Comparison of the Resistance Ratio and Proportion Methods for Antimicrobial Susceptibility Testing of *Mycobacterium tuberculosis*

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Abstract

This study compared two *in vitro* antimicrobial susceptibility methods for determining drug susceptibilities of *Mycobacterium tuberculosis* isolated from newly diagnosed pulmonary tuberculosis patients to four front-line drugs. Of 250 strains of *M. tuberculosis* tested, 74.4 per cent were susceptible by the resistance ratio method, with 72.0 per cent by the proportion method. The results showed high agreement for both methods ($P<0.0001$) and agreement rates to streptomycin, isoniazid, rifampicin and ethambutol were 96.8, 98.0, 94.8 and 96.8 per cent, respectively. For drug resistance patterns, both methods showed the highest resistance to one drug, followed by two, three, and four drugs, respectively. Of the single drug resistance, both methods gave the highest resistance to streptomycin, followed by resistance to isoniazid, rifampicin and ethambutol, respectively. The correlation between both methods for determining susceptibility of *M. tuberculosis* to four drugs was not statistically significantly different by Mc Nemar χ^2 ($p>0.05$). Thus, the resistance ratio method may be substituted. However, WHO recommended the use of the proportion method to be used for determining drug susceptibility of *M. tuberculosis*. The susceptibility testing result can be used as the guidance for proper treatment and is valuable for confirmation of drug resistance in patients showing unsatisfactory response to treatment, useful for identifying primary and acquired drug resistance trends in a community and for minimizing the spread of drug-resistant strains.

Key word : *Mycobacterium tuberculosis,* Antimicrobial Susceptibility Testing, Resistance Ratio, Proportion Method

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Tuberculosis (TB) has re-emerged worldwide as an important public health problem. One-third of the world's population has been infected with *Mycobacterium tuberculosis*. Much concern has been expressed about the dramatic outbreaks of multidrug-resistant tuberculosis (MDR-TB) in HIV-infected TB patients in various parts of the world (1-5). These outbreaks have been characterized by delayed diagnosis, an ineffective treatment system, high mortality and significant rates of nosocomial transmission. It was recommended in the WHO Global Tuberculosis Programme(6) that one important measure for strengthening of the National Tuberculosis Programme was to focus on the global surveillance of drug resistance.

The strategy for achievement of this objective is to implement a surveillance for anti-tuberculosis drug resistance in a number of countries under the guidance of a network of Supranational Reference Laboratories (SRLs) for standardized susceptibility testing using internationally accepted methods and to be assured that data collected are representative of the population being studied and can allow distinction between primary and acquired drug resistance(7). In Thailand, the surveillance of drug resistance of *M. tuberculosis* began in 1998(8, 9). As a result, 25.4 per cent were resistant to one or more drugs, 5.6 per cent were resistant to streptomycin, 6.2 per cent to isoniazid, 2.0 per cent to rifampin, 3.0 per cent to ethambutol and 2.0 per cent were multidrug-resistant.

The commonly used methods for the testing of drug susceptibility of *M. tuberculosis* are resistance ratio, proportion, absolute concentration and radiometric methods. Currently, in spite of new technologies for susceptibility testing of *M. tuberculosis*, the resistance ratio (RR) method was used routinely at the National Reference Laboratory Center of the Tuberculosis Division of Thailand until 1997, then changed to the proportion (PR) method in 1998. There have been few data about agreement rates of these two conventional methods. Thereby, this study was conducted to determine the correlation of the RR and the PR methods for susceptibility testing of *M. tuberculosis* to four front-line drugs, i.e., streptomycin, isoniazid, rifampicin and ethambutol.

MATERIAL AND METHOD

Specimen collection, microscopic examination and culture

The sputum specimens were collected from newly suspected TB patients at the TB Division, Ministry of Public Health, Bangkok. These patients were over 14 years old with chest symptoms suggestive of suspected tuberculosis, and had never received anti-tuberculous drugs. All sputum specimens were processed for direct smear examination by fluorescence microscopy(10) after auramine-rhodamine staining and were cultured onto two Lowenstein-Jensen (L-J) slopes. Mycobacterial cultures were incubated at 37°C for 8 weeks and were examined weekly for growth. Positive slides were confirmed by Ziehl-Neelsen staining. Bacterial colonies were identified as *M. tuberculosis* by conventional methods(11).

