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Original Article

Resazurin microtiter assay for detection of drug resistance and determination of critical concentration of cycloserine resistance of *Mycobacterium tuberculosis*

Chao Li[1,2*], Guilian Li[2*], Ruibai Wang[2], Lingyun Ji[1,2], Yongliang Lou[1], Jianxin Lu[1], Kanglin Wan[1,2]

[1]*Key Laboratory of Laboratory Medicine of Ministry of Education of The People's Republic of China, College of Laboratory Medicine and Life Sciences, Wenzhou Medical University, Wenzhou 325035, Zhejiang, China;* [2]*State Key Laboratory of Infectious Disease Prevention and Control, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China.* [*]*Equal contributors.*

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Abstract: We evaluated the feasibility of resazurin microtiter assay (REMA) for detecting cycloserine resistance of *Mycobacterium tuberculosis*. The cutoff value of the critical concentration of cycloserine resistance in vitro about *M. tuberculosis* isolates was determined innovatively by drawing the receiver operating characteristic (ROC) curve for the first time. The spending time of REMA ranged from 6 to 9 days. The ROC curve analysis showed that the area under the curve (AUC) was 0.950, and *P* value was <0.001. The critical concentration of cycloserine in vitro was >16 μg/ml based on the ROC curve. At this point, the sensitivity of REMA was 79.17%, and the specificity was 100% when compared to the proportion method (PM). Resazurin method was found to be simple, reliable and inexpensive to perform for the rapid detection of anti-tuberculous drugs.

Keywords: Cycloserine, antibiotic resistance, *Mycobacterium tuberculosis*, REMA, ROC curve

Introduction

Mycobacterium tuberculosis still remains a serious pathogen caused the tuberculosis (TB) disease which seriously threatens to the public health. China is one of 27 drug-resistant TB high-burden countries in the world [1]. It is estimated to contribute 22% of the global burden of Multi-drug resistant tuberculosis (MDR-TB) in China [2, 3]. MDR-TB is becoming more prevalent because of the mechanisms including drug-inactivating enzymes, cell wall permeability changes, targeted gene mutations and variety in metabolic pathways.

Early detection of drug resistance of *M. tuberculosis* is benefit to appropriate therapy of the TB patients, which has an important impact for the better control and management for this disease. The developments of rapid methods about drug susceptibility testing (DST) are very important due to the increasing rates of MDR-TB and the extensively drug-resistant tuberculosis (XDR-TB) described worldwide recently [4]. Evaluating and interpreting the minimum inhibitory concentration (MIC) for detecting drug sensitivity of *M. tuberculosis* are also vital to prevent the spread across nations and areas, particularly in developing countries, where people are dealed with different treatment regimens. Cycloserine is a new broad spectrum antibacterial agent and belongs to second-line anti-TB drugs suggested by World Health Organization (WHO). In recent years, chemotherapy containing cycloserine in the treatment of MDR-TB had achieved better effects in Iran and India [5, 6]. It is restricted in clinical treatment due to the complications, allergies and neurotoxicity when blood cycloserine concentration is above 30 mg/L [7]. These side effects increase the cost of treatment and also improve the difficulty of applying cycloserine reasonably. Therefore DST method is the key link of detecting cycloserine drug-resistance for MDR-TB

chemotherapy. The resazurin microtiter assay (REMA) has been used successfully to detect the drug resistance of MDR-TB to isoniazid and rifampin [8, 9].

In this study, the MIC of 145 drug-resistant *M. tuberculosis* isolates was undertaken by setting twelve different cycloserine drug concentrations. We aimed to introduce a scientific method and establish the cutoff value that could separate negative from positive results; the value, achieved with ROC curve, represented the critical concentration which could define susceptible and resistant strains [10]. Considering that there was no susceptibility interpretations of the MIC values for cycloserine, our research would contribute to determining cycloserine resistance quickly. The conventional 1% proportion method (PM) on Löwenstein-Jensen medium was used as reference test. Then drawing ROC curve with the MICs of $\log_2$ transformation to get the cutoff value, which was in the highest accordance with the PM regarded as the gold standard test method.

