Original Article

Colorimetric Methods for Rapid Determination of Pyrazinamide Resistance

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Abstract

Background: Because pyrazinamide (PZA) is only effective for *Mycobacterium tuberculosis* at an acidic pH, susceptibility tests are more difficult to perform than those for other anti-tuberculosis (TB) drugs. The purpose of our work was to investigate the effectiveness of colorimetric methods to detect PZA susceptibility and to detect pncA gene mutations in resistant isolates by sequence analysis. **Methods:** In this study, 30 clinical isolates and 2 reference isolates were used, 15 of which were resistant to PZA. The PZA susceptibility of all the isolates was determined by the BACTEC MGIT 960 reference method. As colorimetric methods, Resazurin Microtiter Assay (REMA), Nitrate Reductase Assay (NRA), Malachite Green Decolorization Assay (MGDA), and Crystal Violet Decolorization Assay (CVDA) methods were included in the study. In addition, mutations in the *pncA* gene were investigated using sequence analysis in PZA-resistant isolates. **Results:** As a result of the comparison of the colorimetric methods with the reference method, agreement was determined as 93.3% in REMA and NRA, 90% in MGDA, and 93.3% in CVDA. In 13 of 15 resistant isolates, the *pncA* gene mutation was detected by sequence analysis. **Conclusions:** As a result of the work, the results from the colorimetric methods were found to be at a high level of concordance with the reference method. They are also inexpensive and easily applicable methods.

Keywords: Colorimetric, nicotinamide, pncA, pyrazinamide, tuberculosis

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Introduction

The number of primary drugs in tuberculosis (TB) treatment is quite small. Increasing resistance to these few primary drugs is a major problem that reduces treatment options. Pyrazinamide (PZA) is one of the primary drugs used in the treatment of TB.[1-4] PZA is a nicotinamide (NIC) analog that was chemically synthesized in 1936, and its activity on TB was shown in 1952.[5,6]

Initially, although PZA resistance was shown to be due to pyrazinamidase and nicotinamidase deficiency in *Mycobacterium tuberculosis*, subsequent studies have shown that the lack of these enzymes is due to mutations in the *pncA* gene-encoding enzymes.[7,8] Mutations in *rpsA* and *panD* have also been shown to be the cause of resistance.[9-12] Despite detected gene mutations, there are a small number of PZA-resistant strains that do not have mutations in *pncA*, *rpsA* or *panD*.[4]

The major challenge in detecting PZA resistance is that PZA is ineffective against *M. tuberculosis* in the culture medium and at neutral pH. It can kill semi-dormant bacilli only in acidic pH (pH 5.5) media. To combat this issue, automated systems have been developed to detect PZA resistance. BACTEC MGIT 960 system has come to the forefront as a method that does not require inoculation for a subculture, which can be achieved quickly. However, this is also a costly system.

Studies in this area have shown that NIC, a PZA analog, can be used to detect PZA resistance. NIC is a PZA analog, and although it shows anti TB activity, it is a chemical that shows

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antagonism toward Isoniasid (INH) when both are used together.[13-15] For this reason, it is not possible for NIC to enter TB treatment regimens.[16] Both NIC and PZA are prodrugs and are converted by the nicotinamidase enzyme, also called pyrazinamidase, to the active forms of nicotinoic acid and pyrazinoic acid. However, physiological pH values that don't inhibit bacterial proliferation are appropriate to convert PZA to pyrazinoic acid (pH 5.5) and to convert NIC to nicotinic acid. Several studies have shown that the PZA resistance status can be reliably detected using high-dose NIC.[17,18] In this case, the limitations of PZA used in susceptibility tests can be overcome with NIC.[19]

In recent years, various studies using colorimetric sensitivity methods such as Nitrate Reductase Assay (NRA), resazurin microtiter assay (REMA), malachite green decolorization assay (MGDA) and crystal violet decolorization assay (CVDA) have frequently resulted in easy, inexpensive and reliable sensitivity detection methods that can be performed with minimal laboratory facilities. In these methods, the basic principle is that the proliferating bacilli cause a color change by metabolizing the color indicator substance in the medium. In many studies, colorimetric methods seem to work well in detecting resistance to INH, rifampin (RIF), ethambutol, and streptomycin.[20-23] In fact, favorable results were also received incolorimetric studies using NIC in this respect.[24-26]

The aim of this study was to investigate the efficacy of four colorimetric methods (REMA, NRA, MGDA, CVDA) using NIC in the detection of PZA resistance. In addition, PZA has been investigated in mutations in *pncA* genes in resistant isolates.

