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Detection of Rifampicin Resistance in *Mycobacterium tuberculosis* by Using Middlebrook 7H9 Broth Medium with 2,3-Diphenyl-5-Thienyl-(2)-Tetrazolium Chloride

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Background: A simple and cost-effective method is needed for the detection of rifampicin resistance in *Mycobacterium tuberculosis* in resource-limited settings. We suggest a broth medium-based method using 2,3-diphenyl-5-thienyl-(2)-tetrazolium chloride (STC) for detection of rifampin resistance of tubercle bacilli within a reasonable time frame.
Methods: The type strain (*M. tuberculosis* H37Rv) and 45 cultured clinical strains of *M. tuberculosis* (35 rifampin-susceptible and 10 rifampin-resistant) were used. Phenotypes of rifampicin resistance were tested by the Korea Institute of Tuberculosis, and confirmed by GenoType MTBDRplus (Hain Lifescience, Germany). Susceptibility tests were performed using STC-containing OADC-enriched Middlebrook 7H9 broth (BD, USA).
Results: All tests were finished in 3 to 6 days. The same results were obtained with the standard and current methods for all 45 clinical isolates (100% sensitivity and specificity for resistance detection).
Conclusion: The current method using STC is a good alternative for detecting *M. tuberculosis* rifampin resistance in a cost-effective and timely fashion, which is particularly important in resource-limited settings. **(Ann Clin Microbiol 2018;21:47-50)**

Key Words: Drug susceptibility test, *Mycobacterium tuberculosis*, Rifampin resistance

INTRODUCTION

Tuberculosis (TB) is still one of the main infectious diseases claiming human lives with 1.3 million people in 2016 [1]. Once developed, the disease cannot be cured unless appropriate anti-TB drug administrations. Resistance to rifampicin, one of the most important drugs, is considered as a surrogate marker for multidrug-resistant (MDR)-TB because most rifampicin resistant strains simultaneously contain isoniazid resistance. In 2016, there were 600,000 new cases with resistance to rifampicin, of which 490,000 had MDR-TB [1]. Even though an appropriate drug combination was administered for the drug-resistant TB, the treatment is often less effective, and takes long time. Furthermore, when the correct diagnosis for the drug-resistant TB is not carried out in a timely fashion, ineffective drugs would be administered for some period, and the patients can spread drug-resistant tubercle bacilli to other people during inappropriate drug administration. That's why the rapid and correct detection of rifampicin resistance is so important, and Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) assay is widely used in the world. However, the assays based on the molecular biologic methods are expensive and requires special equipment to perform those tests. These tests may not be feasible in developing countries. In fact, more than 95% of TB deaths occur in low- and middle-income countries of the world, and most TB patients almost half (47%) of MDR-TB cases were in India, China and the Russian Federation [1,2].

Therefore, a method that is economic and easy-to-perform, and does not require any special equipment is necessary for resource poor settings where TB patients are more prevalent. There has been some studies about the drug susceptibility tests of mycobacteria and fungi using oxidation-reduction dye, 2,3-diphenyl-5-thienyl-(2)-tetrazolium chloride (STC; Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) [3-7]. Here, we suggest a broth

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medium-based method using STC to detect rifampin resistance of tubercle bacilli within a reasonable time frame.

MATERIALS AND METHODS

1. Tested strains

Type strain (*Mycobacterium tuberculosis* H37Rv) and 45 cultured clinical strains of *M. tuberculosis* (35 rifampin susceptible, and 10 rifampin resistant) were included. All strains except type strain have been isolated from patients in Pusan National University Yangsan Hospital from June to December, 2015. When received clinical specimens, mycobacterial cultures had been performed following a routine laboratory procedure by using BACTEC MGIT 960 System (BD, Sparks, MD, USA). After growth, the liquid culture was used to inoculate into liquid media for the detection of rifampin resistance (described below), and the remaining liquid media were sent to the Korean Institute of Tuberculosis (KIT; Osong, Korea) for phenotypic susceptibility testing. Because most strains were susceptible to rifampin, we used some known resistant strains stored in a deep freezer, which were all from clinical specimens at Pusan National University Yangsan Hospital. Stocked strains were restored in MGIT 960 System, and treated in the same manner described above. All the strains were tested with GenoType MTBDRplus (Hain Lifescience, Nehren, Germany) to detect genotypic resistance. The phenotypic and genotypic results were completely identical, so we used these results as a standard to compare the current method's results.

