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ORIGINAL ARTICLE

Evaluation of the Colour Test for Detection of Multidrug-resistant Mycobacterium Tuberculosis

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ABSTRACT

Background: *The timely and cost-effective diagnosis of cases of tuberculosis is of utmost importance, particularly in developing countries where the disease poses a public health threat. The emergence of multi-drug resistant (MDR) Mycobacterium tuberculosis strains, further compounds the diagnosis and appropriate therapy. Rapid, resource-efficient and specific diagnosis of MDR M. tuberculosis is of utmost priority to control this global public health problem. Available diagnostic tests either provide accuracy at the expense of resources (the genotypic methods commonly used such as PCR), or compromise timeliness and specificity while providing a reasonable cost (phenotypic methods such as the proportional method).* ***Objectives:*** *The aim of this study was to evaluate the performance of the colour Test in detection of drug resistance among Mycobacterium tuberculosis isolates to Isoniazid (INH), Rifampicin (RMP) and Ciprofloxacin (CIP), with reference to the golden standard proportion method.* ***Methodology:*** *The colour Test combines the thin-layer agar technique with a simple colour-coded quadrant format, selective medium to reduce contamination and colorimetric indication of bacterial growth to simplify interpretation.This study was conducted on 50 M. tuberculosis strains of Egyptian cases diagnosed as pulmonary tuberculosis, in the National Central Laboratory, Ministry of Health, Egypt, during the period between October 2014 to June 2015.* ***Results:*** *This study shows that the sensitivity and specificity of the colour Test for RMP and INH were 100% and 90.3% respectively. The agreement of the colour Test with the PM was almost perfect agreement (87.6%). For CIP sensitivity and specificity were 96.7% and 90% respectively.* ***Conclusion:*** *The study demonstrates that the colour Test is reliable compared with the proportion method and offers an added advantage of a shorter turnaround time. It is additionally a simple, easy to interpret test. As such, the colour Test offers promise in the pursuit of simple, reliable, rapid and cost-effective techniques for the diagnosis of multi-drug resistant TB.*

INTRODUCTION

Tuberculosis remains one of the world's deadliest communicable diseases, particularly in developing countries where resources for early detection and treatment are lacking. While Egypt is not on the World Health Organization (WHO) list of the 22 high TB-burden countries, yet it contributes to 3% of the total TB cases in WHO's Eastern Mediterranean Region [1]. According to the WHO country profile report in 2011, it is estimated that in Egypt, 17 cases per 100 000 population develop active pulmonary smear positive TB annually[2].The number of reported cases of tuberculosis in Egypt according to World Health Statistics was 8,453 new cases in 2012 [3]. The spread of TB is further compounded by the appearance of drug-resistant strains and especially multidrug-resistant strains to most effective (first-line) anti-tuberculous drugs [4].

Globally, more than half a million new multidrug-resistant tuberculosis (MDR-TB) cases are estimated to emerge annually. Extensively drug-resistant tuberculosis (XDR-TB) is a subset of MDR-TB caused by strains resistant to second-line medicines. It is defined as resistance to any fluoroquinolone and at least one of three injectable second-line medicines: kanamycin, amikacin and capreomycin), with significantly worse outcomes [1]. MDR-TB cases are difficult to treat and cure rates are low, whereas XDR-TB cases are virtually untreatable, since none of the standard drugs or the reserve drugs are effective [5]. Understandably, there is a global need for new, reliable and affordable methods of detection of drug resistance, as rapid identification of MDR- and XDR-TB is vital for the prompt initiation of adequate treatment and interruption of further transmission of resistant strains [6].

Two drug susceptibility testing (DST) strategies are currently in routine use: phenotypic and genotypic (molecular) methods. Phenotypic methods are in general simple to perform and might be closer to implementation on a routine basis in clinical laboratories, but some methods can take weeks to yield

reliable results. Molecular tests have the advantage of a shorter turnaround time, yet require expensive equipment and most also require specialized expertise. Therefore new simple and rapid methods are needed especially for low-income countries with a high incidence of TB and a growing problem of drug resistance [7].

