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RESEARCH ARTICLE

Colorimetric Assays as the Diagnostic Modality of Pulmonary and Extra-pulmonary Tuberculosis in Resource-limited Settings

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ABSTRACT

Objectives: Diagnosis of tuberculosis (TB) is challenging, especially in a resource-limited setting. Non-commercial alternatives such as colorimetric assays must be used for effective diagnosis and prompt treatment. In this study we aimed to determine the diagnostic performance, time to detection, cost, contamination rate, and ease of performance of the oxidation-reduction assays; Resazurin tube assay (RETA) and Malachite Green decolorization assay (MGDA) and their modifications using para-nitro benzoic acid (PNB) for detection of TB against the results on Lowenstein-Jensen medium (LJ) medium.

Methods: Two-hundred-seventeen samples were subjected to colorimetric assays by incubating the inoculated media at 37^0C. Dyes were added on days 10, 14, 18, 28, and 42 days of incubation, and color change was noted after 24 hours and was compared with results on the LJ medium.

Results: Diagnostic performance of colorimetric assays increased from day 10 to day 28 (maximum at day 28), while it was the same on days 28 and 42. The sensitivity, specificity, and diagnostic accuracy of RETA were 87.6%, 95.7%, and 88.6%, while that of MGDA were 81.7%, 95.5%, and 86.5%, respectively. On day 18, more than 51% of the samples were positive by colorimetric assays, whereas only 20.4% were positive by LJ culture. In addition, the colorimetric assays were as economical as LJ culture.

Conclusion: Colorimetric methods can potentially become non-commercial alternatives for rapidly detecting *Mycobacterium. tuberculosis*, especially in TB laboratories with limited resources. The ease of interpreting the results and its cost-effectiveness is an additional advantage. *J Microbiol Infect Dis 2022; 12(3):108-115.*

Keywords: *Mycobacterium tuberculosis, colorimetric assays, LJ culture, malachite green, resazurin*

INTRODUCTION

Tuberculosis (TB) is a dreaded disease, and despite advances in anti-tuberculosis chemotherapy, it continues to be one of the world's biggest threats [1]. Timely diagnosis is crucial for effective control of TB; smear microscopy and culture are the mainstays for diagnosis. The limitation of microscopy is low and variable sensitivity (0-40%) and its inability to differentiate between *Mycobacterium tuberculosis* (*M. tuberculosis*) and non-tuberculous mycobacteria (NTM) [2,3]. The use of culture methods involving solid media requires 3-4 weeks to detect mycobacterial growth, plus an additional period for species identification [4]. World Health Organization has been actively involved in developing new tools for improved diagnosis of TB [5,6]. The exorbitant cost of these systems precludes their use in resource-poor settings. The thin-layer agar (TLA) culture and microscopic observation drug susceptibility (MODS) assays have good sensitivity and specificity and decreased time to detection (11.5 and 7 days, respectively) as compared with culture on Lowenstein-Jensen (LJ) medium. These methods, however, require trained personnel

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and specific equipment in addition to the regular TB culture infrastructure [7,8]. Colorimetric methods such as Resazurin Tube Assay (RETA), Malachite Green Decolorization Assay (MGDA), Nitrate Reductase Assay (NRA), and Crystal Violet Decolorization Assay (CVDA) detect live bacteria through enzymatic activity. Results are obtained by directly interpreting the medium color, and testing can be performed with minimal TB culture infrastructure at a relatively low cost. These methods are currently used for rapid identification and drug sensitivity testing of *M. tuberculosis* [9-14]. Resazurin, an oxidation-reduction dye and an active compound in Alamar blue, has been used as resazurin salt in Resazurin Microtiter Assay (REMA), Microdilution Resazurin Assay, and as Alamar blue in Microplate Alamar Blue Assay (MABA). Thus, REMA and MABA are based on the same chemistry [15]. Malachite Green, another oxidation-reduction dye, is a triphenylmethane dye that is cheap and readily available [16]. It has been reported that the growth of the *M. tuberculosis* complex is inhibited by para nitro benzoic acid (PNB) at a concentration of 500 µg/ml, whereas NTM is resistant to this concentration [17,18]. Limited studies are available on the use of colorimetric assays to diagnose TB. The present study was done to evaluate RETA and MGDA and their modifications using PNB (RETAp and MGDAp) for rapid detection of *M. tuberculosis* in pulmonary and extra-pulmonary samples and to determine the diagnostic performance, cost, ease of performance, contamination rate, and time to detection of these oxidation-reduction assays against the results on LJ medium.