Material and methods

Strains

A total of 145 *M. tuberculosis* isolates were obtained from patients with pulmonary TB from provincial TB hospitals in China, including Fujian, Guizhou, Hunan, Henan, Sichuan and Xizang provinces. Drug susceptibility testing was performed using the Löwenstein-Jensen proportion method (PM); the critical concentration for the PM was described previously [11]. The *M. tuberculosis* standard strain H37Rv was included as a control.

Resazurin microtiter assay (REMA)

REMA method was carried out as described by Martin and Palomino [12]. Middlebrook 7H9 broth was prepared as manufacturer's instruction and 10% OADC (oleic acid, albumin, dextrose, and catalase) was added and mixed fully. Then, 100 µl broth was dispensed in each well of the 96-well plate. Working solution of drug was prepared at 2.56 mg/ml and serial two-fold dilutions of drug were prepared directly on the plate to make the 128-0.063 µg/ml drug concentration range of cycloserine. A total of 100 µl of diluted (1:20 ratio) bacterial suspension were added in each well. A growth control containing no drug and a sterile control without inoculum were also included for each isolate. The plates were incubated in a humid chamber at 37°C. 70 µl indicator (20 µL of 0.02% resazurin solution and 50 µL of sterile 5% Tween-80) was added to the first growth control well without drug and the plate were re-incubated for 12 h at 37°C. The change in color from blue (oxidized state) to pink (reduced state) indicated the growth of *M. tuberculosis*. When the first drug-free control well turned to pink, added the 70 µl indicator to the all tested wells corresponding isolate. If the first control well remained blue, the next drug-free control well was examined for growth and the indicator was added to all wells if the color turn was observed. The microplate was resealed and incubated for an additional 12 h at 37°C, after which all well colors were recorded. The MIC was defined as the lowest concentration of drug that prevented this change in color. *M. tuberculosis* H37Rv and the drug-free wells were used as control. Tween-80, Middlebrood 7H9 powder and OADC nutritional supplements were purchased from Difco (Detroit, MI, USA). Cycloserine was purchased from Sigma-Aldrich (St. Louis, MO, USA). All prepared drug-containing mediums were stored for not more than 2 weeks at 4°C before use. PM was performed on the Löwenstein-Jensen medium according to laboratory's standard procedure [13] and 30 µg/ml cycloserine was used according to the WHO Guidelines [14].

Statistical methods

Receiver operating characteristic (ROC) curve, was a graphical plot that illustrated the performance of a binary classifier system as its discrimination threshold was varied in statistics. The ROC curve was created by plotting the sensitivity against the 1-specificity at various threshold settings. The area under the curve (AUC) was equal to the probability that a classifier would rank a randomly chosen positive instance higher than a randomly chosen negative one [15]. The bigger the AUC was, the higher the judgment value was. Area under the curve (AUC), *P* value, and cutoff point were obtained from the curve [10]. All the MIC values were subject to $\log_2$ transformation in the study. The ROC curve was analyzed by the MedCalc Software 15.8.

Table 1. MICs of Cycloserine of the 145 *M. tuberculosis*

No. of strains	Group*	MIC (μg/ml)									
		128	64	32	16	8	4	2	1	0.5	0.25
24	CS-resistant	1	6	12	5	0	0	0	0	0	0
121	CS-susceptible	0	0	0	58	43	16	3	1	0	0

*Grouping according to the PM results; CS = cycloserine; critical concentration of PM was for 30 μg/ml.