2. Susceptibility tests

Culture media of our method were prepared as follows. First, STC stock solution (50 mg/mL) was prepared by adding of 50 mg of STC into 1 mL of distilled water, and filter-sterilized. Second, rifampicin stock was prepared by adding 10 mg of rifampicin (Sigma, St. Louis, MO, USA) into 10 mL dimethyl sulfoxide (Junsei Chemical, Tokyo, Japan). Third, Middlebrook 7H9 broth medium with OADC enrichment (BD) was prepared by the manufacturer's instruction, and 1 L of 7H9 medium and 11 mL of STC stock solution were mixed. Test procedures were as follows. MGIT 960 tube suspension was used within 4 hours after positive culture signals have detected in the MGIT 960 machine. We mixed 900 μL of 7H9 culture medium and 100 μL of cultured suspensions in 4 sets of Eppendorf tubes for one strain. To test drug resistance, rifampin stock solution was added into 4 tubes in a different volume (20, 10, 0.5, and 0 μL) to become 2, 1, 0.5, and 0 μg/mL of rifampicin as final concentrations. All tubes were kept after capping in a 37°C incubator until black- or violet-colored precipitates were seen in the rifampin-free tube for a maximum of 8 days. When the rifampin-free tube developed dark precipitates, the other tubes

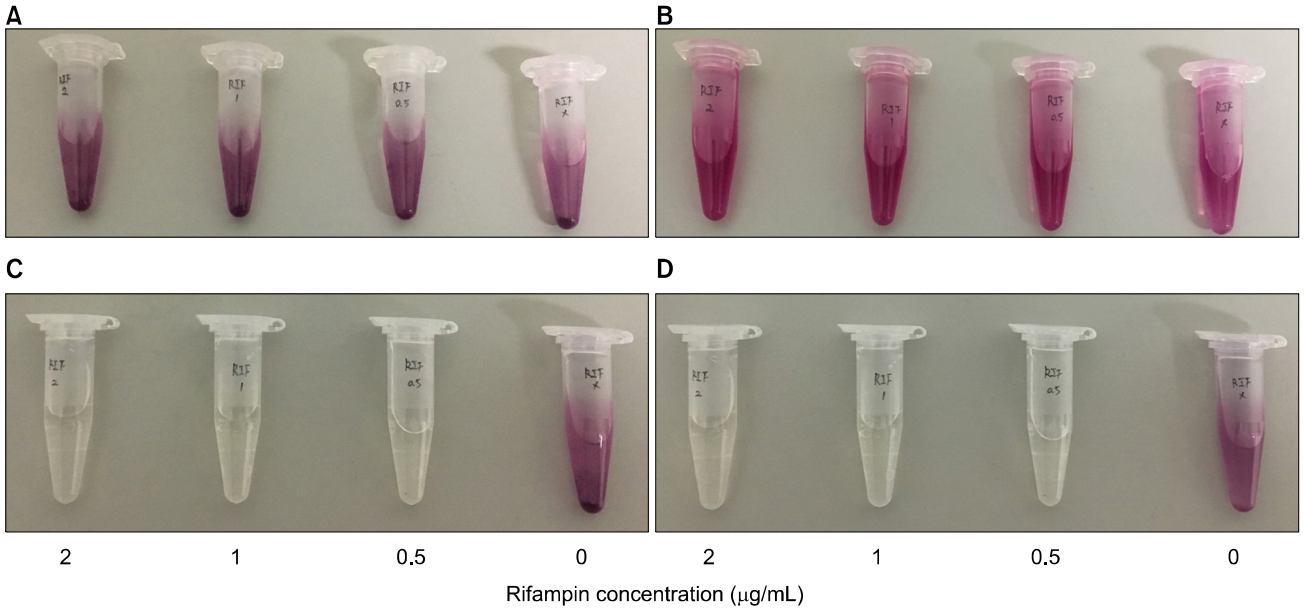

Fig. 1. Eppendorf tubes showing rifampin resistance. (A, C) shows insoluble STC precipitates, and (B, D) shows pink colored solution after adding the solubilizing agent. (A) resistant, or dark precipitates in the bottoms of each tube; (C) susceptible, or no precipitate in left 3 tubes; (B) resistant, or pink colored media; (D) susceptible, or no color change in left 3 tubes.

were observed whether the tube developed dark precipitates (growth of bacteria or drug-resistant) or not (no growth of bacteria or drug-susceptible). The dark precipitates were dissolved and the solution changed to pink color by adding 250 μL of the solubilizing agent to each tube and incubating for 2 h (Fig. 1). All tests were performed in duplicate, and we considered the test failure when dark precipitates were not found in drug-free medium after 8 days of incubation. The isolate was considered resistant when tubes containing 1 μg/mL were changed to pink color, and other tubes containing different concentrations of rifampicin were used as a reference only.