Methods

Supply of isolates

In this study, 30 clinical isolates were used from which 50% were resistant and 2 reference strains (*M. tuberculosis* H37Rv anti-TB drugs susceptible isolate and *M. tuberculosis* ATCC 35828 PZA resistant isolate). PZA susceptibility of the isolates was determined by using the BACTEC MGIT 960 reference method [Table 1]. All the isolates were passaged on Löwestein–Jensen (LJ) medium and stored at 37°C for 3 weeks before study. The fresh colonies obtained were used for the study.

Preparation of medium

Middlebrook 7H9 (Sigma-Aldrich, Steinheim, Germany) was used in the study. It was prepared as recommended by the manufacturer and 10% oleic acid, albumin, dextrose, and catalase and 0.1% casitone were added. For the medium to be used for the NRA test, potassium nitrate was added at a final concentration of 1 mg/ml. One hundred microliters of the prepared medium was distributed to 96-well sterile microplates in each well.

Preparation of bacterial inocula

Colonies from fresh cultures in LJ medium were transferred to Falcon tubes with sterile glass beads and Middlebrook 7H9 medium. The supernatant obtained after vortexing was adjusted to Mc Farland 1 (approximately 10^8 CFU/ml) and diluted 1:15 with Middlebrook 7H9 medium. All procedures were carried out in the class 2 level biosafety cabinet using personal protective equipment.

Nicotinamide preparation

The NIC (Sigma-Aldrich, Steinheim, Germany) stock solution was prepared with distilled water to be 40 mg/ml. After being sterilized by filtration, it was stored at −20°C until use.

Detection of minimum inhibitory concentration Resazurin microtiter assay

Flat-bottom sterile microplates with 96 wells were used for the test. There was one control well and seven test wells at the plates for each bacterium. The prepared medium was distributed to 100 µl in each well. Only the medium and bacteria inoculum was placed in the control well. NIC was added to the test wells, and the concentration was adjusted to be between 2000 and 31.25 µg/ml by pipetting. The prepared bacterial inoculum was distributed to 100 µl in each well. The plates were incubated at 37°C for 7 days. Then, 30 µl of 0.02% resazurin (Sigma-Aldrich, Steinheim, Germany) solution was added to all the wells, and the plates were again incubated at 37°C. The plates were checked every day during the incubation. In the control well, the test was terminated when blue color turned in to a red-pink color accepted as a growth indicator. Because there was no growth, the final well where the blue did not turn red-pink was identified as a minimum inhibitory concentration (MIC) value [Figure 1].

Detection of minimum inhibitory concentration with nitrate reductase assay

Flat-bottom sterile microplates with 96 wells were used for the test. On the plates, three control wells and five test wells were used for each bacterium. The medium with potassium nitrate prepared was distributed in each well at 100 µl. Only the medium and bacteria inoculum were placed in the control well. NIC were

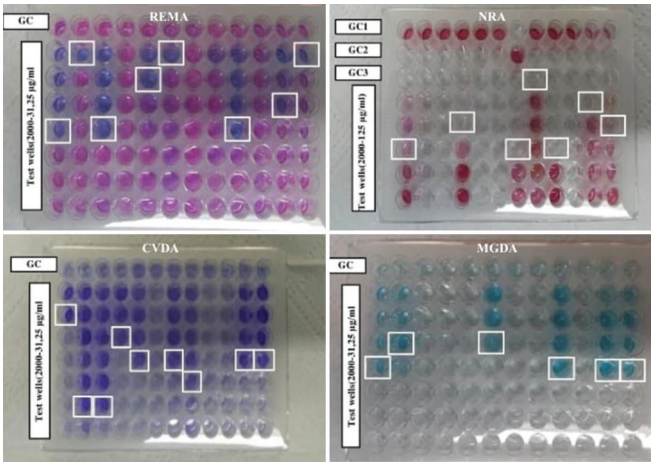

Figure 1: Plaque appearance of the colorimetric methods used. GC: Growth control; Wells marked with white square: Minimum inhibitory concentration value

Table 1: Pyrazinamide susceptibility status of isolates and nicotinamide minimal inhibitory concentration values obtained in colorimetric tests

