 profiles were characterized by low rifabutin MICs. The susceptibility of rifabutin conflicted among the remaining three strains that exhibited ΔS1 profile. In detail, one strain had a mutation at codon 511

**Table 1** Comparison of *rpoB* genotype, susceptibility of rifampicin and rifabutin, and the LiPA profiles

| Isolate phenotype and mutation position[a] | Isolates (*n*) | MIC (μg/ml) | | LiPA |
|---|---|---|---|---|
| | | Rifampicin | Rifabutin | |
| DS-TB | | | | |
| Wild type | 30 | ≤0.03 to 0.03 | ≤0.015 | WT |
| MDR-TB[b] | | | | |
| 511Leu → Pro | 1 | 0.5 | 0.03 | ΔS1 |
| 513Gln → Lys | 2 | 8, 16 | 4, 16 | ΔS1 |
| 516Asp → Val | 17 | 4 to ≥256 | 0.015 to 0.25 | R2 |
| 522Ser → Leu | 1 | 2 | 0.06 | ΔS3 |
| 525ACG insertion | 1 | 32 | 32 | WT[c] |
| 526His → Tyr | 2 | 32, 64 | 8, 64 | R4a |
| 526His → Asp | 3 | 64, 128, 128 | 16, 64, 128 | R4b |
| 526His → Ser | 3 | 2, 4, 64 | 2, 4, 32 | ΔS4 |
| 526His → Arg | 1 | 32 | 32 | ΔS4 |
| 526His → Pro | 2 | 8, 64 | 4,32 | ΔS4 |
| 526His → Leu | 1 | 256 | 64 | ΔS4 |
| 526His → Cys | 1 | 4 | 1 | ΔS4 |
| 526His → Arg, 529Arg → Gln | 1 | 64 | 64 | ΔS4 |
| 531Ser → Leu | 54 | 0.5 to ≥256 | 0.5 to ≥256 | R5 |
| 533Leu → Pro | 1 | 32 | 32 | ΔS5 |
| 512Ser → Ile, 526His → Pro | 1 | ≥256 | ≥256 | ΔS1 + ΔS4 |
| 516Asp → Glu, 522Ser → Leu | 1 | 128 | 0.25 | ΔS2 + ΔS3 |
| 516Asp → Ala, 533Leu → Pro | 1 | 128 | 64 | ΔS5[c] |
| Mixed peak in 516 (GAC (Asp) → GTC (Val)), 526 (CAC (His) → CAA (His), 530 (CTG (Leu) → ATG (Met)), and 531 (TCG (Ser) →, TTC (Leu)) | 1 | 256 | 256 | ΔS2 + ΔS4 + R5[c] |
| 572Ile → Phe | 1 | 1 | 1 | WT |
| Non-RRDR | 2 | 16, 128 | 2, 128 | WT |

*WT* wild-type S profile, *DS-TB* drug-sensitive tuberculosis, *MDR-TB* multi-drug-resistant tuberculosis

[a] Numbers correspond to *Escherichia coli* RNA polymerase amino acid positions

[b] Resistant to rifampicin at 1.0 μg/ml by the Clinical and Laboratory Standards Institute method of proportion in 7H10 agar and mycobacterial growth-indicator tube–aspartate aminotransferase (MGIT-AST) method or 40 μg/ml by WelPack method

[c] The LiPA also did not reveal the correct type of mutation

and appeared to have a low rifabutin MIC, but the remaining two strains, at codon 513, were characterized by high rifabutin MICs. Thus, except for ΔS1, profiles of the LiPA could predict rifabutin susceptibility rather faithfully (Table 1).

According to previous studies, rifabutin MICs against rifampicin-susceptible strains were ≤0.06 μg/ml [13], and all strains susceptible to 1 μg/ml of rifampicin and 12% of the strains resistant to 10 μg/ml of rifampicin were susceptible to 0.5 μg/ml of rifabutin [14]. In the study by Uzun et al. [15], all rifampicin-susceptible strains and 12% of rifampicin-resistant strains were also susceptible to rifabutin (MIC, ≤1 μg/ml). All 30 DS-TB strains and 20 of 98 MDR-TB strains were susceptible to rifabutin (MIC, <0.5 μg/ml) in our study. Clinical outcome regarding the efficacy of rifabutin therapy for isolates of MDR-TB with the MICs of ≤0.5 μg/ml has not yet been obtained, but the proposed critical concentration for rifabutin (≤ 0.5 μg/ml) in this study was the same as that recommended by The Clinical and Laboratory Standards Institute (CLSI) using agar-plate testing. However, the relevant critical concentration of rifabutin should be determined by future clinical outcome study.

Our data indicated that all MDR-TB strains with an R2 profile, which was associated with a specific point mutation (Asp516Val), were almost always identified as rifabutin susceptible. The LiPA may offer improvement in the management of MDR-TB, as these vulnerable patients can commence treatment with rifabutin before the strain's isolation. This study further confirmed that rifabutin remains active against MDR-TB strains harboring certain genetic alterations. We also indicate that the LiPA is useful for rapid detection of strains susceptible to rifabutin in MDR-TB before examining susceptibility testing.

**Acknowledgments** This work was supported by a Health and Labour Science Research Grant for Research on Emerging and Re-Emerging Infectious Diseases from the Ministry of Health, Labour and Welfare, Japan, and a Grant for a National Hospital Organization respiratory network study "Study of Respiratory Diseases (Tuberculosis, Lung Cancer, Diffuse Lung Diseases and Respiratory

Insufficiency) using a Network of 54 Hospitals of National Chest Diseases in Japan".

