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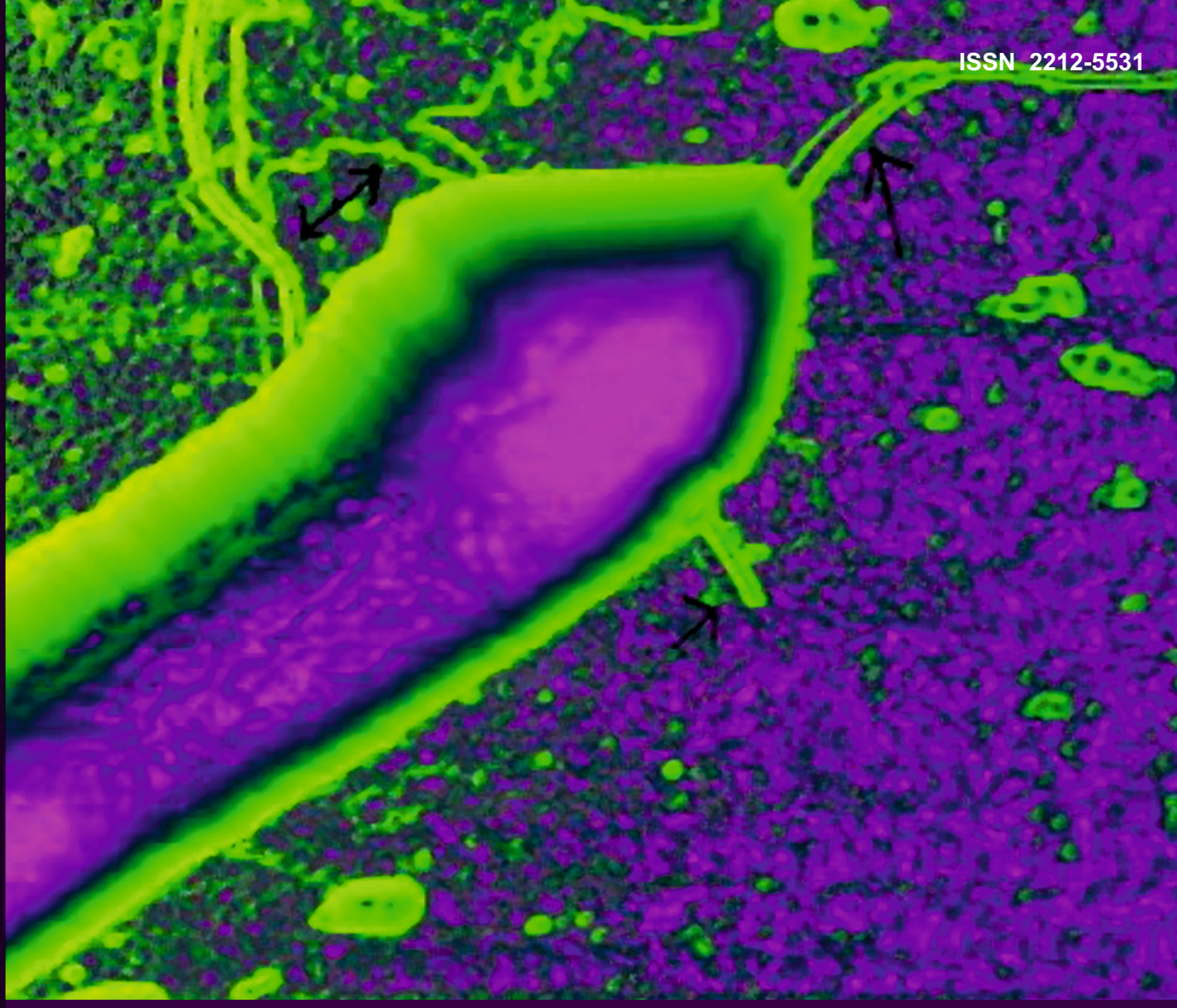

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Crystal Violet Decolorization Assay for Rapid Detection of Multidrug-resistant *Mycobacterium tuberculosis* Isolates: A Multicenter Study

Ahmet Yilmaz Coban[1], Gumral Alakbarova[2], Luqman Satti[3], Nadia Tayyab[3], Gohar Zaman[3], Nora Morcillo[4], Belen Imperiale[5], Ingrid Wainmayer[6], Norberto Simboli[6]