Isolate number	PZA susceptibility (MGIT 960)	REMA (μg/ml)	NRA (μg/ml)	MGDA (μg/ml)	CVDA (μg/ml)
1	R	>2000	1000	>2000	>2000
2	R	2000	>2000	2000	>2000
3	R	>2000	>2000	>2000	1000
4	R	2000	>2000	>2000	>2000
5	S	250	250	250	250
6	R	>2000	2000	500	>2000
7	S	250	250	250	125
8	S	250	500	250	250
9	S	250	500	250	125
10	S	250	500	250	250
11	S	250	250	250	62,5
12	S	250	500	250	250
13	S	250	250	250	250
14	S	1000	500	1000	250
15	R	250	1000	125	250
16	S	250	500	250	250
17	S	250	1000	250	62,5
18	R	1000	>2000	>2000	>2000
19	R	>2000	>2000	>2000	>2000
20	R	1000	1000	1000	500
21	R	1000	250	125	125
22	S	250	250	250	62,5
23	R	2000	1000	500	1000
24	S	250	250	250	250
25	R	2000	2000	500	500
26	R	1000	>2000	>2000	>2000
27	R	>2000	>2000	>2000	>2000
28	R	>2000	2000	1000	500
29	S	250	500	250	250
30	S	250	500	250	62,5
H37Rv	S	125	500	250	125
ATCC 35828	R	>2000	2000	>2000	>2000

Shaded areas show incompatible results with reference method. PZA: Pyrazinamide, REMA: Resazurin microtiter assay, NRA: Nitrate reductase assay, MGDA: Malachite green decolorization assay, CVDA: Crystal violet decolorization assay

added to the test wells, and the concentration was adjusted to be between 2000 and 125 μg/ml by pipetting. The prepared bacterial inoculum was distributed in each well to 100 μl. The plates were incubated at 37°C for 7 days. Then, 50 μl of Griess reagent (consisting of 1 part of 50% HCl, 2 parts of 0.2% sulfanilamide, and 2 parts of 0.1% n-1-naphthylethylenediamine dihydrochloride) were added to the first control wells. When a red-purple color was observed on the control wells, the same amount of Griess was added to the test wells. The change in color in the test wells was indicative of bacterial replication and therefore resistance, and the final well without any color change was accepted as the MIC value. In the absence of discoloration of the control well, the procedure was repeated using control wells 2 and 3 for days 10 and 14 [Figure 1].

Detection of minimum inhibitory concentration with malachite green decolorization assay

The test wells were prepared as with the REMA method. After incubation at 37°C for 7 days, 30 ml of malachite green (Sigma-Aldrich, Steinheim, Germany) at a concentration of 0.05 mg/ml was added to all the wells. The plates were again incubated at 37°C. The plates were checked every day during the incubation. In the control well, the fading or disappearance of green color was accepted as a reproductive indicator, and the test was terminated. Because there was no growth, the last well where the green color did not fade or disappear was determined as the MIC value [Figure 1].

Detection of minimum inhibitory concentration with crystal violet decolorization assay

The test wells were prepared as in the REMA method. After incubation at 37°C for 7 days, 25 ml of crystal violet (Sigma-Aldrich, Steinheim, Germany) at a concentration of 0.025 mg/ml was added to all the wells. The plates were again incubated at 37°C. The plates were checked every day during the incubation. In the control well, the fading or disappearance of the blue color was accepted as a reproductive indicator, and the test was terminated. Because there was no

growth, the last well where the blue color did not fade or disappear was determined as the MIC value [Figure 1].

Detection of the distribution of *pncA* Gene mutations

Sequence analysis was performed to detect *pncA* gene mutations in 15 isolates resistant to PZA. DNA extraction was performed using a DNA extraction kit (PureLink™ Genomic DNA Mini Kit, Thermo Fisher Scientific, Waltham, Massachusetts, USA) from fresh colonies grown on a Middlebrook 7H9 medium. The polymerase chain reaction (PCR) method was used to investigate the *pncA* gene. For the detection of the *pncA* gene, 200 nM HPLC primers PF 5'GGCGTCATGGACCCTATATC 3 'and PR 5'CAACAGTTCATCCCGGTTC 3' were used.[27] The prepared PCR mixture was placed on a thermal cycler (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Thirty-five cycles were studied in the amplification program. Each cycle was applied for 3 min at 95°C for the first denaturation, 30 s at 95°C for denaturation, 30 s at 57°C for primer binding, and 1 min at 72°C for extension. Finally, the PCR was terminated by a final extension of 10 min at 72°C. The agarose gel electrophoresis was performed on the obtained PCR products. The results were analyzed in the imaging device under a UV transilluminator source. The examination of the DNA region revealed that the target region was amplified. The resulting PCR products were purified with the NucleoSpin Gel and PCR clean up kit (Macherey Nagel, Duren, Germany) according to the manufacturer's recommended protocol. Sequencing was then performed with the ABI 3130XL Genetic Analyzer (Applied Biosystems, Foster City, California USA) automated sequencing device using the BigDye Cycle Sequencing Kit v3.1 sequence kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and the primers used in the PCR process.