**Conflict of interest statement** None of the authors has any financial interest or financial conflict with the subject matter or materials discussed in this manuscript.

## References


1. Drobniewski FA, Wilson SM. The rapid diagnosis of isoniazid and rifampin resistance in *Mycobacterium tuberculosis*—a molecular story. J Med Microbiol. 1998;47:189–96.
2. Rossau R, Traore H, De Beenhouwer H, Mijs W, Jannes G, de Rijk P, et al. Evaluation of the INNO-LiPA Rif.TB assay, a reverse hybridization assay for the simultaneous detection of *Mycobacterium tuberculosis* complex and its resistance to rifampin. Antimicrob Agents Chemother. 1997;41:2093–8.
3. Davies G, Cerri S, Richel L. Rifabutin for treating pulmonary tuberculosis.Cochrane Database Syst Rev 2007; 17: (4) CD005159.
4. Luna-Herrera J, Reddy MV, Gangadharam PR. In vitro and intracellular activity of rifabutin on drug-susceptible and multiple drug-resistant (MDR) tubercle bacilli. J Antimicrob Chemother. 1995;36:355–63.
5. Bodmer T, Zürcher G, Imboden P, Telenti A. Mutation position and type of substitution in the β-subunit of the RNA polymerase influence in vitro activity of rifamycins in rifampicin-resistant *Mycobacterium tuberculosis*. J Antimicrob Chemother. 1995;35:345–8.
6. Cavusoglu C, Hilmioglu S, Guneri S, Bilgic A. Characterization of *rpoB* mutations in rifampin-resistant clinical isolates of *Mycobacterium tuberculosis* from Turkey by DNA sequencing and line probe assay. J Clin Microbiol. 2002;40:4435–8.
7. Saribaş Z, Kocagöz T, Alp A, Günalp A. Rapid detection of rifampin resistance in *Mycobacterium tuberculosis* isolates by heteroduplex analysis and determination of rifamycin cross-resistance in rifampin-resistant isolates. J Clin Microbiol. 2003;41:816–8.
8. Williams DL, Spring L, Collins L, Miller LP, Heifets LB, Gangadharam PRJ, et al. Contribution of *rpoB* mutations to development of rifamycin cross-resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 1998;42:1853–7.
9. Yang B, Koga H, Ohno H, Ogawa K, Fukuda M, Hirata Y, et al. Relationship between antimycobacterial activities of rifampicin, rifabutin and KRM-1648 and *rpoB* mutations of *Mycobacterium tuberculosis*. J Antimicrob Chemother. 1998;42:621–8.
10. Cavusoglu C, Karaca-Derici Y, Bilgic A. In vitro activity of rifabutin against rifampicin-resistant *Mycobacterium tuberculosis* isolates with known *rpoB* mutations. Clin Microbiol Infect. 2004;10:662–5.
11. Wallace RJ Jr, Nash DR, Steele LC, Steingrube V. Susceptibility testing of slowly growing mycobacteria by a microdilution MIC method with 7H9 broth. J Clin Microbiol. 1986;24:976–81.
12. Kim B-J, Lee S-H, Lyu M-A, Kim S-J, Bai G-H, Kim S-J, et al. Identification of mycobacterial species by comparative sequence analysis of the RNA polymerase gene (*rpoB*). J Clin Microbiol. 1999;37:1714–20.
13. Heifets LB, Lindholm-Levy PJ, Iseman MD. Rifabutine: minimal inhibitory and bactericidal concentrations for *Mycobacterium tuberculosis*. Am Rev Respir Dis. 1988;137:719–21.
14. Heifets LB, Iseman MD. Determination of in vitro susceptibility of mycobacteria to ansamycin. Am Rev Respir Dis. 1985;132:710–1.
15. Uzun M, Erturan Z, Anğ O. Investigation of cross-resistance between rifampin and rifabutin in *Mycobacterium tuberculosis* complex strains. Int J Tuberc Lung Dis. 2002;6:164–5.

# 遺伝子を用いた抗酸菌鑑別同定試薬 INNO-LiPA MYCOBACTERIA v2の有用性の検討

[1]吉田志緒美 [1]鈴木 克洋 [1]露口 一成 [4]岩本 朋忠
[2]富田 元久 [1]岡田 全司 [3]坂谷 光則

**要旨**：〔目的〕遺伝子を用いた抗酸菌鑑別用同定試薬INNO-LiPA MYCOBACTERIA v2（INNO-LiPA法）のわが国における有用性の検討。〔対象〕NHO近畿中央胸部疾患センターにおいて新規に分離された抗酸菌122株。〔方法〕INNO-LiPA法と3種類の同定キット（コバス アンプリコア マイコバクテリウム法，アキュプローブ法とDDH法）との結果を比較検討した。同定不能もしくはデータ間で違う結果を示した株についてはシークエンス解析を行った。〔結果〕122株のうち112株が3種類の同定キットのいずれかとINNO-LiPA法の結果が一致した（91.8%）。相違を認めた10株のうち6株はINNO-LiPA法とシークエンス解析の結果が一致した。しかし2株のうち1株はDDH法の結果と一致し*M. fortuitum*，もう1株はコバス アンプリコア マイコバクテリウム法とアキュプローブMAC法の結果と一致し*M. intracellulare*と判定された。INNO-LiPA法と3種類の同定キットの結果がともにシークエンス解析結果と異なる株は2株認められた（*M. paraffinicum*，*M. mucogenicum*近縁種）。〔考察〕INNO-LiPA法は正確性，迅速性に優れており有益性が証明された。培養および生化学的性状試験と併行して実施することにより総合的な抗酸菌同定が可能であると考えられた。