Mycobacterium tuberculosis microcolony detection on thin-layer agar (TLA) is a rapid inexpensive, non-commercial, easily implemented technique that only requires a 37°C incubator and an ordinary light microscope to confirm diagnosis. It has the same sensitivity in detecting *M. tuberculosis* as conventional culture methods, but is much more rapid [5]. The TLA-Colour Test has been developed for the identification of *M. tuberculosis* complex and detection of resistance to isoniazid (INH), rifampicin (RMP) and ciprofloxacin (CIP). The Colour Test combines the thin-layer agar technique with a simple colour-coded quadrant format, selective medium to reduce contamination and colorimetric indication of bacterial growth to simplify interpretation. It allows the detection of resistance to the first-line drugs and second-line drugs at the same setting, which helps in early detection of MDR-TB and screening for XDR-TB [7, 8]. Importantly, it offers the advantage of reducing the time of diagnosis compared to the standard proportion method.

This study aimed to evaluate the performance of TLA- colour test in detection of drug resistance among Mycobacterium tuberculosis isolates for detecting drug resistance among *M. tuberculosis* isolates to Isoniazid (INH), Rifampicin (RMP) and Ciprofloxacin (CIP), with reference to the golden standard proportion method, with emphasis on its sensitivity, specificity and time-efficiency.

METHODOLOGY

In this study, drug susceptibility testing for RMP, INH and CIP were done by Colour test thin-layer agar (TLA) and by the proportional method (PM) as a gold standard. These 2 tests were performed on 50 *M. tuberculosis* isolates from sputum samples of Egyptian cases diagnosed as pulmonary TB in the National Central Laboratory, Ministry of Health, Egypt, during the period of October 2014 to June 2015.

1- The Proportion Method (PM)

The proportion method was done for INH, RMP and CIP in the National Central Laboratory, Egypt as described by Canetti et al. [9] and results were confirmed in Supranational TB laboratory; The Tropical Institute of Medicine, Antwerp, Belgium.

Strains showing resistance to CIP in addition to at least one of first-line drugs (INH and RMP) were further tested for amikacin (AMK) resistance by the proportional method to screen for XDR-TB.

Inoculum preparation:

Bacterial suspensions for drug susceptibility testing were made by scraping the growth from a three weeks' old culture with a loop and bacterial suspension was made with sterile distilled water. It was then vortexed and the turbidity was adjusted to McFarland scale 1. Preparation of dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} with sterile distilled water was done. Then 3 dilutions; 10^{-1}, 10^{-3} and 10^{-5}, were tested.

Preparation and Inoculation of L-J medium for sensitivity testing:

The recommended critical concentrations of the anti-tuberculous drugs were added to the L-J medium before pouring in the screw capped bottles, then inspissated at room temperature overnight then left in the refrigerator till used. Four different drug-containing (RMP, INH, CIP and AMK) and one drug-free (control) bottles, for each dilution of microbial suspension (10^{-1}, 10^{-3}, 10^{-5}), were prepared and labelled for each strain. So for each strain there were three rows of the three dilutions of the microbial suspension, each row has two different drug-containing bottles and one drug-free control bottle. Afterwards the bottles were inoculated with 0.1ml of each dilution of the bacterial suspension of each strain. The dilution of 10^{-1} was for the first row, 10^{-3} for the second row and 10^{-5} for the third row.

Drug concentrations used in proportional method were 40 g/ml for RMP, 0.2 g/ml for INH, 2 g/ml for CIP and 30 g/ml for KAN.

Interpretation of results:

After 28 days of incubation, the colonies on the drug-containing and drug-free control bottles were encountered to calculate the proportion of resistant mycobacteria.

The first row with bacterial suspension of 10^{-1} dilution was interpreted with the control 3 and the second row with bacterial suspension of 10^{-3} dilution was interpreted with control 5. This means that the number of colonies growing on the anti-microbial containing tubes was compared to the number of colonies on the following control tube. The first control medium should have at least 100 colonies for the test to be valid.

According to the following formula, the proportion of the bacilli resistant to a given drug was calculated:

$$\%\ \text{of resistance} = \frac{\text{No. of colonies on drug media}}{\text{No. of contents on drug medium}} \times 100$$

The first reading was after 28 days from cultivation. The strain was considered to be **"resistant"** when the proportion of bacteria in drug-containing medium to that of drug free medium exceeded 1%. While if it was less than 1%, a second reading was made on day 42, to determine whether the strain was in fact "**susceptible**".