Bacterial suspension for susceptibility testing

The susceptibility tests were set up within 3 weeks after colonies were visible on L-J slopes. A suspension was prepared by adding representative colonies, 1 loopful into a screw-capped tube containing 7-8 glass beads (3 mm in diameter) and 1 drop of 0.1 per cent Tween-80. This was vortexed for 30 seconds to produce a uniform suspension. A suspension was prepared in 7 ml of sterile water, and kept on the bench to let the coarse particles settle down. Then, 3 ml of the upper suspension was removed to a clean screw-capped tube, and sterile distilled water was added to adjust by standard suspension 1 mg/ml of tubercle bacilli (McFarland No. 1).

Resistance ratio method (RR)(12)

Principle

The RR method compares the resistance of the patient isolate with that of a standard laboratory strain. Parallel sets of media, containing 2-fold dilutions of the drug, are inoculated with a standard inoculum prepared from both the unknown (patient) and known (standard) strain of the tubercle bacilli. Resistance is expressed as the ratio of the minimal inhibitory concentration (MIC) of the test (patient) strain divided by the MIC of the standard strain in the same set.

Procedure

Sensitivity test was done on L-J medium. Drug-containing L-J slopes were made by adding appropriate amounts of the drugs aseptically to the medium before inspissation. The drug concentrations were as follows: streptomycin, 16 and 32 μg/ml; isoniazid, 0.5 and 1 μg/ml; rifampicin, 32 and 64 μg/ml; and ethambutol, 4 and 8 μg/ml. The medium was dispensed in 7-ml amounts in 30 ml screw-capped bottles and was inspissated once for 50 min at 85°C. Bacterial suspension was judged by McFarland No. 1, and then 1 drop of suspension from a Pasteur pipette was spread on the surface of each slope of the test. The slopes were incubated at 37°C. A reading was made at 2 weeks to give a preliminary indication of the presence of resistance strains, but the definitive reading was made at 4 weeks, and a report that a strain was susceptible should not be given earlier. For all tests, growth was defined as the presence of 20 or more colonies. The resistance ratio was the minimal concentration inhibiting growth of the test strain divided by the minimal concentration inhibiting growth of the standard strain, H37Rv, in the same set of tests.

Proportion method (PR)[13]

Principle

The PR method enables precise estimation of the proportion of organisms resistant to a given drug. Several 10-fold dilution of inoculum are planted onto both control and drug-containing media; at least one dilution should yield isolated countable (50-100) colonies. When these numbers are corrected by multiplying by the dilution of inoculum used, the total number of viable colonies observed on the control medium, and the number of mutant colonies resistant to the drug concentrations tested may be determined. The proportion of bacilli resistant to a given drug is then determined by expressing the resistant portion as a percentage of the total population tested[13].

Procedure

Tests were performed using a standard variant of the proportion method[14]. Drug-containing L-J slopes made with the critical drug concentrations for streptomycin, isoniazid, rifampicin, and ethambutol were 4, 0.2, 40, and 2 μg/ml, respectively. The control medium without drug was prepared at the same time as the drug-containing media. The standardized bacterial suspension was diluted in sterile distilled water to give six ten-fold dilutions. Of the six dilutions prepared, only two bacterial suspensions required for inoculation were the dilution 10^{-2} and 10^{-4} of bacilli for each slope. One standard loopful was inoculated onto drug-free as well as drug-containing L-J slopes. The slopes were incubated at 37°C, and the results were read on the 28th day. The colonies were counted only on the slopes seeded with the lowest inoculum that had produced growth. The average number of colonies obtained for the 2 control slopes indicates the number of culturable particles contained in the inoculum. The average number of colonies obtained for the drug-containing slopes indicates the number of resistant bacilli contained in the inoculum. The ratio between the second figure and the first indicates the proportion of resistant bacilli existing in the strain. The proportions were reported in terms of percentages. If the calculation was 1 per cent or more than interpreted resistance.

Statistical analysis

The agreement rates of the RR and PR methods were compared using Kappa analysis with SPSS version 7.5 program. Statistical comparison was performed by using the McNemar chi square test with SPSS version 7.5 program; a P of < 0.05 was considered significant.