[1]Department of Nutrition and Dietetics, Faculty of Health Sciences, Akdeniz University, Antalya, Turkey, [2]Thalassemia Center, Medical Microbiology Laboratory, AZ1072 Baku, Azerbaijan, [3]Department of Microbiology, Armed Forces Institute of Pathology, Rawalpindi, Pakistan, [4]Laboratory Department, Mycobacteriology Laboratory, Dr. Cetrangolo Hospital, Florida, Buenos Aires Province, [5]Institute of Experimental Medicine (IMEX-CONICET)-National Academy of Medicine, [6]Mycobacteria Service, INEI ANLIS "Dr. Carlos G. Malbran," Buenos Aires, Argentina

Abstract

Background: Effective control of tuberculosis is achieved by early diagnosis and drug susceptibility testing for initiation of appropriate treatment. The performance of crystal violet decolorization assay (CVDA) for susceptibility testing of *Mycobacterium tuberculosis* to isoniazid (INH) and rifampicin (RIF) was compared in a multicenter study. **Methods:** Seventy-two *M. tuberculosis* isolates were tested in two phases by CVDA. **Results:** In Phase I, the specificity, sensitivity, positive predictive value (PPV), negative predictive value (NPV), and agreement for INH were 100%, respectively. Specificity, sensitivity, PPV, NPV, and agreement for RIF were 98.2%, 100%, 94.1%, 100%, and 98.6%, respectively. In Phase II, specificity, sensitivity, PPV, NPV, and agreement were 98%, 100%, 95.4%, 100%, and 98.6% for INH, respectively. Specificity, sensitivity, PPV, NPV, and agreement for RIF were 96.3%, 88.2%, 88.2%, 96.3%, and 94.4%, respectively. Results in the study were obtained on average 10.9 ± 3.1 days in Phase I and 9.8 ± 2.2 days in Phase II. **Conclusion:** CVDA can be performed for drug susceptibility testing in developed and developing countries. In addition, further studies with larger sample size are needed for evaluation of this method.

Keywords: Crystal violet decolorization assay, isoniazid, multidrug resistant, *Mycobacterium tuberculosis*, rifampicin

Introduction

Tuberculosis (TB) is still one of the most important infectious diseases in the world. Effective control of TB is achieved by early diagnosis and drug susceptibility testing for initiation of appropriate treatment.[1] Following streptomycin (STR) introduction as an anti-TB agent in the late 1940s, drug-resistant *Mycobacterium tuberculosis* was reported in a very short time. Even drug-resistant TB was not taken into consideration until multidrug-resistant-TB (MDR-TB) showed a big explosion in the USA and Europe 1990s.[2] MDR-TB is defined as TB resistant at least to both isoniazid (INH) and rifampicin (RIF). MDR-TB compared drug-susceptible TB is a global health threat results from treatment and diagnosis difficulties.[3] Accurate and rapid diagnosis of drug-resistance TB can provide early initiation of effective treatment and therefore reduce the spread of drug resistance and improve healing rate. Early and rapid diagnosis of TB and MDR-TB is a global priority.[4] Colorimetric assays are based on the principle of color change of the indicator dye added to antibiotic-containing and antibiotic-free medium. *M. tuberculosis* bacilli change the color of medium by metabolizing the dye during growth. When compared with conventional antibiotic susceptibility testing, these assays give rapid and reliable results for detecting resistance.[5] One of these tests is the crystal violet decolorization assay (CVDA) based on the loss of color due to the reduction and sequestering the dye by living bacilli

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Address for correspondence: Prof. Dr. Ahmet Yilmaz Coban, Department of Nutrition and Dietetics, Faculty of Health Sciences, Akdeniz University, Antalya, Turkey.
E-mail: cobanay2003@gmail.com

ORCID:
Ahmet Yilmaz Coban: 0000-0002-8815-6063

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in the medium.[1] In this multicenter study, we compared the performance of CVDA for susceptibility testing of *M. tuberculosis* to INH and RIF.

Methods

Study centers

The study was performed in 4 centers. A total of 24 isolates (19 clinical isolates and 5 standard strains) were tested at C1. Only one of these isolates was resistant to INH. All isolates were sensitive to RIF. In addition, five reference strains were used as controls in C1. In C2, 20 isolates were tested, including 6 multidrug resistant (MDR), 2 INH resistant, and 11 INH and RIF susceptible isolates. One isolate excluded from the study due to contamination.