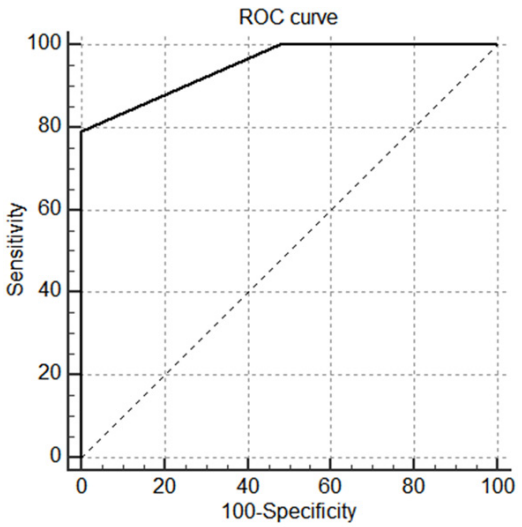

Figure 1. Receiver operating characteristic (ROC) curve for cycloserine determination; area under the curve (AUC) = 0.950, P<0.001, Standard Error = 0.02, Youden index J = 0.79.

Results

MICs of cycloserine

The MICs of 145 drug-resistant clinical isolates were shown in **Table 1**. According to the PM test, 24 isolates were resistant to cycloserine and 121 isolates were susceptible. Among the resistant strains, the MICs of 19 isolates determined by REMA were >16 μg/ml, including 32 μg/ml for 12 isolates, 64 μg/ml for 6 isolates, and 128 μg/ml for 1 isolates. Among the other 121 cycloserine sensitive strains, the MICs were 16 μg/ml for 58 isolates, 8 μg/ml for 43 isolates, 4 μg/ml for 16 isolates, 2 μg/ml for 3 isolates, 1 μg/ml for 1 isolate.

ROC curve analysis

ROC curve analysis (**Figure 1**) showed an AUC of 0.950, a *P* value of 0.000. The corresponding critical concentration of cutoff value was >16 μg/ml. On the basis of the critical concentration, the sensitivity and specificity of REMA were 79.17% and 100% to PM (**Table 2**), respectively. For all of the 145 strains, the REMA took about 6 to 9 days, and PM needed 30 days.

Discussion

At present the development of better and faster DST methods is an urgent priority for dealing with drug-resistant *M. tuberculosis,* especially for second-line drugs with the spread of MDR TB and XDR TB in the world. The goal of DST is to provide a better management and treatment of the patients infected with *M. tuberculosis*. The DST can also decrease the risk of disease and possible amplification of drug resistance. The methods, such as the PM and the BACTEC MGIT 960 system [16, 17], are currently available for DST of *M. tuberculosis*. However the former is cheap but time-consuming, the latter is fast but too costly to be applicable in most high incidence TB areas. There is obviously a great need for faster methods for DST of *M. tuberculosis*.

Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) was a blue dye; it was usually available commercially as the sodium salt. Resazurin was used as an oxidation-reduction indicator in cell viability assays for bacteria [18], a colorimetric liquid culture-based drug susceptibility assay for *M. tuberculosis* as well. In our study, the DST of cycloserine of 145 *M. tuberculosis* isolates was performed by REMA. The REMA was very similar to the Alamar blue assay and the correlation with the PM was perfect [4, 19-21]. Resazurin has been identified as the main component of Alamar blue [22]. The REMA plate method has proven to be, in recent experience, a reliable method for the detection of MDR TB [23]. And the method had been endorsed by the World Health Organization [23]. Resazurin was very cheap, reducing the cost of the DST. Each strain called for $2 by our rough estimate, which meant that resazurin would be easily extended in some low-resource countries. Results were easily determined visually by reading the change to a stable color from blue to pink. The time was required between 6 and 9 days. No special equipment was required to perform REMA, giving the opportunity for its widespread application again. It owned an advantage that it needn't be absorbed by the

Table 2. Sensitivity and specificity of REMA for Cycloserine susceptibility testing of *Mycobacterium tuberculosis*

REMA	Proportional method (PM)		Sensitivity	Specificity
	Resistant	Susceptible		
Resistant	19	0	79.17%	100%
Susceptible	5	121		

Note: REMA = Resazurin microtiter assay; the critical concentration of Cycloserine resistance was >16 μg/ml.

bacterial cell [24]. One disadvantage, however, was biosafety, since the plates required the use of liquid medium and may generate aerosols.