**キーワーズ**：抗酸菌，INNO-LiPA MYCOBACTERIA v2，同定検査，16S rRNA遺伝子，ITSシークエンス解析

## はじめに

抗酸菌同定検査において生化学的同定検査を実施するには多大な労力と菌量が必要である。また培養にかかる期間も結核菌だと小川培地で3～8週間は必要であり，治療方針を決定するうえでも迅速な同定検査は必須である。近年，遺伝子検査の手法を応用した抗酸菌の迅速同定検査が日常的に用いられるようになり，さまざまな測定原理から開発された菌同定用キットが市販されている。抗酸菌を正確かつ迅速に同定する性能を兼ね備えたこれらのキットは，先人により高い評価と共に各種問題点も報告されている[1)~7)]。

コバス アンプリコア マイコバクテリウム法はpolymerase chain reaction（PCR）を用いてDNAを増幅後，ハイブリダイゼーションすることで臨床検体や菌株を対象として*M. tuberculosis* complex，*M. avium*，*M. intracellulare*の3菌種が同定できる[1)~3)]。前倉らによると肺結核患者における塗抹陽性検体の94.4%はコバス アンプリコア マイコバクテリウム法で陽性であったが，塗抹陰性検体の場合は70.8%が陽性となった[4)]。

アキュプローブ法は検体の16S rRNAをターゲットとして，菌種特異的DNAプローブとハイブリダイゼーションさせてから，専用の検出器を用いて化学発光を検出するキットである[5)]。結核菌群と*M. avium* complex（MAC），*M. kansasii*，*M. gordonae*の4種類のキットがあり，アキュプローブ陽性となる感度は結核菌群と*M. gordonae*で100%，MAC 95.2%，*M. kansasii* 44.0%という報告[5)]や，結核菌群87.2%，MAC 78.6%，*M. kansasii* 91.7%，*M. gordonae* 85.9%とする報告[6)]などがある。

DDH法は核酸の相同性を利用し，ハイブリダイズし

独立行政法人国立病院機構近畿中央胸部疾患センター[1]臨床研究センター，[2]研究検査科，[3]内科，[4]神戸市環境保健研究所

連絡先：吉田志緒美，独立行政法人国立病院機構近畿中央胸部疾患センター臨床研究センター，〒591-8555 大阪府堺市北区長曽根町1180（E-mail: dustin@kch.hosp.go.jp）
(Received 28 Aug. 2008/Accepted 30 Oct. 2008)

たDNAの比率をビオチン-アビジン反応を用いてそれぞれの基準DNA株と被検菌DNAのDNA塩基配列の相同性を測定するキットで18菌種の同定を一度に行うことが可能である[7]。同法は「全染色体DNAの類似度(similarity)が70%以上であれば，同じ菌種としてよい」という細菌分類学の菌種同定基準を利用している。そのため相対類似度で算出された数値から供試菌がどの菌種の基準株に最も近いかという結果は得られるが，同定不能となる菌種が多い傾向がある[8][9]。また「肉眼的に明らかな発色が確認された場合には吸光度を測定せずにウェルの菌種と同定してもよい」としていることも誤判定を生じやすい原因である。

INNO-LiPA MYCOBACTERIA v2（INNO-LiPA法）は16S-23S internal transcribed spacer（ITS）領域をターゲットとしPCR法で増幅されたDNAを対象に，ラインプローブアッセイを用いて検出する。一度に15菌種の同定と*Mycobacterium*属に共通の*Mycobacterium* genusのプローブがあることから抗酸菌の確定が可能である[10]。

INNO-LiPA法の検討はこれまでにいくつか報告はあるが[10]~[13]，現時点でわが国における検討報告はなされていない。今回われわれはINNO-LiPA法の迅速性ならびに正確性について上記の先に市販されている同定キットの結果と比較しその有用性を検討した。

## 方　法

### 対象

独立行政法人近畿中央胸部疾患センターにおいて新規に分離培養される菌株のうち大半を占める抗酸菌は結核菌群であるが，多くの菌種が存在し，日常検査で判定に苦慮する割合が高いのは非結核性抗酸菌（NTM）である。今回NTMに対する同定結果の比較に重点をおき，結核菌群の菌株数を絞って検討を試みた。したがって2006年2月1日から6月30日までの期間に分離された結核菌群7株，NTM 115株の合計122株を対象とした。すべての菌株同定は同定検査結果とあわせて小川培地上でのコロニー性状の観察をもって最終判定とした。

検体内に複数の菌が混在する場合に同時に鑑別が可能かどうかを検討するため，臨床検体から複数の菌種が認められた3つの混合培養も検討に加えた。これらはあらかじめ固形培地上で異なるコロニー性状をもつと判定され，おのおのに純培養を行って3種類の同定キットにて同定検査を実施，複数菌混在であることを確認した。