METHODS

This study was a cross-sectional study conducted in the Department of Microbiology for 12 months (June 2017 to May 2018).

Samples

A total of 217 samples, 84 retrospectively collected and decontaminated sputum samples stored at -70 ^{0}C and 133 prospectively collected pulmonary and extra-pulmonary samples from patients with a presumptive diagnosis of TB, were included. Samples included sputum (114), endometrial curettage (21), bone marrow (04), cervical lymph node biopsy (03), gastric biopsy (03), urine (30), bronchoalveolar lavage (BAL) (16), pus/purulent exudates (07), pleural fluid (06), CSF (04), ascitic fluid (05), synovial fluid (02), pericardial aspirate (01), and semen (01).

Processing of samples

Sputum was collected as a spot sample and a next day early morning sample in suspected cases of pulmonary TB [19). The treating physician collected extra-pulmonary samples, except urine, under strict aseptic conditions and sent them to the laboratory. Urine was collected on three separate days as a whole morning sample by the patient. Samples were processed on the same day, and in case of delay in processing, the samples were stored at 4 ^{0}C for a maximum of 24 hrs. All samples were processed in class IIA biosafety cabinets. Samples from sterile sites were concentrated by centrifugation at 3000 rpm for 15 minutes, while the samples from the non-sterile sites were decontaminated by N-acetyl-L-cysteine and sodium hydroxide (NALC-NaOH) method for respiratory samples [20) while non-respiratory samples were subjected to decontamination by modified Petroff's method [21]. The sediment obtained after concentration and decontamination was reconstituted in 2 ml of sterile phosphate-buffered saline, subcultured onto blood agar and incubated overnight for sterility check. Simultaneously, smears for microscopy were made and interpreted according to standard protocols [19].

Culture

The sterile sediment on blood agar was then subjected to culture on LJ medium, RETA and RETAp, and MGDA and MGDAp.

LJ culture: 100 µl of sediment was placed onto LJ medium (HiMedia) prepared according to the standard protocol [22]. LJ cultures were incubated at 37°C and examined weekly for six weeks. The strains that grew on the LJ medium were subjected to the MPT 64 Rapid antigen test for confirmation as *M. tuberculosis*.

Colorimetric assay (RETA/MGDA)

Media: Middlebrook 7H9 medium (HiMedia) supplemented with oleic acid, albumin, dextrose, and catalase, with 0.5% glycerol, 0.01% casitone, and polymyxin B, amphotericin B, nalidixic acid, trimethoprim-azlocillin (PANTA) with and without PNB (500 µg/ml) was used in both the assays. Ten 1.5 ml microtubes containing 500 µl of

supplemented liquid medium were labeled; four as RETA and four as MGDA (days 10,14,18 and 28), and two microtubes containing 500µl of the same medium with PNB were labeled as RETAp and MGDAp [23].

Inoculation of media

Hundred µl of the decontaminated/ concentrated sample was inoculated into each of the ten tubes and incubated at 37 ^{0}C.

Addition of dyes

On day 10, 25 µl of resazurin (0.25%) solution was added to one tube of RETA and 25µl of malachite green (5%) to one tube of MGDA, and the tubes were incubated overnight (37 ^{0}C). Reading was taken the next day. In case of no color change, a similar methodology was carried out on days 14, 18, 28, and 42.