RESULTS

Test results for all strains were proved to be valid, and the drug-free media developed dark precipitated in 3 to 6 days, which means that the rifampicin resistance could be revealed in less than 1 week. Specifically, the resistance detection dates of 35 susceptible and 10 resistant strains were 4.1±0.9 days and 4.5±1.2 days, respectively (P=0.2889 in unpaired Student t-test). Among 45 clinical isolates, all 35 susceptible isolates have shown the same results in the current method (specificity of resistance detection 100%), and all 10 resistant isolates have shown the same results (sensitivity of resistance detection 100%, Table 1 and Fig. 1).

DISCUSSION

Many *in vitro* diagnostic methods using molecular technique have been developed for mycobacterial detection and identification as well as drug susceptibility testing [8,9]. The sensitivity and specificity were good enough to be used in clinical settings, and operation process is so simple. However, they cost too much to be used in TB high burden countries. We think that one important issue is that TB is much more prevalent in high burden countries, and that most of them have limitation of resources that can afford to use. Therefore, we should provide a simple, rapid and economic method to detect MDR-TB, or at least to detect rifampin-resistant TB. The current study have shown preliminary results that rifampin resistance can be detected easily. In the previous studies, it was demonstrated that STC can be used for detecting microbial growth including *M. tuberculosis*. The current method using STC is simple and economic because it uses only small volume of media and reagents. It can detect rifampicin resistance within a week. It does not require any sophisticated equipment nor technique. One problem of the current study is that the number of tested strains was small. But the results were perfect regarding the rifampin resistance. In fact, the discrimination power between resistant and susceptible strains is high for rifampicin [10]. And the current method adopted the similar protocol of the broth test method using Middlebrook 7H9 medium, except adding the STC as a color growth indicator. Therefore, the high concordance rates of the current method to the standard method is fully expected. In conclusion, the current method using STC is a good alternative for detecting rifampin resistance or predicting MDR-TB in an economic and timely fashion when drug resistance detection of *M. tuberculosis* is necessary in a resource-limited settings.

Table 1. Results of STC-based rifampin resistance detection method

Tubes of rifampin concentration (mg/mL)	No. of tubes with dark precipitates	
	Rifampin-susceptible (35)	Rifampin-resistant (10)
0	35	10
0.5	5	10
1.0*	0	10
2.0	0	10

*Benchmark concentration of defining resistance.

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=국문초록=

2,3-Diphenyl-5-Thienyl-(2)-Tetrazolium Chloride 첨가 Middlebrook 7H9 액체배지를 이용한 결핵균의 리팜핀 내성 검출

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배경: 경제적 자원이 부족한 환경에서 간단하고 저렴하게 결핵균의 리팜핀 감수성 검사를 수행할 수 있는 방법이 필요하다. 저자들은 2,3-diphenyl-5-thienyl-(2)-tetrazolium chloride (STC)를 첨가한 액체배지를 이용하여 결핵균의 리팜핀 내성을 비교적 빨리 확인할 수 있는 방법을 제안한다.

방법: 결핵균 표준균주(*Mycobacterium tuberculosis* H37Rv)와 45개의 임상균주(리팜핀 내성 35주, 감수성 10주)를 사용하였다. 통상적인 감수성검사는 결핵연구원에서 시행하였고, 내성유전자검사는 GenoType MTBDRplus (Hain Lifescience, Germany)를 이용하였다. 본 실험에서의 리팜핀 내성검사는 STC가 포함된 OADC 첨가 Middlebrook 7H9 액체배지(BD, USA)를 이용하였다.

결과: 모든 감수성검사는 3-6일 소요되었고, 모든 결과는 통상적인 감수성검사 결과와 일치하였다(내성 검출 민감도 및 특이도 100%).

결론: 본 시험법은 자원이 부족한 상황에서 신속하고 경제적으로 결핵균의 리팜핀 내성 검출을 위한 대안으로 활용할 수 있을 것이다. [Ann Clin Microbiol 2018;21:47-50]

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