### 同定キット

NHO近畿中央胸部疾患センターにおいて日常検査に使用している遺伝子を用いた同定キットを使用した。

結核菌群の同定には結核菌群同定用アキュプローブ結核菌群同定キット（極東製薬工業）と結核菌群同定試薬キャピリアTB（日本ベクトン・ディッキンソン）を用いた。*M. avium*と*M. intracellulare*の同定にはコバス アンプリコア マイコバクテリウム アビウムとコバス アンプリコア マイコバクテリウム イントラセルラー（コバス アンプリコア マイコバクテリウム法：ロシュ・ダイアグノスティクス），MACの同定にはアキュプローブ マイコバクテリウム アビウム コンプレックス（アキュプローブMAC法：極東製薬工業）を用いた。*M. kansasii*および*M. gordonae*の同定には，研究用試薬であるアキュプローブ マイコバクテリウム カンサシとアキュプローブ マイコバクテリウム ゴルドネ（共に極東製薬工業）を用いた。上記以外の菌種の同定にはDDHマイコバクテリア '極東'（DDH法：極東製薬工業）を用いた。すべての方法は添付の仕様説明書に準拠して行った。DDH法はDNAの精製が不十分な場合に同定不能の結果が得られることもあるため同定不能の結果が得られた場合には再検査を行った。

### DNAの抽出

小川培地発育菌から白金耳で径2～3 mmのコロニー2個分の菌量を採取し，1.5 mlマイクロチューブに分注したインスタジーンDNA精製マトリックス（BIO-RAD）200 $\mu$lに懸濁した。56℃，15～30分処理後10秒間vortexし，正確に100℃，8分間処理した後直ちに氷水中で急冷した。10秒間vortexし，12000 rpm，3分遠心した上清をINNO-LiPA法ならびにシークエンス解析法に用いた。

### INNO-LiPA法

INNO-LiPA MYCOBACTERIA v2（INNO-LiPA法：INNOGENETICS）は，発色確認用コントロールと抗酸菌特異的プローブ（MYC genus）および菌種鑑別のための22本のITS遺伝子プローブが固相化されたストリップ状のキットである。プローブは12菌種のプローブに加えて，3種類のsubtypeが鑑別可能な*M. kansasii*プローブ，4種類のMAIS complexプローブ，*M. abscessus*を含んだ3種類の*M. chelonae* complexプローブが配置されている。同キットの使用説明書に準拠して16S-23S ITS領域の遺伝子のPCR増幅を行い，得られたPCR増幅産物をLiPA検体として使用した。LiPA検体をハイブリダイズさせ，得られた発色パターンによって抗酸菌の同定を行った（Table 1）。

INNO-LiPA法においてはPCRの後，すべてのPCR産物を電気泳動し得られたバンドから増幅の確認を行った。またDDH法と同様にMYC genusにしか発色が見られない場合，再検査を行った。

### 16S rRNA遺伝子，ITS領域のシークエンス解析

3種類の同定キットとINNO-LiPA法により同定不能であった株，ならびに結果の乖離が認められた株に対し

**Table 1** Interpretation of *Mycobacterium* species by using the INNO-LiPA MYCOBACTERIA v2

| Line | Probe | Taxa reacting with the probe |
|---|---|---|
| 1 | Conjugate Control | |
| 2 | MYC genus | Presence of *Mycobacterium* in the test sample |
| 3 | MTB | *M. tuberculosis* complex: *M. tuberculosis*, *M. bovis*, *M. microti*, *M. africanum* |
| 4 | MKA-1 | *M. kansasii* (group I)* |
| 5 | MKA-2 | *M. kansasii* (group II)* |
| 6 | MKA-3 | *M. kansasii* (group III, V, VI)*, *M. gastri* |
| 7 | MXE | *M. xenopi* |
| 8 | MGO | *M. gordonae* |
| 9 | MGV | *M. genavense* |
| 10 | MSI | *M. simiae* |
| 11 | MMU | *M. marinum*+*M. ulcerans* |
| 12 | MCE | *M.celatum* |
| 13 | MAIS | *M. avium*, *M. intracellulare*, *M. scrofulaceum*, MAC, *M. malmoense* |
| 14 | MAV | *M. avium*, *M. paratuberculosis*, *M. silvaticum* |
| 15 | MIN-1 | *M. intracellulare* (sqv. Min-A, -B, -C, and-D) |
| 16 | MIN-2 | *M. intracellulare* (sqv. Mac-A) |
| 17 | MSC | *M. scrofulaceum* |
| 18 | MML | *M. malmoense* |
| 19 | MHP | *M. haemophilum* |
| 20 | MCH-1 | *M. chelonae* complex (group I, II, III, IV, *M. abscessus*)* |
| 21 | MCH-2 | *M. chelonae* complex (group III, *M. abscessus*)* |
| 22 | MCH-3 | *M. chelonae* complex (group I)* |
| 23 | MFO | *M. fortuitum*–*M.peregrinum* complex |
| 24 | MSM | *M. smegmatis* |

*group is based on sequevar derived from 16S–23S nucleotide sequences. sqv., sequevar

て，データベースが豊富な16S rRNA遺伝子のシークエンスを，さらに16S rRNA遺伝子の相同性解析で同定が困難な菌株に対してはITSシークエンス解析を追加し菌種を決定した。PCR反応は岩本らの方法[14]に準じ，Takara Ex Taq（タカラバイオ）を用いて，94℃ 30秒，55℃ 30秒，72℃ 1分を35サイクル行った。16S rRNA遺伝子の超可変部AとBを含む領域をプライマー 285F［5'-GAG AGT TTG ATC CTG GCT CAG-3'］と264R［5'-TGC ACA CAG GCC ACA AGG GA-3'］を用いてPCR増幅産物を得た。ITS領域全長の増幅にはITS1［5'-GAT TGG GAC GAA GTC GTA AC-3'］とITS2［5'-AGC CTC CCA CGT CCT TCA TC-3'］を用いた。PCR産物を精製した後BigDye Terminator Ready Reaction Cycle Sequencing Kit（Applied Biosystems Japan）を用いて16S rRNA遺伝子の部分配列とITS全長の塩基配列を得た。得られた塩基配列は，Ribosomal Differentiation of Microorganisms: RIDOMを用いて相同性検索を行い，99%以上の塩基配列一致をもって同一菌種と決定した。