Interpretation of the results

•*In tubes containing resazurin indicator, blue color (oxidized state) was interpreted as no growth and pink color (reduced state) as growth. (Figure 1).*

•*In tubes containing malachite green, dark green color (oxidized state) was interpreted as no growth and light green or no color (reduced state) as growth. (Figure 2).*

•*In case of color change in any of the tubes, a sterility check was done on blood agar, and smear microscopy was performed to rule out contamination. The tubes were reported as contaminated if they showed a negative smear for acid-fast bacilli (AFB) with a concomitant growth on blood agar.*

•*Results were considered indeterminate in case the blood agar was sterile with no AFB seen on smear microscopy, or blood agar showed bacterial growth, and AFB was seen on smear microscopy.*

•*In case of no growth on blood agar and smear microscopy positive for AFB, the tube was reported as positive.*

•*Any color change in the tube containing PNB was considered indicative of NTM, while no color change in the tube was considered indicative of M. tuberculosis.*

•*A culture was considered negative if no growth was observed after six weeks on LJ and oxidation-reduction assays. Diagnosis of TB was considered as confirmed when a patient had a positive culture in any of the three culture methods used.*

Quality Control

A quality control for media and colorimetric method was done using a standard isolate of *M. tuberculosis* (H37Rv).

Ethical Approval

The study was approved by the institutional ethical committee (No: SIMS 1 131/IEC-SKIMS/2017 Dt 14.06.2017)

Statistical analysis

In this comparative study, the new methods were compared with conventional LJ media to determine the diagnostic performance of the test method. All the categorical variables have been shown in terms of frequency and percentage. In addition, paired comparisons were performed, excluding the specimens showing contamination in any of the culture media. Sensitivity and predictive values were calculated using MedCalc software.

RESULTS

Cumulative results of RETA, MGDA, and LJ culture media are shown in Table 1. On day 28, 82 samples were positive by RETA (81 positives for *M. tuberculosis* while 1 for NTM by RETAp), 79 were positive by MGDA, and 31 were positive by LJ media. There was a gradual increase in the yield of positive results from day 10 to day 28 for all three methods. Maximum positivity was observed on day 28 (82 by RETA and 79 by MGDA). No difference was found in the results of the colorimetric assays on day 28 and day 42, while LJ showed maximum yield on day 42. Overall, the sensitivity, specificity, and diagnostic accuracy of RETA were 87.7%, 95.7%, and 88.6%, while that of MGDA were 81.7%, 95.5%, and 86.5%, respectively (Table 2).

Taking the cumulative percentage of the positive samples from day 10 to day 42, we observed that more than 52% (44/82 by RETA and 41/79 by MGDA) of positive results were available by the colorimetric methods by day 18. While only 20.4% (17/83) were positive by LJ culture (Fig. 3). The cost of performance of the colorimetric assays for an individual sample in the present study was 27.73 INR (0.37 USD) and 24.47 INR (0.32 USD) for LJ culture media. The overall contamination rates

in this study for RETA, MGDA, and LJ culture were 12%, 3.7%, and 8.8%, respectively.

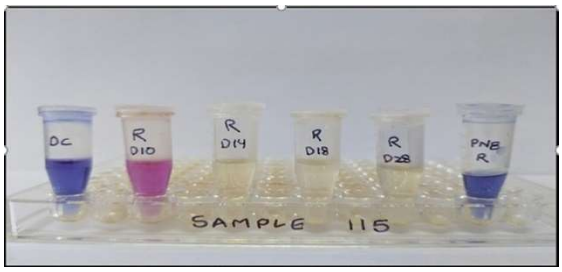

Figure 1. RETA on day 10 showing color change in comparison to dye control.

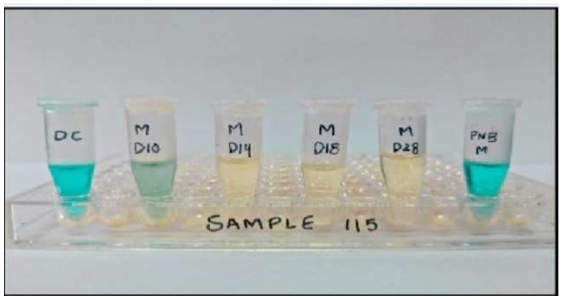

Figure 2. MGDA on day 10 showing complete decolorization.