A total of 21 isolates and the reference strain H37Rv were tested in C3. One INH resistant and 1 RIF resistant isolate were excluded from the study due to lack of growth and a total of 19 isolates including 8 MDR, 1 INH resistant, and 10 susceptible isolates were evaluated.

In the C4, 2 MDR isolates, 1 INH resistant isolate, 6 susceptible isolates, and H37Rv reference strain were tested. This multicenter study consisted of two phases (Phase I and II). The reference test method was Bactec Mycobacteria Growth Indicator Tube (MGIT) 960 in all centers. Ethical approval was not required for the study.

Preparation of crystal violet

Crystal violet (CV) stock solution was prepared in sterile distilled water and sterilized by filtration. Final concentration was adjusted to 25.0 μg/ml and solution kept on +4°C until used.[1,6]

Preparation of bacterial inoculums

Bacterial inoculums were prepared from freshly grown on Lowenstein–Jensen media and the turbidity of the supernatant was adjusted to a McFarland No. 1 standard.[1,6]

Preparation of test tubes with and without drugs for Phase I

In Phase I, all tubes were prepared as previously described.[6] Briefly, after preparation, 1 mL of 7H9S broth was dispensed into screw cap tubes. For each isolates, 3 tubes (tube 1; 0.125 μg/mL INH, tube 2; 0.50 μg/mL RIF and tube 3; growth control) were used. All tubes were stored at +4°C until use (not exceed 1 month).

Preparation of test microplates for Phase II

All tests were performed in 96-well microtiter plates. All wells were filled with 0.1 mL of Middlebrook 7H9S broth. Antibiotic test concentrations were prepared by the serial two-fold dilution. Seven dilutions of each antibiotic and a growth control well were prepared for each isolate. The antibiotics concentrations were 2.00–0.03 μg/mL for INH and RIF, except C3 (2.00–0.06 μg/mL).All prepared microtiter plates were stored at −80°C until use.[7]

Performing the test

Phase I: An INH test tube (0.125 μg/mL), a RIF test tube (0.50 μg/mL), and a drug-free growth control tube were used for each isolate. Fifty microliters of a bacterial suspension (McFarland no 1) was inoculated into the three tubes and the tubes were be incubated at 37°C. On the 5th or 7th day of incubation, 100 μl of CV stock solution (25.0 μg/mL) was then added to all tubes and incubated for an additional 24–48 h. As CV (blue/purple) was decolorized by the growth of bacteria, the isolates were considered to be resistant to that drug if the color of CV was lost. If a color did not decolorize in the growth control tube, incubation was prolonged until decolorization [Figure 1]. Decolorization was measured the complete disappearance of blue color.[6]

Phase II: The bacterial suspension (McFarland no 1) was diluted at a 1:10 ratio and 100 μl of bacterial suspension was inoculated into each well. After bacterial inoculation, all plates were incubated at 37°C. On the 5th or 7th day of incubation, 25.0 μL of CV stock solution (25.0 mg/L) was added into all wells. After that incubation was continued until decolorization in the growth control well. Minimum inhibitory concentration (MIC) was defined as the lowest drug concentration without decolorization. If the MIC value was over the breakpoint value, the isolate was considered to be resistant to tested antibiotic [Figure 2]. Breakpoints values were 0.125 and 0.5 μg/mL for INH and RIF, respectively.[7]

Results

C1: A total of 24 *M. tuberculosis* isolates were tested, including five reference strains and 19 clinical isolates. Tested reference strains of *M. tuberculosis* were ATCC 35822 (INH resistant), ATCC 35838 (RIF resistant), ATCC 35820 (STR resistant), ATCC 35837 (ethambutol [ETM] resistant), and H37Rv (susceptible to anti-TB drugs). Susceptibility testing of the isolates with primary anti-TB drugs was performed by Bactec MGIT 960 as reference method, and one of these clinical isolates was resistant to INH, and the remaining 18 isolates were susceptible to INH and RIF.