## 結　果

供試菌122株のうち112株においてINNO-LiPA法と3種類の同定キットの結果が一致した。対象菌株のうち結核菌群の7株はすべて，結核菌群同定用アキュプローブ結核菌群同定キット，キャピリアTBとINNO-LiPA法の結果が一致した。NTM 115株のうちアキュプローブMAC法でMACと同定され，コバス アンプリコア マイコバクテリウム法により*M. avium*と同定された24株はINNO-LiPA法ではMAISとMAVプローブのバンドを認めた。一方アキュプローブMAC法でMAC，コバスアンプリコア マイコバクテリウム法により*M.intracellulare*と同定された7株がMAISとMIN-1プローブに反応していたが，菌株23のみMIN-1に反応を示さず結果に乖離が見られた。DDH法を実施した83株のうち再検査を実施しても同定不能となった株は6株認められた。これら6株のうち3株はINNO-LiPA法でもMYC genusにしか反応が見られなかった。一方DDH法で菌種同定ができたがINNO-LiPA法との間に結果の食い違いが見られた株は3株認められた。したがって3種類の同定キットのいずれかとINNO-LiPA法との間で同定不能や結果が異なった10株に対してシークエンス解析を行った。

6株（菌株2，19，14，22，7，6）はシークエンス解析結果とINNO-LiPA法の結果が一致した。菌株2と19はINNO-LiPA法でMKA-3の反応を認め*M. kansasii* 3と判定され，シークエンス解析からそれぞれアキュプローブ カンサシで陰性となる*M. kansasii* sqv. ⅢとⅥと判定された。菌株14はINNO-LiPA法，シークエンス解析ともに*M. gordonae*と判定された。一方，菌株5と23の2株は同定キットの結果とシークエンス解析結果が一致した。菌株5は，INNO-LiPA法でMYC genusに反応が認

**Table 2** Discrepant and unidentified results in identification of *Mycobacterium* species, including 9 isolates of *M. lentiflavum*.

| Isolate No. | Cobas Amplicor system | AccuProbe | DDH | INNO-LiPA | 16S rRNA gene | | ITS | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Identity (%) | | Identity (%) |
| 9 isolates | Negative | Negative | Unidentified** | MYC genus | *M. lentiflavum* DSM44418T | 429/429 (100) | | |
| 2 | Negative | Negative | Unidentified** | *M. kansasii* 3 | *M. kansasii* Borste 8875/99, sqv. VI-3 | 441/441 (100) | *M. kansasii*, MkaF | 277/277 (100) |
| 19 | Negative | Negative | Unidentified** | *M. kansasii* 3 | *M. kansasii* Borste 539/99, sqv. III | 440/440 (100) | *M. kansasii*, MkaC | 279/279 (100) |
| 14 | Negative | NT | Unidentified** | *M. gordonae* | *M. gordonae* Borste 11340/99, sqv. III | 440/440 (100) | *M. gordonae*, MgoC | 270/270 (100) |
| 22 | Negative | NT | Unidentified** | MYC genus | *M. interjectum* ATCC51457T | 430/430 (100) | | |
| 7 | Negative | NT | Unidentified*** | *M. abscessus* | *M. abscessus* or *M. chelonae* (*M. abscessus* by ITS) | 428/428 (100) | *M. abscessus* DSM44196 | 294/294 (100) |
| 6 | Negative | NT | *M. fortuitum* | *M. chelonae* | *M. abscessus* or *M. chelonae* (*M. chelonae* by ITS) | 428/428 (100) | *M. chelonae* Mche B | 293/294 (99.7) |
| 5 | Negative | NT | *M. fortuitum* | MYC genus | *M. fortuitum* DSM46621T | 428/428 (100) | | |
| 23 | *M. intracellulare* | MAC* | NT | MAIS | *M. intracellulare* ATCC35770 sqv. III | 442/442 (100) | | |
| 13 | Negative | NT | Unidentified*** | *M. fortuitum* | *M. mucogenicum* ATCC49650T | 423/428 (98.8) | | |
| 18 | Negative | NT | *M. scrofulaceum* | *M. intracellulare* 2 | *M. paraffinicum* DSM44181T | 439/439 (100) | | |

*M. avium complex **slow growers ***rapid growers NT: not tested T, Type strain sqv., sequevar

められたが菌種の特定には至らず，DDH法とシークエンス解析では*M. fortuitum*と同定された。菌株23はコバス アンプリコア マイコバクテリア法で*M. intracellulare*，アキュプローブMAC法でMAC，INNO-LiPA法でMAISと判定され，シークエンス解析で*M. intracellulare* ATCC 35770 sqv. Ⅲ（Mac-D）と100%相同と判定された。

シークエンス解析結果といずれかの方法の結果が異なった株（菌株13，18）は各々*M. mucogenicum*近縁種と*M. paraffinicum*と同定された。

小川培地上で遅発育菌と観察され，コバス アンプリコア マイコバクテリウム法，アキュプローブ法，DDH法でも同定不能となり，INNO-LiPA法でMYC genusにしかバンドの発色が見られなかったがシークエンス解析で*M. lentiflavum*と同定された株が9株認められた（Table 2）。