DISCUSSION

In this study, an increasing trend in the diagnostic performance of the colorimetric assays from day 10 to day 28, with the maximum diagnostic accuracy of 88.6% by RETA and 86.5% by MGDA, was seen on day 28 (Figure 4). The findings were concurrent with the results observed by Yap Baum et al. wherein RETAp had an excellent specificity (99.3%) but low sensitivity (71.2%) at day 18 and extending incubation to 28 days increased the sensitivity to 82.6% [24]. However, there was no increase in the performance of these tests when incubated for more than 28 days. Parrissa et al. monitored the treatment of patients with pulmonary TB wherein the sensitivity and specificity of the Alamar Blue assay were 95% and 93%, and that of Malachite Green was 93% and 92%, respectively [25]. Our study showed a PPV of 94.2% by RETA, which is similar to the study conducted by Yap Baum et al [24]. However, the low NPV (84.9%) in the current study as compared to Yap Baum et al. may be due the variation in the prevalence of the disease; lower NPV is seen in areas with higher prevalence [26].

The performance of the colorimetric methods for primary detection of TB on day 18 seems to be slightly lower than the performance of other non-commercial methods like MODS and TLA culture, with sensitivities of 92% and 87% and specificities of 96% and 98%, respectively [7], while the performance of colorimetric assays at 28 days is comparable [24]. Nonetheless, compared to these assays, colorimetric methods provide a significant advantage in terms of ease of interpretation because they rely on a color change instead of observation of microcolonies under the microscope and thus are much less prone to inter-user variability. The time to positivity of the colorimetric assays was less (28 days) as compared to conventional LJ media (42 days). Similar findings were reported by Parrisa et al [25]. The cost of colorimetric assays (0.37 USD) in our study was substantially less as compared to MODS (6.53 USD), BACTEC 460 TB (21 USD), and the MGIT system (23 USD) used for the diagnosis of TB [27]. The low-cost factor has a significant advantage, especially in resource-limited settings

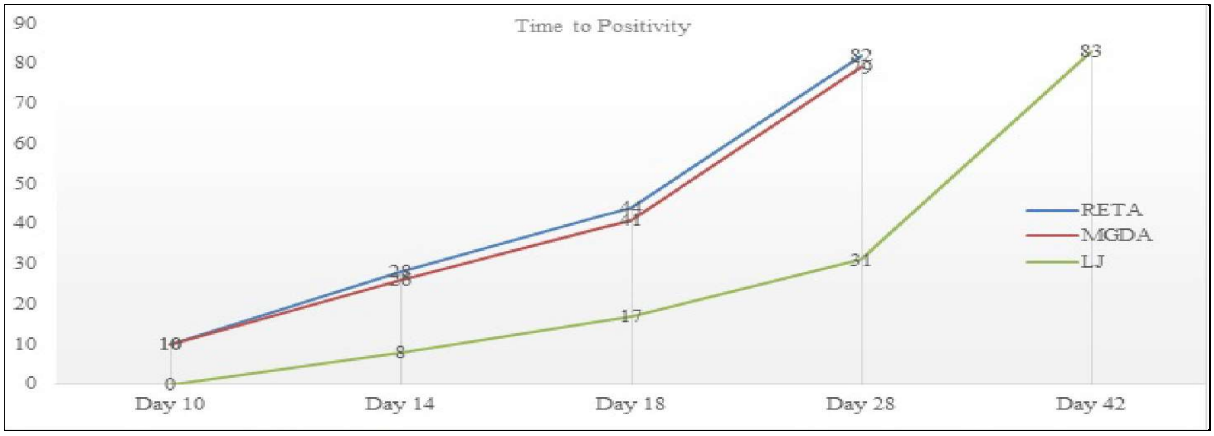

Figure 3. Time to positivity of colorimetric assays in comparison to LJ culture media results.