In Phase I, ATCC 35822 *M. tuberculosis* and one clinical isolate were resistant to INH by both CVDA and reference

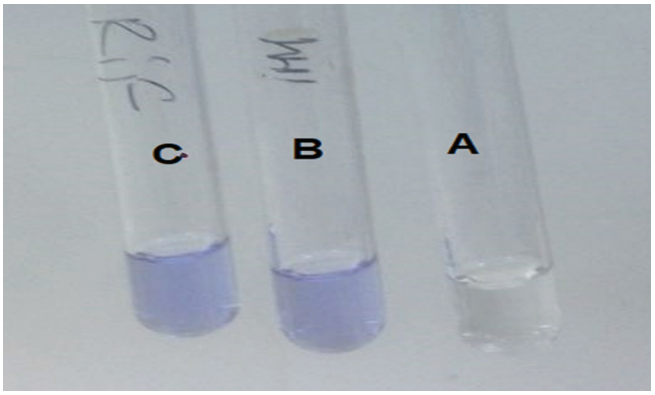

Figure 1: (A) Growth control (decolorized). (B) Susceptible to isoniazid (not decolorize). (C) Susceptible to rifampicin (not decolorize)

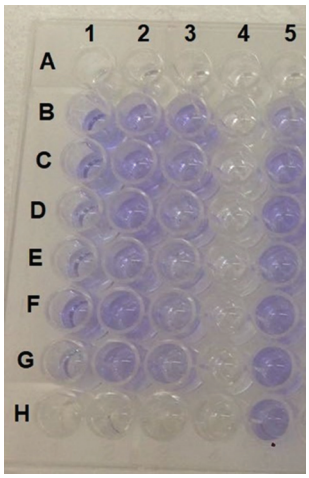

Figure 2: A1-5: Growth control (decolorized). Isolate 1, 2, and 3: Minimum inhibitory concentration value is G1, 2 and 3 (0.06 μg/ml). Isolate 4: Minimum inhibitory concentration value is ≥2.00 μg/ml (resistant). Isolate 5: Minimum inhibitory concentration value is ≤0.03 μg/ml

method. Others were identified susceptible to INH and RIF by both methods. The results were obtained on average of 10 ± 0.78 days (9–11 days).

In Phase II, MIC values of INH in ATCC 35838, ATCC 35820, ATCC 35837, and H37Rv reference strains were ≤0.03 μg/mL; MIC of ATCC 35822 strain was ≥2.00 μg/mL. MIC values were determined ≤0.03 μg/mL in 10 isolates, 0.06 μg/mL in 5 isolates, 0.125 μg/mL in 3 isolates, and ≥2.00 μg/mL in 1 isolate. The MIC value of RIF for ATCC 35822, ATCC 35820, and H37Rv standard strains were 0.06 μg/mL; ≤0.03 μg/mL for ATCC 35837; and ≥2.00 μg/mL for ATCC 35838. The MIC values for RIF were found as ≤0.03 μg/mL for 5 isolates; 0.03 μg/mL for 1 isolate; 0.06 for 5 isolates; and 0.125 μg/mL for 8 isolates. Results were obtained on the 8th day except for the ATCC 35838 strain that was obtained on the 10th day.

C2: Although 20 isolates were tested; 1 isolate was excluded from the study due to contamination. Drug susceptibility testing by MGIT 960 showed 6 MDR-TB isolates, 2 resistant to INH, and the remaining 11 isolates were susceptible to both drugs.

In Phase I, 11 isolates were susceptible to INH and RIF by both methods. Two isolates were found resistant to INH and susceptible to RIF in two methods. Five out of six MDR isolates were defined MDR by CVDA. One isolate found RIF susceptible by CVDA whereas it was resistant by MGIT 960. The results were obtained on average 10.1 ± 1.32 days (9–13 days).

In Phase II, MIC values of INH were ≤0.03 μg/mL for 4 isolates, 0.03 μg/mL for 4 isolates, 0.06 μg/mL for 3 isolates, 0.50 μg/mL for 2 isolates, 1.00 μg/mL for 2 isolates, 2.00 μg/mL for 2 isolates, and ≥2.00 μg/mL for 2 isolates. MIC values of RIF were detected as ≤0.03 μg/mL for 4 isolates, 0.03 μg/mL for 2 isolates, 0.06 μg/mL for 5 isolates, 0.50 μg/mL for 2 isolates, 2.00 μg/mL for 2 isolates, and ≥2.00 μg/mL for 4 isolates. The results were obtained on average 12.05 ± 1.5 days (9–14 days).