2- The Colour Test method:

Principle of the test:

The colour test TLA is a phenotypic method for detecting drug resistance in *M. tuberculosis.* Thin-layer agar technique with a simple colour-coded bisected petri dish format was done on the Middlebrook 7H11agar. Bisected petri dishes were prepared. Each drug (INH, RMP and CIP) was added to one of the prepared halves. The remaining half contained no drug concentration and acted as control for growth detection. This method was done as described by Toit et al. [7]

Constituents of thin-layer agar (TLA) media:

- Middlebrook 7H11 Agar base (Sigma, Germany).
- Oleic Acid Dextrose Catalase (OADC): (Becton Dickinson)
- Mycobacteria Selectatab: (Kirchner, Mast Laboratories Ltd, Merseyside, United Kingdom).
- 2,3 diphenyl-5-(2-thienyl) tetrazolium chloride (Scientech lab, Egypt). This is an oxidation-reduction indicator. It resulted in the growth of red TB colonies, thus making them visible to the naked eye at early stages of growth.
- Glycerol (Al Gomhoreya Company, Egypt).
- Food colours (yellow, green and blue): Dr Oetker (Leeds, United Kingdom).
- Drugs :
  - INH (Sigma, Germany)
  - RMP (Sigma, Germany)
  - CIP (Eipico, Egypt).

Inoculation of the media:

The inoculum was prepared from fresh L-J medium adjusted to a MacFerland tube no.1 as follows:

1. A full loop of bacterial growth was adjusted to MacFerland tube no. 1 and used to inoculate the prepared media (4 drops for each half).
2. The plates were sealed with parafilm and incubated at 37°C. They were checked every other day until at least 50 colonies appeared on the drug-free control half.

Interpretation of the colour Test results:

1. Growth of *M. tuberculosis* was detected as red colonies due to redox reaction of tetrazolium chloride.
2. Growth of isolates in the control half is mandatory to confirm test validity.
3. Growth of more than 50 red colonies in any drug supplemented half is considered as a resistance to this drug.
4. ZN stained films were done for the isolated colonies for confirmation.

3- Statistical analysis

1. Drug susceptibility patterns for RMP, INH and CIP were recorded by both of PM and the Colour Test.
2. The performance of the colour test TLA was evaluated taking PM (the routinely used method) as the gold standard.
3. The programme used was **Diagnostic and agreement statistics (DAG)** (*www.mhri.edu.au/biostats/DAG-Stat*).
4. Sensitivity and specificity were calculated according to the following equations:

$$\%\ \text{of sensitivity} = \frac{\text{True positive}}{(\text{True positive} + \text{false negative}} \text{ x } 100$$

$$\%\ \text{of specificity} = \frac{\text{True negative}}{(\text{True negative} + \text{false positive}} \text{ x } 100$$

Worthy of note is that sensitivity indicates the ability to identify correctly those who have the disease (with less likelihood of getting false negatives), while specificity indicates the ability of the test to correctly identify those that do not have the disease (with less likelihood of getting false positives).

RESULTS

This study was conducted on 50 *M. tuberculosis* strains of Egyptian cases diagnosed as pulmonary tuberculosis, in the National Central Laboratory, Ministry of Health, Egypt, during the period of October 2014 to June 2015.

The colour Test TLA was performed on the 50 strains as a phenotypic susceptibility testing to detect resistance to RMP, INH and CIP. The proportional method (PM) was performed as the gold standard to evaluate the results of the colour test.

Table 1: Drug resistance among the tested strains by proportion method and Colour test:

Drugs	*Proportion method*		*Colour Test*	
	Number (50)	**%**	**Number (50)**	**%**
RMP	31	62%	28	56%
INH	31	62%	28	56%
CIP	20	40%	19	38%

(CIP= Ciprofloxacin, INH= Isoniazid, RMP= Rifampicin)

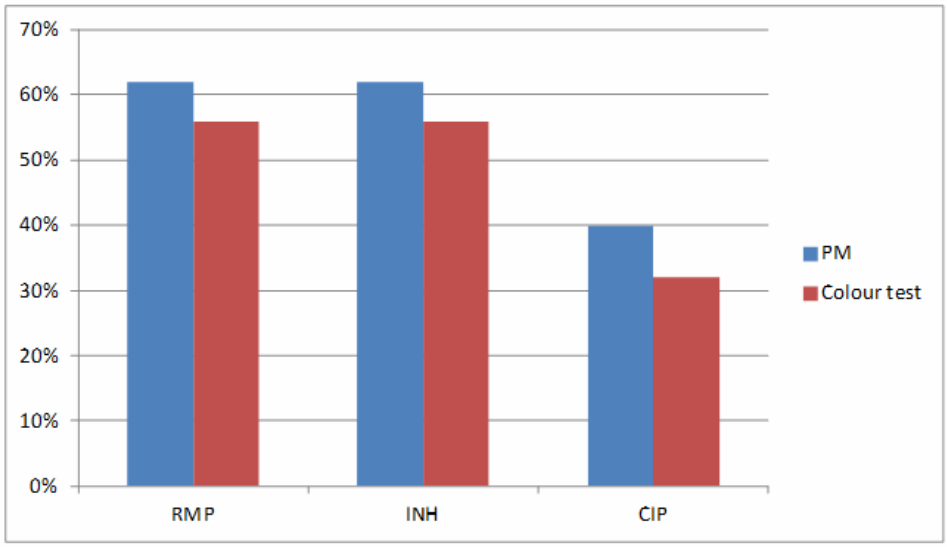

Fig. 1: Drug resistance among the tested strains by proportion method and colour test.