Table 1: Cumulative results of colorimetric assays and LJ culture media.

Variables	Day 10	Day 14	Day 18	Day 28	Day 42
Resazurin					
Positive	10	28	44	82	82
Negative	199	174	152	109	109
Contaminated	8	15	21	26	26
Total	217	217	217	217	217
Malachite Green					
Positive	10	26	41	79	79
Negative	199	183	168	130	130
Contaminated	8	8	8	8	8
Total	217	217	217	217	217
LJ Media					
Positive	0	8	17	31	83
Negative	217	207	190	171	115
Contaminated	0	2	10	15	19
Total	217	217	217	217	217

Table 2. Sensitivity, specificity, and diagnostic accuracy of colorimetric methods.

Method	Sensitivity	Specificity	PPV	NPV	Diagnostic Accuracy
Resazurin					
Day 10	10.84	99.07	85.71	58.15	59.16
Day 14	32.93	99.04	95.83	63.58	67.64
Day 18	49.38	98.99	97.22	68.06	73.89
Day 28	87.65	95.74	94.2	84.91	88.57
Day 42	87.65	95.74	94.2	84.91	88.57
Malachite Green					
Day 10	10.84	99.07	85.71	58.15	59.16
Day 14	30.49	99.10	95.45	64.33	67.88
Day 18	43.90	98.20	93.94	68.12	72.54
Day 28	81.71	95.50	92.42	83.46	86.53
Day 42	81.71	95.50	92.42	83.46	86.53

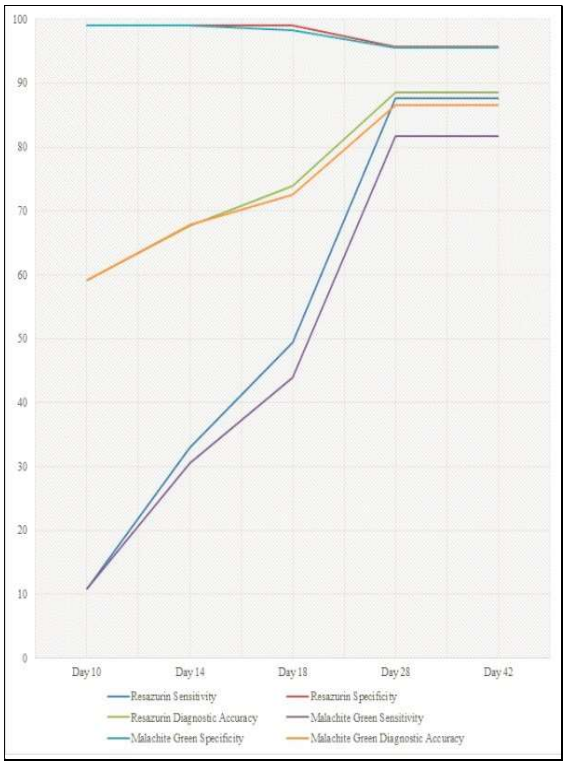

Figure 4. Performance of RETA and MGDA in comparison to LJ culture.

The contamination rates of RETA and LJ media were in accordance with other studies [28]. The lower rate of contamination of MGDA can be attributed to the broad-spectrum antimicrobial activity of malachite green. Variation in contamination rates in different studies can be explained by the final concentration of sodium hydroxide used during decontamination and the nature of the samples; most of the extra-pulmonary samples are sterile and show low contamination rates [29].

MGDA and RETA require media, reagents, and equipment easily available in routine mycobacteriology laboratories. The media can be stored in microfuge tubes, placed in racks at -20 °C, and used on the day of inoculation after thawing. Incorporating PNB-containing tubes helps in simultaneously identifying isolates as MTB/NTM. In addition, using tubes rather than microtiter plates prevent carryover contamination and is safer and comparatively cheap.