C3: A total of 21 isolates and the H37Rv reference strain were tested, but decolorization was not observed in two isolates and they were excluded from the study due to lack of growth in Phase I and Phase II. For that reason, 19 isolates were evaluated in the study.

In Phase I, 8 MDR isolates, 1 INH resistant isolates, and 10 drug-susceptible isolates were tested. The results obtained by CVDA for INH and RIF were found in full agreement with the reference method MGIT 960. Results were obtained on average 13.8 ± 4.7 days (7–24 days) in the study.

In Phase II, MIC value of INH were found ≤0.06 μg/mL for 10 isolates, 0.125 μg/mL for 1 isolate, 0.25 μg/mL for 2 isolates, 0.50 μg/mL for 2 isolates, 1.00 μg/mL for 1 isolate, and ≥2.00 μg/mL for 3 isolates. One isolate was determined as resistant to INH by MGIT 960, whereas it was susceptible by CVDA (MIC value was 0.125 μg/mL). MIC values of RIF were determined as ≤0.06 μg/mL in 11 isolates, 0.50 μg/mL in 2 isolates, 1.00 μg/mL in 2 isolates, and ≥2.00 μg/mL in 4 isolates. Two isolates were found resistant to RIF by MGIT 960, but it was susceptible by CVDA (MIC values were 0.50 μg/mL). Results were obtained on average 10.3 ± 2.74 days (6–13 days).

C4: A total of 9 clinical isolates and one standard strain were tested. MGIT 960 was used as reference method for susceptibility testing of all isolates. Two isolates were resistant to both INH and RIF and one isolate was resistant to INH.

In Phase I, the results of the tested isolates were concordant for INH and RIF. Two isolates were resistant to INH and RIF and one isolate was resistant only to INH by CVDA and MGIT 960. The other 6 isolates were susceptible to both drugs. In Phase I, results were obtained on the 9th day.

In Phase II, MIC values of INH were 0.015 μg/mL for 5 isolates, 0.03 μg/mL for 2 isolates, 1.00 μg/mL for 2 isolates, and 2.00 μg/mL for 1 isolate. All results were concordant with MGIT 960. MIC values of RIF were 0.015 μg/mL for 1 isolate, 0.06 μg/mL for 1 isolate, 0.125 μg/mL for 2 isolates, 0.25 μg/mL for 1 isolate, 0.50 μg/mL for 1 isolate, 1.00 μg/mL for 2 isolates, 2.00 μg/mL for 1 isolate, and ≥2.00 μg/mL for 1 isolate. Two isolates were susceptible by MGIT 960, whereas they were determined as resistant by CVDA (MIC values were 1.00 and 2.00 μg/mL). In Phase II, results were obtained on the 9th day.

Evaluation of the results from all centers

In Phase I, the specificity, sensitivity, positive predictive value (PPV), negative predictive value (NPV), and agreement for INH were 100%, whereas they were 98.1%, 100%, 94.4%, 100%, and 98.6% for RIF, respectively [Table 1].

In Phase II, specificity, sensitivity, PPV, NPV, and agreement for INH were 98%, 100%, 95.4%, 100%, and 98.6%, whereas they were 96.3%, 88.2%, 88.2%, 96.3%, and 94.4%, for RIF,

Table 1: Comparison of crystal violet decolorization assay and reference method in Phase I

Drug	CVDA	Reference method (BACTEC MGIT 960)		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Agreement (%)
		R	S					
INH	R	22	0	100	100	100	100	100
	S	0	50					
RIF	R	16	0	100	98.2	94.1	100	98.6
	S	1	55					

PPV: Positive predictive value, NPV: Negative predictive value, INH: Isoniazid, RIF: Rifampicin, CVDA: Crystal violet decolorization assay, R: Resistant, S: Susceptible

Table 2: Comparison of crystal violet decolorization assay and reference method in Phase II

Drug	CVDA	Reference method (BACTEC MGIT 960)		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Agreement (%)
		R	S					
INH	R	21	0	100	98	95.4	100	98.6
	S	1	50					
RIF	R	15	2	88.2	96.3	88.2	96.3	94.4
	S	2	53					

PPV: Positive predictive value, NPV: Negative predictive value, INH: Isoniazid, RIF: Rifampicin, CVDA: Crystal violet decolorization assay, R: Resistant, S: Susceptible

respectively [Table 2]. Results were obtained on average 10.9 ± 3.1 days in Phase I and 9.8 ± 2.2 days in Phase II.