Table (1) and figure (1) show that the colour test missed the detection of 3 resistant strains to RMP and missed 3 resistant strains to INH. Regarding CIP, 1 strain was not detected by the colour test.

Table 2: Susceptibility testing results of RMP by proportional method and Colour test:

		Proportional Method (Gold Standard)		*Total*
		Sensitive	**Resistant**	
Colour test	**Sensitive**	19 (TN)	3 (FP)	22
	Resistant	0 (FN)	28 (TP)	28
Total		19	31	50

(FN= False negative, FP = False positive, TN = True negative, TP = True positive).

This table shows that the sensitivity and specificity of the colour test for RMP were 100% and 90.3% respectively. The agreement of the colour test with the PM was almost perfect agreement (87.6%).

Table 3: Susceptibility testing results of INH by the proportional method and the Colour test:

		Proportional Method (Gold Standard)		*Total*
		Sensitive	**Resistant**	
colour test	**Sensitive**	19 (TN)	3 (FP)	22
	Resistant	0 (FN)	28 (TP)	28
Total		19	31	50

(FN= False negative, FP = False positive, TN = True negative, TP = True positive).

The table shows that the sensitivity and specificity of the colour test for INH were 100% and 90.3% respectively. The agreement of the colour test with the PM was almost perfect agreement (87.6%).

Table (4): Susceptibility testing results of CIP by the proportional method and Colour test:

		Proportional Method (Gold Standard)		*Total*
		Sensitive	**Resistant**	
colour Test	**Sensitive**	29 (TN)	2 (FP)	31
	Resistant	1 (FN)	18 (TP)	19
Total		30	20	50

(FN= False negative, FP = False positive, TN = True negative, TP = True positive).

The table shows that the sensitivity and specificity of the colour test for CIP were 96.7% and 90% respectively. The agreement of the colour test with the PM was almost perfect agreement (87.4%).

Table 5: Comparison between results of Colour test for the 3 tested drugs:

Drug	*Sensitivity*	*Specificity*	*Agreement*	*PPV*	*NPV*
RMP	100%	90.3%	87.6%	90.3%	100%
INH	100%	90.3%	87.6%	90.3%	100%
CIP	96.7%	90%	87.4%	90%	96.6%

The table shows that the sensitivity of the Colour test for CIP (96.7%) was less than the sensitivity of the test for RMP and INH (100% for each one).

Table 6: Pattern of resistance among the tested strains:

	Proportional Method		*Colour Test*	
Type	**Number**	**Percentage**	**Number**	**Percentage**
• MDR:	27	54%	24	48%
Resistant to RMP + INH only	13	26%	11	22%
Resistant to RMP + INH + CIP	14	28%	13	24%
• **XDR:** (Resistant to RMP + INH + CIP + KAN)	2	4%	-	-

(CIP= Ciprofloxacin, INH= Isoniazid, MDR= Multi-drug resistant, PM= Proportional method, RMP= Rifampicin, XDR= Extensively-drug resistant).

Table 6 shows that 16 strains were resistant to RMP, INH and CIP (second line anti-tuberculous drug) by the proportional method. Those strains were further tested for susceptibility to one injectable drug of second-line drugs, Kanamycin (KAN), using the proportional method. Two strains out of the 16 strains were resistant to KAN, so they were considered XDR-TB.

DISCUSSION

The rapid diagnosis of MDR tuberculosis is of utmost importance to ensure early and appropriate intervention and prevent spread of the disease. This is particularly important in low-resourced countries where there is a pressing need for rapid detection of MDR with the least possible expense. In the last few years, several methods have been proposed for the rapid detection of drug resistant tuberculosis compared with conventional drug susceptibility testing that can take several weeks. These methods include new phenotypic techniques which are easier to perform and which shorten the turnout time for the diagnosis of MDR-TB [10].

The Colour test TLA is simple and easy to perform; it does not need special equipment for reading the results as the plate format is easy to read. It allows testing for both first-line and second-line-drugs in the same setting, helping in early detection of MDR-TB and screening for XDR-TB strains.