The use of LJ media as a gold standard for comparison might be a limitation in this study. However, future studies may be undertaken using commercial automated and/or molecular methods as a gold standard.

CONCLUSION

Colorimetric assays are cost-effective, easy to perform, and have easy to interpret. These assays lead to a faster turnaround time than LJ and can be used in laboratories with limited resources. Additionally, to simplify these processes further and reduce the lab personnel's burden, only two readings can be taken on days 18 and 28. Further, these assays can be incorporated as an adjunct to LJ medium for routine diagnosis of TB.

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REFERENCES

1. Global Tuberculosis Report 2021 [Internet]. [cited 2022 Sep 5]. Available from: https://www.who.int/teams/global-tuberculosis-programme/tb-reports/global-tuberculosis-report-2021

2. Liu KT, Su WJ, Perng RP. Clinical utility of polymerase chain reaction for diagnosis of smear-negative pleural tuberculosis. J Chin Med Assoc 2007; 70(4):148-151; discussion 146-147.

3. Haldar S, Sharma N, Gupta VK, Tyagi JS. Efficient diagnosis of tuberculous meningitis by detection of *Mycobacterium tuberculosis* DNA in cerebrospinal fluid filtrates using PCR. J Med Microbiol 2009; 58:616-624.

4. Rouillon A, Perdrizet S, Parrot R. Transmission of tubercle bacilli: The effects of chemotherapy. Tubercle 1976; 57(4):275-299.

5. TB/HIV research priorities in resource-limited settings: report of an expert consultation [Internet]. [cited 2022 Sep 5]. Available from: https://www.who.int/publications/i/item/WHO-HTM-TB-2005.355.

6. The Stop TB strategy: building on and enhancing DOTS to meet the TB-related Millennium

Development Goals [Internet]. [cited 2022 Sep 5]. Available from: https://apps.who.int/iris/handle/10665/69241.

7. Leung E, Minion J, Benedetti A, Pai M, Menzies D. Microcolony culture techniques for tuberculosis diagnosis: a systematic review. Int J Tuberc Lung Dis 2012; 16(1):16-23, i-iii.

8. Moore DA, Evans CA, Gilman RH, et al. Microscopic-observation drug-susceptibility assay for the diagnosis of TB. N Engl J Med 2006; 12;355(15):1539-1550.

9. De Souza AO, Santos Júnior RR, et al. Antimycobacterial activity of 4'-bromo-[1,1'-biphenyl]-4-yl 4-x-phenylmethanone derivatives, and their acute toxicity and cytotoxicity. Pharmazie 2001; 56(11):871-874.

10. Affolabi D, Odoun M, Sanoussi N, et al. Rapid and inexpensive detection of multidrug-resistant *Mycobacterium tuberculosis* with the nitrate reductase assay using liquid medium and direct application to sputum samples. J Clin Microbiol 2008; 46(10):3243-3245.

11. Martin A, Portaels F, Palomino JC. Colorimetric redox-indicator methods for the rapid detection of multidrug resistance in *Mycobacterium tuberculosis*: a systematic review and meta-analysis. J Antimicrob Chemother 2007; 59(2):175-83.

12. Palomino JC, Martin A, Portaels F. Rapid drug resistance detection in *Mycobacterium tuberculosis*: a review of colourimetric methods. Clin Microbiol Infect 2007; 13(8):754-762.

13. Angeby KA, Klintz L, Hoffner SE. Rapid and inexpensive drug susceptibility testing of *Mycobacterium tuberculosis* with a nitrate reductase assay. J Clin Microbiol 2002; 40(2):553-555.

14. Primm T, Franzblau S. Recent Advances in Methodologies for the Discovery of Antimycobacterial Drugs. Curr Bioact Compd 2007; 30;3(3):201-208.

15. Franzblau S. A rapid, microplate-based assay for evaluating the activity of drugs against Mycobacterium leprae, employing the reduction of Alamar Blue. Lepr Rev 2000; 71(Supplement).