Results

In the 15 resistant isolates tested with REMA, the MIC values were >2000 μg/ml in 6 isolates, 2000 μg/ml in 4 isolates, 1000 μg/ml in 4 isolates, and 250 μg/ml in 1 isolate. In PZA-susceptible isolates, the MIC value was determined to be 1000 μg/ml in 1 isolate and 250 μg/ml in 14 isolates. In the determination of the MIC values using the MGDA method, the MIC values of the 15 PZA-resistant isolates were found to be >2000 μg/ml for 7 isolates, 2000 μg/ml for 1 isolate, 1000 μg/ml for 2 isolates, 500 μg/ml for 3 isolates, and 125 μg/ml for 2 isolates. In PZA-susceptible isolates, the MIC value was determined to be 1000 μg/ml for 1 isolate and 250 μg/ml for 14 isolates. In the CVDA and MIC values, the results of 15 resistant isolates were found to be >2000 μg/ml in 8 isolates, 1000 μg/ml in 2 isolates, 500 μg/ml in 3 isolates, 250 μg/ml in 1 isolate, and 125 μg/ml in 1 isolate. In PZA-susceptible isolates, the MIC value was determined to be 250 μg/ml in 9 isolates and 125 μg/ml in 6 isolates [Tables 1 and 2].

As shown in Table 2, the breakpoints were 250 μg/ml for REMA, MGDA and CVDA. The breakpoint for NRA was 500 μg/ml. The completion times of the tests for REMA, NRA, MGDA, and CVDA were 10.7, 8, 12.2, and 11.3 days, respectively. Agreement with the reference method was 93.3% for REMA, NRA, and CVDA, and 90% for MGDA [Table 3].

The *pncA* genes of the PZA-resistant isolates were detected using the PCR method in 670 bp fragment length bands. The mutations in the *pncA* gene were determined by comparing it to the wild-type *M. tuberculosis pncA* gene sequence using BLAST [Table 4].

Table 2: Distribution of minimal inhibitory concentration values of isolates

MIC	>2000 μg/ml	2000 μg/ml	1000 μg/ml	500 μg/ml	250 μg/ml	125 μg/ml	62.5 μg/ml	31.25 μg/ml
REMA								
Resistant (*n*=15)	6	4	4	0	1	0	0	0
Susceptible (*n*=15)	0	0	1	0	14	0	0	0
H37Rv (*n*=1)	-	-	-	-	-	1	-	-
ATCC 35828 (*n*=1)	1	-	-	-	-	-	-	-
NRA								
Resistant (*n*=15)	7	3	4	0	1	0	-	-
Susceptible (*n*=15)	0	0	1	8	6	0	-	-
H37Rv (*n*=1)	-	-	-	1	-	-	-	-
ATCC 35828 (*n*=1)	-	1	-	-	-	-	-	-
MGDA								
Resistant (*n*=15)	7	1	2	3	0	2	0	0
Susceptible (*n*=15)	0	0	1	0	14	0	0	0
H37Rv (*n*=1)	-	-	-	-	1	-	-	-
ATCC 35828 (*n*=1)	1	-	-	-	-	-	-	-
CVDA								
Resistant (*n*=15)	8	0	2	3	1	1	0	0
Susceptible (*n*=15)	0	0	0	0	9	6	0	0
H37Rv (*n*=1)	-	-	-	-	-	1	-	-
ATCC 35828 (*n*=1)	1	-	-	-	-	-	-	-

REMA: Resazurin microtiter assay, NRA: Nitrate reductase assay, MGDA: Malachite green decolorization assay, CVDA: Crystal violet decolorization assay, MIC: Minimal inhibitory concentration, MIC: Minimum inhibitory concentration

Table 3: Comparison of the results obtained by colorimetric methods with the results of the reference method (MGIT 960 method)