By the Colour Test TLA, results of drug susceptibility testing showed that 28 (56%) out of the 50 *M. tuberculosis* strains, were resistant to RMP and 28 (56%) were resistant to INH, while strains showing resistance to CIP were 19 (38%). On the other hand, by proportional method (PM) 31 (62%) strains were resistant to RMP, 31 (62%) resistant to INH and CIP resistant strains were 20 (40%) out of the 50 *M. tuberculosis* strains. Taking PM as the standard test to evaluate the performance of colour test, the sensitivity of colour test TLA was 100% for RMP and INH, and 96.7% for CIP. On the other hand its specificity was 90% for RMP, INH and CIP. The agreement of the colour test for each of the 3 drugs was 87.6% and the PPV was 90%. The NPV was 100%, 100% and 96.6% for RMP, INH and CIP respectively. As such, it can be concluded that the colour test is comparable with the proportional method in detecting anti-tuberculous drug resistance.

The main advantage offered by the colour test is time-efficiency. In our study, the colour test TLA gave results after 28 days, which although still faster than the proportion method in detection, yet is much longer than reported by Toit et al. [7] and Robledo et al. [11]. Indeed, Toit et al.[7] detected MDR strains by the colour test in 13 days, while Robledo et al. [11] reported that TLA method showed a rapid turnaround time (11 and 11.5 days for RMP and INH respectively). The discrepancy of turnaround time could be due to using isolated strains in our study, and not fresh specimens as done in the aforementioned studies. Further studies will be required

to determine if there is a difference in the effectiveness of the colour test performance on fresh strains as opposed to isolates.

The source of the studied strains (either obtained from fresh samples or isolates) may not only have an effect on the time required for growth, but may also impact the specificity of the tests. While our results are consistent with several other groups, slight differences are noted when the specimens are obtained from fresh samples as compared to isolates. For example, the same sensitivity results were obtained by Hernandez-Sarmiento et al. [12], when they performed TLA method on 100 patients diagnosed with TB. Taking the proportion method as the gold standard, they reported a sensitivity of 100% to each of RMP and INH. Regarding the specificity, it was 100% for each of RMP and INH, which is higher than the specificity reported in this study (90% for RMP and INH). Working on sputum specimens instead of isolates can explain the difference.

In this study, 24 (48%) strains were MDR-TB by the colour test TLA, while 27 (54%) strains were MDR-TB by the proportion method. Two strains (4%) were XDR-TB out of the 50 tested strains by the proportion method. This is in accordance with the study conducted by Campbell et al. [13] on 314 *M. tuberculos*is isolates, where they recorded that 163 (52%) were MDR-TB and 10 (3%) were diagnosed as XDR-TB. Similarly, the study conducted by Robledo et al., [11] on 95 sputum specimens in Colombia to evaluate the TLA test, reported that the sensitivity for RMP and INH were 100%. Also negative predictive values were 100% for RMP and INH in both studies.

In addition to the comparison of the colour test with the proportion method for detection of anti-tuberculous drug resistance, other groups conducted similar analyses using alternative techniques. Thus, in parallel with the current study, Martin et al.[14] studied 147 isolates of *M. tuberculosis*. Using the TLA method, they tested for resistance to RMP and CIP. The BACTEC MGIT960 was used as gold standard for RMP while the proportion method was used as gold standard for CIP. The sensitivity and specificity for RMP and CIP were 100%. This shows agreement with our results using the colour test method for DST. Furthermore, Toit et al.[7] reported agreement between the colour test and BACTEC MGIT 960 being 96%, 98% and 94% for RMP, INH and CIP respectively, while this study reported the agreement of the Colour Test with PM as 87.6%, 87.6% and 87.4% for RMP, INH and CIP respectively. Slight differences in the results can be explained by the use of different types of tests as gold standards for comparison.

CONCLUSION

In conclusion, the results of the current study corroborate those of several other groups in that the colour Test is a reliable, relatively rapid, time and resource-efficient way of detecting MDR *M. tuberculosis*. It is comparable to the proportion method, but simpler, less time-consuming and does not require special equipment or technology. It also allows the testing of first and second line drugs in the same setting. Further research will be required to determine the difference between the results obtained from fresh samples compared to isolated strains. Nonetheless, the significance of using reliable, simple, rapid techniques for detection of MDR TB is recognized as a global priority in the fight against tuberculosis, particularly in low-resourced, developing countries where tuberculosis poses an imminent threat to public health.

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