RESULTS

During the 6-month period from February through July 1999, a total of 5,340 new patient cases had attended the Bangkok Chest Clinic. Among these patients, 726 aged more than 14 years old had been diagnosed with TB by clinical manifestation, smear AFB and radiological examination. Sputum samples from these TB patients were divided according to AFB results into 4 groups as negative, 1+, 2+, and 3+ for 285, 93, 134, and 214 samples, respectively. For culture examination, 13 of 726 samples were contaminated, 472 samples were culture positive, and 241 were culture negative. Rate of detection of *M. tuberculosis* was 60.7 per cent by AFB microscopy and 65.0 per cent by culture.

Antimicrobial susceptibility studies

Only 250 of 472 clinical isolates (53%) with biochemically confirmed *M. tuberculosis* were subjected for susceptibility studies. The susceptibilities of these strains to each drug tested by the RR and PR methods are listed in Table 1. The results

Table 1. Susceptibilities of *M. tuberculosis* (n=250) to the four front-line drugs determined by the resistance ratio (RR) and the proportion (PR) methods.

Drugs[a]	RR method		PR method	
	Susceptible	Resistant	Susceptible	Resistant
S	204	46	204	46
H	207	43	208	42
R	233	17	230	20
E	241	9	237	13

[a] Drugs were used at the following concentrations (in micrograms per milliliter, μg/ml): S (streptomycin); 16 and 32 (RR method), 4 (PR method); H (isoniazid), 0.5 and 1.0 (RR method), 0.2 (PR method); R (rifampicin), 32 and 64 (RR method), 40 (PR method); and E (ethambutol), 4 and 8 (RR method), 2 (PR method).

Table 2. Comparison between the resistance ratio (RR) and the proportion (PR) methods for susceptible and resistance of 250 clinical isolates of *M. tuberculosis* to all front-line antituberculous drugs.

RR method	PR method		Total
	Susceptible	Resistant	
Susceptible	176	10	186
Resistant	4	60	64
Total	180	70	250

K = 0.8574 P < 0.0001

of both methods were compared for the rates of susceptible and resistance of strains to all 4 drugs (Table 2). For the RR method; 186 of 250 strains (74.4%) were susceptible, and 64 of 250 (25.6%) were resistant. For the PR method; 180 of 250 strains (72.0%) were susceptible, and 70 of 250 (28.0%) were resistant. The results of susceptible and resistant rates of *M. tuberculosis* to these drugs determined by both methods were in high agreement (P<0.0001).

Drug resistance patterns

As shown in Table 3, the RR method identified 25.6 per cent of the isolates as resistant to at least one of the four drugs. Resistance to streptomycin was the highest at 5.2 per cent, while resistances to isoniazid, rifampicin and ethambutol were 3.6, 2.4 and 0.4 per cent, respectively. Resistance to one, two, three, and four drugs was observed in 11.6, 9.2, 3.2, and 1.6 per cent of the isolates, respectively. MDR was found in 4.0 per cent of the isolates. Resistance to streptomycin and others, isoniazid and others, rifampicin and others, and ethambutol and others was found in 18.4, 17.2, 6.8, and 3.6 per cent, respectively.

The PR method identified 28.0 per cent of the isolates as resistant strains. Resistance to streptomycin was the highest at 5.6 per cent, while resistances to isoniazid, rifampicin, and ethambutol were 4.4, 2.4, and 1.6 per cent, respectively. Resistance to one, two, three, and four drugs was observed in 14.0, 8.0, 5.6, and 0.4 per cent, respectively. MDR was found in 4.4 per cent of the isolates. Resistance to streptomycin and others, isoniazid and others, rifampicin and others, and ethambutol and others was found in 18.4, 16.8, 8.0, and 5.2 per cent, respectively.

Agreement between the RR and PR methods

The percentages of agreement between the RR and the PR methods for antimicrobial susceptibilities of 250 *M. tuberculosis* to streptomycin, isoniazid, rifampicin and ethambutol were 96.8, 98.0, 94.8 and 96.8 per cent, respectively. (Table 4). Correlation between both methods for determining susceptibilities of these strains to the four drugs tested is shown in Table 5. There was high agreement between both methods when tested against streptomycin, isoniazid, rifampicin and ethambutol with K = 0.893, 0.929, 0.621 and 0.620, respectively. Statistical comparison using Mc Nemar χ^2 test revealed that there was no statistically significant difference of the susceptibilities with regard to the individual drugs tested (p>0.05).