	Reference method		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Agreement (%)
	Resistant	Susceptible					
REMA							
Resistant	14	1	93.3	93.3	93.3	93.3	93.3
Susceptible	1	14					
NRA							
Resistant	14	1	93.3	93.3	93.3	93.3	93.3
Susceptible	1	14					
MGDA							
Resistant	13	1	86.6	93.3	92.8	87.5	90
Susceptible	2	14					
CVDA							
Resistant	13	0	86.6	100	100	88.2	93.3
Susceptible	2	15					

PPV: Positive productive value, NPV: Negative productive value, REMA: Resazurin microtiter assay, NRA: Nitrate reductase assay, MGDA: Malachite green decolorization assay, CVDA: Crystal violet decolorization assay

Table 4: *pnc A* gene mutations in pyrazinamide resistant *Mycobacterium tuberculosis* isolates

Isolate number	Change in nucleotide sequence	Change in amino acid sequence
1	228, (A→C)	Cys-138→Arg
2	No mutation	No mutation
3	457, (C→G)	Thr-135→Arg
4	637, (G→C)	Ser-67→Trp
6	627, (A→T)	Gly-24→Asp
15	No mutation	No mutation
18	413, (T→C)	Leu-119→Pro
19	241, (C→T)	Val-175→Lys
26	25, (T→G)	Ala-21→Val
27	11, (T→G)	Ala-130→Val
28	22, (T→C)	Thr-38→Asn
20	613, (T→C)	Ala-163→Val
25	644, (G→A)	Val-46→Ala
21	248, (C→T)	Ala-163→Val
23	13, (T→G)	Asp-12→Gly

Ala: Alanin; Arg: Arginin; Asn: Asparagin; Asp: Aspartat; Cys: Sistein; Gly: Glisin; Leu: Lösin; Lys: Lizin; Pro: Prolin; Ser: Serin; Thr: Treonin; Trp: Triptofan; Val: Valin

Discussion

Since PZA is effective only at acid pH, resistance determination is difficult. Automated systems such as the BACTEC MGIT 960 are costly. Therefore, there are few studies on PZA resistance detection. To address this problem, studies have been conducted on the use of NIC, an antagonist of PZA. Studies have shown that PZA resistance status can be reliably detected using a high dose of NIC.[15-19] Colorimetric methods have proved to be an important alternative to reference methods in determining resistance to anti-TB drugs in recent years due to their cheapness and ease of application. Successful results are obtained from colorimetric methods using NIC in detecting PZA resistance. However, the number of studies performed in this area is also very limited. Two studies by Martin *et al*. and a study by Mirabal*et al*. are rare studies in this area.

In a study by Martin *et al*. conducted with REMA using NIC, the critical MIC value was 250 μg/ml, whereas their sensitivity and specificity were found to be 100% and 98%, respectively.[24]

In another study by Martin *et al*., the MIC values were determined to be 1000 μg/ml and 500 μg/ml in the NRA method using NIC. In this study, the sensitivity was 91%, and the specificity was 94%.[25]

In the work of Mirabal *et al*., the NIC was used in both the NRA method and the MGDA. The critical MIC value in the NRA was accepted as 500 μg/ml. When this value was compared with the reference method, the sensitivity was determined to be 93.75%, and the specificity was found to be 97.6%. In MGDA, the critical MIC value was determined to be 250 μg/ml, and when compared with the reference method, the sensitivity was 93.75%, and the specificity was 97.6%.[26]

Another colorimetric method is the CVDA developed by Coban,[23] However, until our study, the PZA susceptibility had not been studied with this method. The agreement with the reference method for INH and RIF resistance detection studies using this method ranged from 94.5%–98.1% for INH and 96.3%–100% for RIF.[23,28-30] This method was used to detect PZA resistance for the first time in our study.

These studies show that colorimetric methods have an important role in anti-TB drug resistance detection. In addition, the fact that NIC is used instead of PZA in these studies removes the disadvantages caused by PZA.

In our study, agreement with the reference method was 93.3% for REMA, NRA, and CVDA, and 90% for MGDA [Table 3]. The results obtained in the REMA, NRA, and MGDA methods are consistent with the results obtained in previous studies.

In our study, MIC values and MIC distribution of the isolates for REMA, NRA, MGDA, and CVDA are as in Tables 1 and 2. According to the data we obtained, we propose critical concentration of 250 μg/ml NIC for detecting PZA resistance in the REMA, MGDA, and CVDA. The critical concentration value for NRA is proposed 500 μg/ml.