複数菌種が混在していた3混合培養はINNO-LiPA法でも複数のバンドパターンが認められた（*M. tuberculosis*＋*M. gordonae*，*M. avium*＋*M. fortuitum*，*M. kansasii*＋*M. gordonae*）。

## 考　察

分子遺伝学的に近縁な菌種であり16S rRNA遺伝子に違いが見られない場合，ITS領域のほうが進化速度は速いため，より多様性のある配列結果が得られる。ITS領域をターゲットとしたINNO-LiPA法はITS領域で高い多型性が知られているMACに対して4種類の亜型プローブを使って亜型判定を可能としている。菌株23はアキュプローブMAC法でMAC，コバス アンプリコア マイコバクテリウム法で*M. intracellulare*，シークエンス解析で*M. intracellulare* ATCC 35770 sqv. Ⅲ（Mac-D）と判定された。INNO-LiPA法では同タイプに対応する亜型プローブは設計されていないためにMAISプローブのみの反応となった。Lebrunらも ATCC 35770の検討で同じくMAISプローブにのみ発色が認められたと報告している[13]。したがって，ITS領域において菌種内多型性を示す菌種に対しては，シークエンス解析で相同性を確認することが重要となってくる。

シークエンス解析といずれの方法とも結果が食い違った2株のうち菌株18はアキュプローブMAC法陰性，DDH法で*M. scrofulaceum*となり，INNO-LiPA法でMAISとMIN-2に発色が見られ*M. intracellulare* sqv. Mac-Aと判定された。遅発育菌である同菌株は16S rRNA解析では100%の相同性で*M. paraffinicum* DSM 44181と判定され，同じく*M. scrofulaceum* DSM 43992とは99%の相同性が見られた。Tortoliらも*M. paraffinicum*はMAISとMIN-2に発色が見られたがアキュプローブMAC法は陰性であったと報告している[10]。一方Lebrunらはアキュプロー

プMAC法陰性，INNO-LiPA法ではMAISのみにバンドに発色があり，シークエンス解析で*M. paraffinicum*と判定されたが同時に*M. scrofulaceum* DSM 43992と98.9%の相同性があったと報告している[13]。今回アキュプローブMAC法でMAC，コバス アンプリコア マイコバクテリウム法により*M. avium*と同定された菌株はすべてINNO-LiPA法で明確にMAVに発色が見られ，アキュプローブMAC法でMAC，コバス アンプリコア マイコバクテリウム法により*M. intracellulare*と判定された菌株も上記の菌株23以外はMIN-1に発色が見られた。したがって唯一MIN-2にバンドを示した菌株18は*M. intracellulare* sqv. Mac-Aとかなり相同性が高い近縁菌種と考えられた。

菌株13は16S rRNAシークエンス解析で*M.mucogenicum* ATCC 49650Tと5bpの違い（98.8%の相同性）が見られ*M. mucogenicum*の近縁種と推定された。小川培地上で迅速に発育しDDH法で同定不能，INNO-LiPA法で*M. fortuitum*と判定されており，結果に乖離が見られた。*M. mucogenicum*は古くは*M.chelonae*-likeとして知られていたが，16S rRNA遺伝子では*M. chelonae*よりも*M.fortuitum*に近い系統に位置しており[15]，現在では*M. chelonae-abscessus*グループと*M.fortuitum*グループに近縁の迅速発育菌として独立したグループと定義されている。Ballardらは同じくATCC49650Tと5 bp違いでなおかつATCC49649と1bp違いの*M. mucogenicum* N248を解析しており，新しいsubspeciesの可能性があると報告している[16]。迅速発育菌は多様性に富んでおり，菌株13も*M. mucogenicum*のvariant typeの可能性が考えられた。

同じく迅速発育菌であった菌株5はINNO-LiPA法ではMYC genusのみ発色が見られ，DDH法で*M.fortuitum*，シークエンス解析で*M.fortuitum* DSM46621とDSM44220に100%の相同性が認められた。PadillaらはINNO-LiPA法で同じタイプのDSM46621株は*M.fortuitum*と同定されたと報告している[12]。われわれの検討では同菌種のDSM44220株（*M.fortuitum subspecies acetamidolyticum*）はDDH法とINNO-LiPA法で*M.fortuitum*と同定できた（データ未掲載）。*M.fortuitum*はITSシークエンス解析でsqv. Ⅰ～Ⅳが認められており高い多型性を示すため[17]，迅速発育菌の詳細な亜型解析にはシークエンス解析が重要であると思われた。

遺伝子を用いた同定キットによる判定と併行して従来法やコロニー性状から菌種を鑑別することは非常に重要である。菌株22はコバス アンプリコア マイコバクテリウム法，アキュプローブ法，DDH法で同定不能となった遅発育菌である。INNO-LiPA法ではMYC genusの反応が見られたが，シークエンス解析では*M. interjectum*と判定された。*M. interjectum*は非光発色性の遅発育菌でかつ16S rDNA配列が特異的であり，遺伝子を用いた同定キットによる菌種同定は困難である[18]。INNO-LiPA法においても該当プローブが固相化されていないため同菌種の同定はできず，培養でのコロニー性状の観察や生化学的性状試験が鑑別上重要になってくる。同様に菌株2と19は，3種類のプローブで*M. kansasii*の亜型を判別可能であるINNO-LiPA法でMKA-3に発色した。16S rRNA遺伝子のシークエンス解析から*M. kansasii* sqv. ⅢとⅥとに判定されたが研究用試薬アキュプローブ カンサシで陰性となるため*M. kansasii*と判定されなかった。日常検査では光発色試験に及んでいなかったが，改めて実施した結果*M. kansasii*と同定できた。