Table 3. Patterns of drug resistance of *M. tuberculosis* (n=250) determined by the resistance ratio (RR) and the proportion (PR) methods.

Pattern	No of strains			
	RR method	%	PR method	%
Resistance	64	25.6	70	28.0
1 drug	29	11.6	35	14.0
S	13	5.2	14	5.6
H	9	3.6	11	4.4
R	6	2.4	6	2.4
E	1	0.4	4	1.6
2 drugs	23	9.2	20	8.0
SH	22	8.8	16	6.4
SR	0	-	1	0.4
SE	0	-	1	0.4
HR	0	-	1	0.4
HE	0	-	0	-
RE	1	0.4	1	0.4
3 drugs	8	3.2	14	5.6
SHR	5	2.0	8	3.2
SHE	2	0.8	4	1.6
SRE	0	-	1	0.4
HRE	1	0.4	1	0.4
4 drugs	4	1.6	1	0.4
SHRE				
MDR-TB	10	4.0	11	4.4
HR	0	-	1	0.4
SHR	5	2.0	8	3.2
HRE	1	0.4	1	0.4
SHRE	4	1.6	1	0.4
S & Others	46	18.4	46	18.4
H & Others	43	17.2	42	16.8
R & Others	17	6.8	20	8.0
E & Others	9	3.6	13	5.2

S, streptomycin; H, isoniazid; R, rifampicin; and E, ethambutol.

DISCUSSION

Since drug-resistant TB has increased in incidence and interfered with TB control programs, particularly in high HIV burden areas. Monitoring of drug resistance patterns in specific locales can help to identify areas where infection control or public health interventions may be necessary to prevent MDR-TB outbreaks. So, all isolates of *M. tuberculosis* should be tested for their susceptibilities to the front-line antituberculous drugs. The results can be used as the guidance for proper treatment. The testing may be valuable for confirmation of drug resistance in patients showing unsatisfactory response to treatment, and may be useful for identifying primary and acquired drug resistance trends in a community.

Of the conventional culture-based techniques for antimycobacterial drug susceptibility testing, the resistance ratio (RR) and the proportion (PR) methods are commonly used in Thailand. The RR method was formerly used in routine susceptibility testing of *M. tuberculosis* at the Laboratory Section of the TB Division. Since 1998, the RR method has been replaced by the standardized PR method for susceptibility testing at this laboratory with monitoring of quality control by the Korea-Supranational Reference Laboratories.

To determine the correlation of the RR and the PR methods for susceptibility testing of *M. tuberculosis* to the four front-line drugs, only 250 of 472 (53%) clinical isolates were enrolled in this study. In general, the percentages of agreement

Table 4. Percentage agreement between the resistance ratio (RR) and the proportion (PR) methods for susceptibility testing of *M. tuberculosis* (n=250) to each drug tested.

Drug	No. of isolates with the following results:		Per cent Agreement
	RR method - S, PR method - S	RR method - R, PR method - R	
Streptomycin	200	42	96.8
Isoniazid	205	40	98.0
Rifampicin	225	12	94.8
Ethambutol	235	7	96.8

S; susceptible, R; resistance.

Table 5. Comparison between the resistance ratio (RR) and the proportion (PR) methods for determining susceptibility of *M. tuberculosis* (n=250) to four front-line drugs.

RR method	PR method		Total	K	p-value
	Susceptible	Resistant			
Streptomycin				0.893	1.000
Susceptible	200	4	204		
Resistant	4	42	46		
Total	204	46	250		
Isoniazid				0.929	1.000
Susceptible	205	2	207		
Resistant	3	40	43		
Total	208	42	250		
Rifampicin				0.621	0.581
Susceptible	225	8	233		
Resistant	5	12	17		
Total	230	20	250		
Ethambutol				0.620	0.289
Susceptible	235	6	241		
Resistant	2	7	9		
Total	237	13	250		

determined by both methods were high with regard to all drugs tested. Since drug susceptibility or resistance depends on the presence or absence of growth on the control and the drug containing media, the inoculum for each culture must be carefully performed. This is done by determining the growth on control cultures seeded with different dilutions of inoculum. Homogenization of the inoculum to eliminate large clumps of cells is essential. The inoculum must be heavy enough to result in at least 200 colonies on control medium to provide statistically significant data, but not so heavy that confluent growth covers the surface of the control culture[15].