When the results obtained with the methods used in our study are compared with the reference method, the compatibility in all the methods is over 90%. It has also been found that CVDA concordance with the reference method is at the same level as other methods used in the study and that PZA resistance can be successfully detected.

Isolate No. 14, which is susceptible according to the reference method, was found to be resistant in REMA and MGDA and was found to be susceptible in NRA and CVDA. Isolate No. 15 resistant to PZA was detected as susceptible according to the REMA, MGDA and CVDA methods, and these three methods were insufficient to detect susceptibility. Resistance in this isolate could only be detected by the NRA method. Although isolate 17 appears to be susceptible in the reference method, it was found to be resistant in NRA. Isolate 21, identified as resistant according to the reference method, was detected as susceptible to NRA, MGDA, and CVDA. The resistance in this isolate could only be detected by the REMA method. Isolate No. 19, the only isolate of PZA mono resistance, was identified as resistant by all the colorimetric methods.

When the tests were evaluated according to the cut-off time, the fastest test was the NRA, with an average of 8 days. The cutoff time in NRA was between 7 and 10 days. The average cut-off times of REMA and CVDA are close to one another. The average time in the REMA is 10.7 days, and in the CVDA is 11.3 days. In both methods, the time of cutoff of the test ranged from 8 to 13 days. In the MGDA, the average cutoff time was 12.2 days, and all tests were completed between 8 and 15 days. In the reference method, BACTEC MGIT 960, it was shown that the cutoff time changed between 7 and 18 days (an average of 12 days). These findings show that colorimetric methods can be an alternative to the reference method for rapid results.

In the clinical isolates resistant to PZA, there was a very good correlation between the *pncA* mutations, the loss of PZase activity and PZA resistance. This correlation suggests that the *pncA* mutation is the main mechanism of PZA resistance in *M. tuberculosis*. In our study, the *pncA* gene sequence was also carried out in 15 isolates with PZA resistance, and the *pncA* mutation was detected in 13 isolates. Mutations in PZA-resistant isolates were distributed throughout the *pncA* gene. However, no mutations could be detected in isolates 2 and 15. The resistance in isolate 15, in which the *pncA* gene mutation could not be detected, could only be detected by the NRA method. For unknown reasons, it was possible that the loss of PZase activity on this isolate is at a limit that could not be detected by the other three methods. A similar situation may be the case with isolate 21, which was found to be only resistant in the REMA method. Recent studies have shown that there may be mutations in the *rpsA* and *panD* genes in resistant isolates without *pncA* mutations. The investigation of other gene mutations in isolates 2 and 15, in which the *pncA* gene mutation could not be detected, can be considered.

Table 5: Comparison of the cost of colorimetric methods and the cost of BACTEC MGIT 960

BACTEC MGIT 960	REMA	NRA	MGDA	CVDA
$ 14.55	$ 1.59	$ 1.84	$ 1.60	$ 1.63

REMA: Resazurin microtiter assay, NRA: Nitrate reductase assay, MGDA: Malachite green decolorization assay, CVDA: Crystal violet decolorization assay

PZA kit for the BACTEC MGIT 960 is usually not available even in laboratories using BACTEC MGIT 960. Whereas, most of the materials (e.g. crystal violet and malachite green) used in colorimetric methods are materials that can be found in every microbiology laboratory and have a long shelf life. As shown in Table 5, the cost of colorimetric methods is extremely low.

As a result, in our study, it has been shown that all of the colorimetric methods used are fast, easy to apply, cheap, do not require an excessive amount of training for practitioner and can be performed with the materials and chemicals available in many microbiology laboratories. However, it should not be forgotten that maximum personal protection measures must be taken against the risk of infection and contamination and necessitate the use of class II biosafety cabinet during the implementation of these methods. REMA and NRA are methods that have been tested in various studies for detecting anti-TB drug susceptibility. Although the preparation of NRA is more difficult than REMA, it is advantageous for quick results. MGDA has been tested with anti TB drugs as an easy-to-use and low-cost method, and good results were obtained.

Although CVDA is a very new method compared to other methods, recent studies indicate that it is a successful method for detecting resistance in anti-TB drugs. This study was conducted to investigate the efficacy of colorimetric methods in detecting PZA susceptibility, and the number of isolates is limited for this reason. Multicenter studies with high numbers of isolates will contribute more to the literature on the effectiveness of colorimetric methods.

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Conflicts of interest

There are no conflicts of interest.

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