In our study, an innovative method was established that critical concentration was analyzed by the area under the ROC curve. And the closer the curve followed the left-hand border and the top border of the ROC space, the more accurate the test was. The following was a rough guide for classifying the accuracy of a diagnostic test based on area under the ROC curve: 1-0.9 = excellent; 0.9-0.8 = good; 0.8-0.7 = fair; 0.7-0.6 = poor; and 0.6-0.5 = fail [15]. Our research revealed that ROC curve analysis showed an AUC of 0.95, a *P* value of 0.000. According to this, it could be considered that REMA owed the high diagnostic value, which was a reliable method for determination of drug-susceptibility. The ROC curve could show this cutoff value, of which corresponding concentration was with the highest sensitivity and specificity compared with the PM as gold standard method. It was >16 μg/ml by the ROC curve analysis in this study. The result represented the "critical concentration" that defined susceptible and resistant strains based on the best fit of the colorimetric results with the conventional method. The sensitivity and specificity of REMA were separately 79.17% and 100% against the PM. And Youden index J was 0.79. The accuracy of the REMA method was very good. And notably, the REMA was a slightly complicated to operate and considering the long time for cell culture, it may happen bacterial contamination, as a result of repeated tests.

Conclusion

In summary, the REMA had been found to be a rapid, simple, and inexpensive technique for the detection of drug resistance, particularly in low-income countries. Our proposed cut-off value as 16 μg/ml could serve as an important reference to determining cycloserine resistance. The study introduced the ROC curve method of reporting critical concentration, which was with innovation. We hoped that it was adopted and popularized by authority.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Kanglin Wan, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, 155, Changbai Road, Changping District, Beijing 102206, China. Tel: 0086 10 589-00779; Fax: 0086 10 58900774; E-mail: wankanglin@icdc.cn

References

[1] Organization WH. Global tuberculosis control: WHO report 2010. World Health Organization, 2010.

[2] Mokrousov I, Jiao WW, Sun GZ, Liu JW, Valcheva V, Li M, Narvskaya O and Shen AD. Evolution of drug resistance in different sublineages of Mycobacterium tuberculosis Beijing genotype. Antimicrob Agents Chemother 2006; 50: 2820-2823.

[3] Zhao Y, Xu S, Wang L, Chin DP, Wang S, Jiang G, Xia H, Zhou Y, Li Q, Ou X, Pang Y, Song Y, Zhao B, Zhang H, He G, Guo J and Wang Y. National survey of drug-resistant tuberculosis in China. N Engl J Med 2012; 366: 2161-2170.

[4] Coban AY, Akbal AU, Uzun M and Durupinar B. Evaluation of four colourimetric susceptibility tests for the rapid detection of multidrug-resistant Mycobacterium tuberculosisisolates. Mem Inst Oswaldo Cruz 2015; 110: 649-654.

[5] Prasad R, Verma S, Sahai S, Kumar S and Jain A. Efficacy and safety of kanamycin, ethionamide, PAS and cycloserine in multidrug-resistant pulmonary tuberculosis patients. Indian J Chest Dis Allied Sci 2006; 48: 183-6.

[6] Masjedi M, Tabarsi P, Chitsaz E, Baghaei P, Mirsaeidi M, Amiri M, Farnia P, Javanmard P, Mansouri D and Velayati A. Outcome of treatment of MDR-TB patients with standardised regimens, Iran, 2002-2006. Int J Tuberc Lung Dis 2008; 12: 750-755.

[7] Nitsche MA, Jaussi W, Liebetanz D, Lang N, Tergau F and Paulus W. Consolidation of human motor cortical neuroplasticity by D-cycloserine. Neuropsychopharmacology 2004; 29: 1573-1578.

[8] Gabrielson J, Hart M, Jarelöv A, Kühn I, McKenzie D and Möllby R. Evaluation of redox indicators and the use of digital scanners and spectrophotometer for quantification of microbial growth in microplates. J Microbiol Methods 2002; 50: 63-73.

[9] Palomino J, Martin A and Portaels F. Rapid colorimetric methods for the determination of drug resistance in Mycobacterium tuberculosis. Res Adv Antimicrob Agents Chemother 2004; 4: 29-38.