However, in practice, both methods vary greatly in drug concentrations, inoculum sizes, and interpretation of the drug resistance results. Since this study was performed by using the same ino-

culum size of each isolate adjusted to McFarland No. 1 for testing by both methods at the same time, no variation in inoculum size occured. The rate of drug resistance by the RR method (25.6%) was slightly less than that of the PR method (28.0%). For the single drug resistance determined by both methods, distribution rate of resistance to all drugs, except ethambutol had no difference. Rate of resistance to ethambutol by the PR method was higher than the RR method, due to the over inoculum size on the medium or an error of the researcher. Siddiqi (16) showed that variations of results have always been a problem for *in vitro* susceptibility testing especially at the lower concentrations. Two concentrations of ethambutol were used, and high concentration had the percentage of resistance less than low concentration. Both methods showed the highest resistance to single drug, followed by two, three, and four drugs, respectively. The rate of MDR-TB in new pulmonary tuberculosis in this study was slightly different between these methods.

This *in vitro* testing showed that all frontline drugs might be effective for the treatment of newly detected pulmonary tuberculosis by short-course chemotherapy. A study of the treatment outcome of new TB patients that had one drug resistance revealed a cure of more than 90 per cent (17). Resistance was fundamentally a phenomenon linked to a large bacterial population. The far greater population of drug resistance was found in cavitary TB patients more than that of non cavitary TB patients(17).

The data of drug resistance in this present study were not compared with that of the Laboratory Section, TB Division since the study design was a cross-sectional study over a short term. There were several important factors of different susceptibility results; i.e., variation in drug stability, and preparation of inoculum size. Susceptibility testing results not only depend on the presence or absence of growth on the control and drug-containing medium, the inoculum for each culture must also be carefully controlled(13).

However, antimicrobial susceptibility test should be performed, preferably with an inexpensive and relatively simple technique. The RR method compares the MIC of the unknown strain with that of the control strain on the same batch of medium. Some workers use the H37Rv strain of *M. tuberculosis* as the control strain but the susceptibility of this strain to drugs does not parallel that of wild tubercle bacilli and may give a misleading ratio. It is better to use the modal resistance method of Marks (18). The unknown strains were compared with the modal resistance. Smooth suspensions must be used. Large clumps or rafts of bacilli gave irregular results and made reading difficult. Drug concentrations of each laboratory must be determined for their own ranges. Although this method gave variable results, it was convenient for inoculum preparation and required a shorter time. Interpretation of the result was rather simple.

For the PR method, several dilutions of the inoculum were made and media containing no drug and standard concentration of drugs were inoculated. This method was technically very difficult. There were also more risks attached to standardizing the inocula than with the RR method. However, even now there are several new methods, e.g., E test(19), Alamar blue(20), DNA probes and molecular fingerprinting(21-23), but these methods are more expensive, and some techniques require specialized equipment and highly skilled personnel, thus they are difficult for use in general laboratories although they provide results within 1-5 days.

The descriptive study of pulmonary TB during the 6 month period studied at the TB Division (data not shown) showed that most patients were less than 45 years old, the 25-34 years was the highest age group and there were more males than females. These patients were of working age, they may have migrated from rural areas to the city and this age group was the highest HIV burden group of the country. The impact of the HIV/AIDS epidemic can result in the resistance of antituberculosis agents, high mortality and delayed diagnosis. In Thailand, HIV seropositivity rate among new TB patients was another indicator reflecting a threat from HIV. Reports from the TB Division and zonal TB centers have shown an obvious increasing trend. Tuberculosis was the main opportunistic infection of hospitalized AIDS patients in Thailand with an average proportion of around 40 per cent, the highest proportion of above 60 per cent was found in Bangkok(24).