16. Laszlo A, Siddiqi SH. Evaluation of a rapid radiometric differentiation test for the *Mycobacterium tuberculosis* complex by selective inhibition with p-nitro-alpha-acetylamino-beta-hydroxypropiophenone. J Clin Microbiol 1984, 19:694-698.

17. Rastogi N, Goh KS, David HL. Selective inhibition of the *Mycobacterium tuberculosis* complex by p-nitro-alpha-acetylamino-beta-hydroxypropio phenone (NAP) and p-nitrobenzoic acid (PNB) used in 7H11 agar medium. Res Microbiol 1989; 140(6):419-423.

18. Martins M C, Ueki S Y M, Palhares M C A, et al. An alternative biphasic culture system for recovery of mycobacteria and for differentiation of species other than *M. tuberculosis* complex from blood specimens. Rev Microbiol 1997; 183-189.

19. Koneman EW. Color Atlas and Textbook of Diagnostic Microbiology 1997 [cited 2022 Sep5]; 63. Available from: https://books.google.com/books/about/Color_Atlas_and_Textbook_of_Diagnostic_M.html?hl=fr&id=OHhhQgAACAAJ.

20. Tripathi K, Tripathi PC, Nema S, Shrivastava AK, Dwiwedi K, Dhanvijay AK. Modified Petroff's method: an excellent simplified decontamination technique in comparison with Petroff's method. Int J Recent Trends Sci Technol. 2014; 10(3):461.

21. Khalifa RA, Nasser MS, Gomaa AA, Osman NM, Salem HM. Resazurin Microtiter Assay Plate method for detection of susceptibility of multidrug resistant *Mycobacterium tuberculosis* to second-line anti-tuberculous drugs. Egypt J Chest Dis Tuberc 2013; 62(2):241-247.

22. Revised National TB Control Programme Training Manual for *Mycobacterium tuberculosis* Culture & Drug Susceptibility testing. Central TB division, Directorate General of Health Services, Ministry of Health and Family Welfare, New Delhi, India, 2009. https://tbcindia.gov.in/WriteReadData/l892s/6995271860Training%20manual%20M%20tuberculosis%20C%20DST. pdf.

23. Tsukamura M. [P-nitrobenzoic acid medium (PNB medium) as an aid to differentiate tubercle bacilli from other mycobacteria. (A review)]. Kekkaku 1984; 59(6):361-368.

24. Boum Y, Orikiriza P, Rojas-Ponce G, et al. Use of colorimetric culture methods for detection of *Mycobacterium tuberculosis* complex isolates from sputum samples in resource-limited settings. J Clin Microbiol 2013; 51: 2273-2239.

25. Farnia P, Mohammadi F, Mirsaedi M, et al. Application of Oxidation-Reduction Assay for Monitoring Treatment of Patients with Pulmonary Tuberculosis. J Clin Microbiol 2004; 42 :3324-3325.

26. Harrell FE Jr, Califf RM, Pryor DB, Lee KL, Rosati RA. Evaluating the yield of medical tests. JAMA 1982; 247(18):2543-2546.

27. Walusimbi S, Kwesiga B, Rodrigues R, et al. Cost-effectiveness analysis of microscopic observation drug susceptibility test versus Xpert MTB/Rif test for diagnosis of pulmonary tuberculosis in HIV patients in Uganda. BMC Health Serv Res 2016; 16: 563.

28. Chihota VN, Grant AD, Fielding K, et al. Liquid vs. solid culture for tuberculosis: performance and cost in a resource-constrained setting. Int J Tuberc Lung Dis 2010; 14(8):1024-1031.

29. Smaoui S, Kammoun S, Marouane C, Slim L, Messadi-Akrout F. Evaluation of the BACTEC MGIT 960 TB with Solid Media for Recovery of Mycobacteria from Extrapulmonary Specimens in South Tunisia. J Med Diagn Meth 2015; 4: 171. doi: 10.4172/2168-9784.1000.171.