コロニーの光発色試験での光発色菌，暗発色菌，非光発色菌の鑑別は純培養を用いるため可変的，主観的であり，熟練を要する。*M. szulgai*は37℃で暗発色性，25℃培養で光発色性になる。*M. simiae*の光発色性の出現は通常1時間の光照射のところ6～24時間の光照射が必要であり注意を要する。培養時のコロニー性状の観察において，S型，R型，その移行型（SR型，RS型）の性状が継代を重ねることで変化してくることがある。また発育速度の観察は，遅発育菌でも大量の菌を接種すれば7日ぐらいで発育は見られる場合はあるし，迅速発育菌での分離培養の時にはコロニーの発生までに時間がかかる場合もある。したがって培養条件により変化する菌の性状を十分考慮して，なるべく初代分離菌について詳細に観察することが望ましい。

INNO-LiPA法の製造元であるINNOGENETICS社の本社がベルギーに位置するため，欧米のAIDS患者から分離された*M. genavense*[19]や，イギリス，スコットランド，ウェールズ，スウェーデン，フランスで分離が増えている*M. malmoense*[20]といった菌種に対する同定が可能となっている。わが国では現時点でのこれらの菌種による感染症の報告は非常にまれであるため，今後これら稀少菌種の同定の際には大きな威力を発揮すると思われる。一方，最近わが国で分離の報告が増加している遅発育菌の*M. lentiflavum*[14]が今回シークエンス解析により9株確認されたが，対応プローブが配置されていないINNO-LiPA法ではMYC genusにしかバンドの発色が見られず同定に至らなかった。臨床での有用性をより高めるために，わが国の抗酸菌分離状況にあわせたINNO-LiPA法の仕様改良を切望したい。

今回有用性が認められたINNO-LiPA法は手技面でもPCR増幅後約3時間で判定可能であり，迅速性が証明された。ハイブリダイゼーションから洗浄，発色までを行う自動化ハイブリダイゼーション装置Auto-LiPAを利用すれば労力の軽減が可能であると思われる。またINNO-LiPA法はストリップ上に得られるバンドの有無で判定するため，DDH法のような読み取り時の測定誤差は少

なくなると考えられる。複数菌混合培養における複数菌種同定も可能であることから，単一分離培養に要する時間や手間が省かれ，迅速に同定結果が得られることが明らかとなった。

抗酸菌における遺伝子検査の進歩は特に目覚ましく，今回用いた検査法も含めて多様な検査キットが市販されている。各種キットの特徴を熟知したうえでそれぞれの施設に適した検査法を選択し，各キット間に生じる結果の乖離や同定不能な株が存在する場合を考慮して菌種同定を行うことが望まれる。またこれらキットは定性用検査であり，検体内の菌量を反映できないため，塗抹・培養検査の結果と同定結果とを鑑みて治療方針を決定することが重要である。特にNTMを分離した場合には非結核性抗酸菌症の診断基準[21)22)]と合わせて総合的に判断するべきである。

## 文　献


1) Ichiyama S, Iinuma Y, Tawada Y, et al.: Evaluation of Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test and Roche PCR-microwell plate hybridization method (AMPLICOR MYCOBACTERIUM) for direct detection of mycobacteria. J Clin Microbiol. 1996 ; 34 : 130−133.

2) Abe C, Hirano K, Wada M, et al.: Detection of *Mycobacterium tuberculosis* in clinical specimens by polymerase chain reaction and Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test. J Clin Microbiol. 1993 ; 31 : 3270−3274.

3) Wobeser WL, Krajden M, Conly J, et al.: Evaluation of Roche Amplicor PCR assay for *Mycobacterium tuberculosis*. J Clin Microbiol. 1996 ; 34 : 134−139.

4) 前倉亮治，横田総一郎，小倉　剛：抗酸菌感染症診断の進歩．分子呼吸病．1998 ; 2 : 346−352.

5) Lebrun L, Espinasse F, Poveda JD, et al.: Evaluation of nonradioactive DNA probes for identification of mycobacteria. J Clin Microbiol. 1992 ; 30 : 2476−2478.

6) Reisner BS, Gatson AM, Woods GL: Use of Gen-Probe AccuProbes to identify *Mycobacterium avium* complex, *Mycobacterium tuberculosis* complex, *Mycobacterium kansasii*, and *Mycobacterium gordonae* directly from BACTEC TB broth cultures. J Clin Microbiol. 1994 ; 32 : 2995−2998.

7) Kusunoki S, Ezaki T, Tamesada M, et al.: Application of colorimetric microdilution plate hybridization for rapid genetic identification of 22 *Mycobacterium* species. J Clin Microbiol. 1991 ; 29 : 1596−1603.

8) 山崎利雄，髙橋　宏，中村玲子：マイクロプレートハイブリダイゼーション法による抗酸菌同定法の検討．結核．1993 ; 68 : 5−11.

9) 斉藤　宏，長友雅彦，中嶌雅信，他：DNA-DNA Hybridizationを原理とする「DDHマイコバクテリア '極東'」を用いた抗酸菌同定とその同定精度の検討．JARMAM. 1994 ; 6 : 23−28.

10) Tortoli E, Mariottini A, Mazzarelli G : Evaluation of INNO-LiPA MYCOBACTERIA v2 : improved reverse hybridization multiple DNA probe assay for mycobacterial identification. J Clin Microbiol. 2003 ; 41 : 4418−4420.