Trends of drug resistance from the report of the Laboratory Section, TB Division, from 1995 to 2000 showed an increasing trend of resistance to streptomycin, isoniazid, and rifampicin. Resistance to two drugs continued to increase, especially to streptomycin and isoniazid. Increasing initial resistance to rifampicin, and MDR-TB in TB patients did

not occur. This may be explained by the infection from another patient excreting a drug resistant organism; many of these patients have acquired resistance as a result of inadequate treatment. The reasons are that patients do not take prescribed medications with sufficient regularity and duration to achieve cure. In particular, regular intake of drugs in the initial 2 month phase is often not achieved. Other factors include drug supply and availability, financial constraints, and inappropriate treatment. WHO recommended that the strategy for improving the treatment system was to implement Directly Observe Therapy Short Course (DOTS). By using DOTS one can almost be assured that TB patients will be cured. DOTS is our only available hope for preventing drug-resistant TB from worsening to terrifying and unimaginable proportions. While there is still time, world leaders must come together to fight the TB epidemic and stop it at the source.

In conclusion, this study showed high agreement between the RR and PR methods ($P< 0.0001$) with agreement rates to streptomycin, isoniazid, rifampicin and ethambutol of 96.8, 98.0, 94.8 and 96.8 per cent, respectively. The correlation between both methods for determining susceptibilities of *M. tuberculosis* to the four front-line drugs tested was not statistically significantly different ($p>0.05$). Thus, the RR method may be substituted. However, WHO recommended the use of the PR method to be used for determining drug susceptibility of *M. tuberculosis*. The drug susceptibility testing result can be used as the guidance for proper treatment and is valuable for minimizing the spread of drug-resistant strains.

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เปรียบเทียบวิธี Resistance ratio และวิธี Proportion ในการทดสอบความไวของเชื้อวัณโรคต่อยาต้านจุลชีพ

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การศึกษาเปรียบเทียบวิธีการทดสอบความไวในห้องปฏิบัติการ 2 วิธี ในการตรวจความไวของเชื้อวัณโรคซึ่งแยกได้จากผู้ป่วยรายใหม่จำนวน 250 ราย ต่อยาต้านจุลชีพหลัก 4 ชนิด พบว่าวิธี Resistance ratio ให้ผลความไวต่อยาที่ใช้ในการทดสอบ 74.4% ในขณะที่วิธี Proportion ให้ผลความไว 72.0% วิธีทั้งสองมีความสอดคล้องกันสูงมาก (P<0.0001) โดยมีอัตราความสอดคล้องต่อยา streptomycin 96.8%, ยา isoniazid 98.0%, ยา rifampicin 94.8% และยา ethambutol 96.8% ส่วนรูปแบบในการดื้อยาพบการดื้อต่อยาหนึ่งตัวมากที่สุด รองลงไปคือการดื้อต่อยา 2 ตัว, 3 ตัว, และ 4 ตัว ตามลำดับ สำหรับการดื้อต่อยาหนึ่งตัวนั้นพบการดื้อต่อยา streptomycin มากที่สุด รองลงไปคือการดื้อต่อยา isoniazid, ยา rifampicin, และยา ethambutol ตามลำดับ ความสัมพันธ์ของวิธีการทดสอบความไวของเชื้อวัณโรค 2 วิธี โดยใช้ Mc Nemar chi-square test ต่อยาแต่ละชนิด พบว่าทั้ง 2 วิธีไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ (P>0.05) ดังนั้นวิธี Resistance ratio จึงอาจใช้แทนกันได้ อย่างไรก็ตามวิธี Proportion ยังถือเป็นวิธีมาตรฐานในการทดสอบความไวของเชื้อวัณโรคตามนโยบายการควบคุมวัณโรคขององค์การอนามัยโลก การทราบผลความไวว่าเชื้อวัณโรคมีการดื้อต่อยาที่ใช้รักษาหรือไม่จะเป็นประโยชน์อย่างยิ่งในการจำแนกชนิดของการดื้อยาเพื่อการดูแลรักษาผู้ป่วยวัณโรคได้อย่างมีประสิทธิภาพ โดยเฉพาะในผู้ป่วยวัณโรคที่มีปัจจัยเสี่ยงที่จะมีเชื้อวัณโรคดื้อยา ซึ่งจะช่วยลดปัญหาการแพร่กระจายของเชื้อสายพันธุ์ที่ดื้อยาในชุมชนได้

คำสำคัญ : เชื้อวัณโรค, วิธีการทดสอบความไว, วิธี resistance ratio, วิธี proportion

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