Discussion

Especially in low- and middle-income countries, TB is still a major infectious disease with high morbidity and mortality.[8] In recent years, an increase in the prevalence of multidrug and extensively drug-resistant TB has complicated TB control.[9]

Accurate, reliable, and rapid culture and susceptibility testing are prerequisites for a successful treatment regimen. Rapid detection of drug susceptibility acts as an important factor to prevent the spread of resistant isolates.[10]

Due to the increasing number of MDR-TB cases in last years, there is an urgent need of rapid, reliable, and inexpensive drug susceptibility testing methods. Colorimetric-based methods are faster than standard culture methods and it is also less expensive than molecular methods.[9] Many colorimetric methods such as the resazurin microtiter assay (REMA) or the resazurin tube assay, the malachite green decolorization assay (MGDA), the nitrate reductase assay (NRA), and the CVDA have been developed. These methods are reliable, rapid, inexpensive, safe and repeatable.[11-18]

The first study of CVDA for antibiotic susceptibility testing was performed in 2014 by Coban[6] and has been validated for INH and RIF susceptibility testing. The sensitivity, specificity, PPV, and NPV for INH were 92.5%, 96.4%, 96.1%, 93.1%, and 94.5% whereas they were 88.8%, 100%, 100%, 94.8%, and 96.3% for RIF, respectively. The results were obtained within 8–9 days. The study concluded that CVDA was a rapid, simple, and inexpensive method for detection *M. tuberculosis* INH and RIF resistance in developing countries.[6]

Coban *et al*.[11] comparatively evaluated REMA, MGDA, microplate NRA, and CVDA for the rapid detection of MDR-TB. Specificity, sensitivity, PPV, NPV, and agreement were 100%, 95%, 100%, 96.7%, and 98% for the INH and 100%, 94.1%, 100%, 97%, and 98% for RIF, respectively.

Coban *et al*.[7] evaluated the CVDA to determine the MIC of primary anti-TB drugs. It was reported that sensitivity, specificity, PPV, NPV, and agreement were 96.3%, 100%, 100%, 96.3%, and 98.1% for INH; 91.3%, 100%, 100%, 93.7% and 96.2% for STM; and 100%, 97.6%, 90.9%, 100%, 98.1% for EMB, respectively. Moreover, all were 100% for RIF. The total agreement for the four antibiotics was obtained 98.1% and mean time to obtain the results was 9.5 ± 0.89 days.[7]

Recently, 11 centers participated in a multicenter study.[1] This study was performed in two phases. In Phase I, Center 1 prepared the test tubes containing INH and RIF and drug-free growth control tubes. These tubes were sent to all centers for performing drug susceptibility testing. The centers inoculated the bacteria into the tubes according to test procedure, and subsequently, they sent the tested bacteria to Center 1. The isolates were again tested with the same procedure in Center 1. Agreements were 96.2%–96.8% for INH and 98.1%–98.7% for RIF in the Phase I and II, respectively. Mean time to obtain the results was 14.3 ± 5.4 days in Phase I and 11.6 ± 3.5 days in Phase II. The study concluded that CVDA is a rapid, safe, and inexpensive method and could be used for rapid detection of MDR-TB. In addition, it was emphasized that it could be adapted for drug susceptibility testing in developed and developing countries.[1]

Multicenter study, especially in the determination of the reproducibility and reliability of the newly developed methods, has great importance. Especially, if the results of all centers are defined as the same or very close, they

provide important information regarding the reliability and application of the method. This is the second multicenter study since CVDA was developed. Coban *et al*.[1] performed the first study and the agreements were generally above 90% in the study. This study is a multicenter international study, in which the four centers participated in. The agreements were found to be over 90%. In this study, agreements were determined 100% for INH and 98.6% for RIF in Phase I. In Phase II, agreements were 98.6% for INH and 94.4% for RIF, respectively.

Conclusion

CVDA is a rapid, safe, inexpensive, and repeatable method. This method can be performed for susceptibility testing in developed and developing countries. However, further studies are needed with more centers and isolates.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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