11) Miller N, Infante S, Cleary T : Evaluation of the LiPA MYCOBACTERIA assay for identification of mycobacterial species from BACTEC 12B bottles. J Clin Microbiol. 2000 ; 38 : 1915−1919.

12) Padilla E, González V, Manterola JM, et al.: Comparative evaluation of the new version of the INNO-LiPA Mycobacteria and GenoType Mycobacterium assays for identification of *Mycobacterium* species from MB/BacT liquid cultures artificially inoculated with mycobacterial strains. J Clin Microbiol. 2004 ; 42 : 3083−3088.

13) Lebrun L, Weill FX, Lafendi L, et al.: Use of the INNO-LiPA-MYCOBACTERIA assay (version 2) for identification of *Mycobacterium avium−Mycobacterium intracellulare−Mycobacterium scrofulaceum* complex isolates. J Clin Microbiol. 2005 ; 43 : 2567−2574.

14) 岩本朋忠，中永和枝，石井則久，他：*Mycobacterium lentiflavum*の菌種内塩基配列変異に関する研究．結核．2008 ; 83 : 417−422.

15) Springer B, Böttger EC, Kirschner P, et al.: Phylogeny of the *Mycobacterium chelonae*-like organism based on partial sequencing of the 16S rRNA gene and proposal of *Mycobacterium mucogenicum* sp. nov. Int J Syst Bacteriol. 1995 ; 45 : 262−267.

16) Ballard J, Turenne CY, Wolfe JN, et al.: Molecular characterization of nontuberculous mycobacteria isolated from human cases of disseminated disease in the USA, Thailand, Malawi, and Tanzania. J Gen Appl Microbiol. 2007 ; 53 : 153−157.

17) Richter E, Niemann S, Rüsch-Gerdes S, et al.: Identification of *Mycobacterium kansasii* by using a DNA probe (Accu Probe) and molecular techniques. J Clin Microbiol. 1999 ; 37 : 964−970.

18) Tortoli E : Impact of genotypic studies on mycobacterial taxonomy : the new mycobacteria of the 1990s. Clin Microbiol Rev. 2003 ; 16 : 319−354.

19) Bogdan C, Kern P, Richter E, et al.: Systemic infection with *Mycobacterium genavense* following immunosuppressive therapy in a patient who was seronegative for human immunodeficiency virus. Clin Infect Dis. 1997 ; 24 : 1245−1247.

20) The Research Committee of the British Thoracic Society : Pulmonary disease caused by *M. malmoense* in HIV negative patients : 5-yr follow-up of patients receiving standardised treatment. Eur Respir J. 2003 ; 21 : 478−482.

21) Griffith DE, Aksamit T, Brown-Elliott BA, et al., on behalf of the ATS Mycobacterial Diseases Subcommittee : An Official ATS/IDSA Statement : Diagnosis, Treatment, and Prevention of Nontuberculous Mycobacterial Diseases. Am J Respir Crit Care Med. 2007 ; 175 : 367−416.

22) 日本結核病学会非結核性抗酸菌症対策委員会，日本呼吸器学会感染症・結核学術部会：肺非結核性抗酸菌症診断に関する指針—2008年．結核．2008 ; 83 : 525−526.

Original Article

# EVALUATION OF THE INNO-LiPA MYCOBACTERIA v2 FOR MYCOBACTERIAL IDENTIFICATION

[1]Shiomi YOSHIDA, [1]Katsuhiro SUZUKI, [1]Kazunari TSUYUGUCHI, [4]Tomotada IWAMOTO, [2]Motohisa TOMITA, [1]Masaji OKADA, and [3]Mitsunori SAKATANI

**Abstract** [Purpose] Evaluation of the INNO-LiPA MYCOBACTERIA v2 (the INNO-LiPA assay) for mycobacterial identification.

[Materials and Methods] The laboratory identifications consisting of Cobas Amplicor systems, AccuProbe, and DDH, are commonly used to identify mycobacterial isolates in Japan. We compared the results between the INNO-LiPA assay and the common methods. A total of 122 clinical isolates from NHO Kinki-chuo Chest Medical Center from 1 February to 30 June 2006 were tested.

[Results] There was agreement between the INNO-LiPA assay and the common methods for 112 mycobacterium isolates. The six discordant isolates have showed same results between sequencings and the INNO-LiPA assay. The one *M.fortuitum* isolates was indicated correctness by DDH and the one *M. intracellulare* isolates was recognized by Cobas Amplicor systems and as MAC by AccuProbe MAC. Moreover, discrepant results between sequencings and mycobacterial identifications including the INNO-LiPA assay were 2 isolates (*M.paraffinicum*, *M.mucogenicum* variant type).

[Conclusion] The INNO-LiPA assay could provide rapid and correct identification results with clear-cut and easy interpretation.

**Key words**: Mycobacteria, INNO-LiPA MYCOBACTERIA v2, Identification, 16S rRNA gene, ITS sequencing

[1]Clinical Research Center, [2]Department of Clinical Laboratory, [3]Department of Respiratory Medicine, National Hospital Organization (NHO) Kinki-chuo Chest Medical Center, [4]Kobe Institute of Health

Correspondence to : Shiomi Yoshida : Clinical Research Center, NHO Kinki-chuo Chest Medical Center, 1180 Nagasone-cho, Kita-ku, Sakai-shi, Osaka 591−8555 Japan. (E-mail: dustin@kch.hosp.go.jp)