[10] McNeil BJ and Hanley JA. Statistical approaches to the analysis of receiver operating characteristic (ROC) curves. Med Decis Making 1984; 4: 137-150.

[11] Barrera L, Cooreman E, de Dieu Iragena J, Drobniewski F, Duda P, Havelkova M, Hoffner S, Kam KM, Kim SJ, Labelle S, Lambregts K, Leimane V, Nunn P, Ramsay A, Raviglione M, Rich M, Ridderhof J, Rodrigues F, Rüsch-Gerdes S, Salfinger M, Scholten J, Selvakumar N, Shinnick T, Shul'gina M, Šķenders G, Sloutsky A, Small P, Van Deun A, Varaine F, Yagui M, Vincent V, Weyer K, Wright A, Zignol M. Policy guidance on drug-susceptibility testing (DST) of second-line antituberculosis drugs. Geneva: World Health Organization; 2008.

[12] Martin A, Camacho M, Portaels F and Palomino JC. Resazurin microtiter assay plate testing of Mycobacterium tuberculosis susceptibilities to second-line drugs: rapid, simple, and inexpensive method. Antimicrob Agents Chemother 2003; 47: 3616-3619.

[13] Organization WH. WHO/IUATLD Global Working Group on anti-tuberculosis drug resistance surveillance. Guidelines for surveillance of drug resistance in tuberculosis. 1996.

[14] Updated interim critical concentrations for first-line and second-line DST (as of May 2012) http://www.stoptb.org/wg/gli/assets/documents/Updated%20critical%20concentration%20table_1st%20and%202nd%20line%20drugs.pdf.

[15] Fawcett T. An introduction to ROC analysis. Pattern Recognition Letters 2006; 27: 861-874.

[16] Zhao LL, Xia Q, Lin N, Liu B, Zhao XQ, Liu Z and Wan KL. Evaluation of BACTEC MGIT 960 system for the second-line drugs susceptibility testing of Mycobacterium tuberculosis in China. J Microbiol Methods 2012; 91: 212-214.

[17] Said HM, Kock MM, Ismail NA, Baba K, Omar SV, Osman AG, Hoosen AA and Ehlers MM. Comparison between the BACTEC MGIT 960 system and the agar proportion method for susceptibility testing of multidrug resistant tuberculosis strains in a high burden setting of South Africa. BMC Infect Dis 2012; 12: 369.

[18] Anoopkumar-Dukie S, Carey JB, Conere T, O'sullivan E, van Pelt FN, Allshire A. Resazurin assay of radiation response in cultured cells. Br J Radiol 2005; 78: 945-947.

[19] Patil SS, Mohite ST, Kulkarni SA and Udgaonkar US. Resazurin tube method: rapid, simple, and inexpensive method for detection of drug resistance in the clinical isolates of mycobacterium tuberculosis. J Glob Infect Dis 2014; 6: 151-156.

[20] Rivoire N, Ravololonandriana P, Rasolonavalona T, Martin A, Portaels F, Ramarokoto H and Rasolofo Razanamparany V. Evaluation of the resazurin assay for the detection of multidrug-resistant Mycobacterium tuberculosis in Madagascar. Int J Tuberc Lung Dis 2007; 11: 683-688.

[21] Coban AY, Cekic Cihan C, Bilgin K, Uzun M, Akgunes A, Cetinkaya E and Durupinar B. Rapid susceptibility test for Mycobacterium tuberculosis to isoniazid and rifampin with resazurin method in screw-cap tubes. J Chemother 2006; 18: 140-143.

[22] O'Brien J, Wilson I, Orton T and Pognan F. Investigation of the Alamar blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur J Biochem 2000; 267: 5421-5426.

[23] Palomino JC, Martin A, Camacho M, Guerra H, Swings J and Portaels F. Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in Mycobacterium tuberculosis. Antimicrob Agents Chemother 2002; 46: 2720-2722.

[24] Mann CM and Markham JL. A new method for determining the minimum inhibitory concentration of essential oils. J Appl Microbiol 1998; 84: 538-544.