

Rapid and low-cost colorimetric method using 2,3,5-triphenyltetrazolium chloride for detection of multidrug-resistant *Mycobacterium tuberculosis*

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A rapid and inexpensive method for the detection of drug resistance in *Mycobacterium tuberculosis* is essential for the effective control of tuberculosis. The aim of this study was to evaluate a colorimetric method using 2,3,5-triphenyltetrazolium chloride (TTC) for antibiotic susceptibility testing of *M. tuberculosis* isolates. Eleven multidrug-resistant (MDR) isolates of *M. tuberculosis* and 12 isolates which were susceptible to rifampicin (RIF) and isoniazid (INH) were used. The test was performed with a critical concentration of 0.2 µg ml⁻¹ for INH and 2.0 µg ml⁻¹ for RIF in 7H9GC broth with 0.625 µg TTC ml⁻¹. Each isolate was inoculated under these conditions and inspected daily for colour changes; the results were obtained after a mean of 4.9 days. The sensitivity and specificity of this method were 100% and 92%, respectively, for both antibiotics. Considering the speed, technical ease and cost-effectiveness of this method, the TTC assay is a good alternative method for drug susceptibility testing of *M. tuberculosis* isolates.

INTRODUCTION

The spread of multidrug-resistant (MDR) *Mycobacterium tuberculosis* is of increasing public health concern in many parts of the world and the rapid detection of MDR isolates is critical for the effective treatment of affected patients (Tenover *et al.*, 1993). The method of proportion, which has been accepted as the gold standard, requires 3 weeks of incubation before an isolate can be determined as susceptible or resistant (Woods, 2000). The Bactec radiometric susceptibility method produces results in 7–10 days, but requires a heavy technical investment and is costly to perform (Ardito *et al.*, 2001). Molecular methods for the characterization of genes that confer resistance to first-line antimicrobial agents such as isoniazid (INH) (Musser *et al.*, 1996) and rifampicin (RIF) (Mokrousov *et al.*, 2003) are available; however, the equipment and specialized skills required to perform such methods make them an impractical option, especially in developing countries.

Colorimetric assays, using reagents such as Alamar blue (Franzblau *et al.*, 1998; Yojko *et al.*, 1995), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

(MTT) (Abate *et al.*, 2004; Martin *et al.*, 2005; Montoro *et al.*, 2005; Mshana *et al.*, 1998), 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) (De Logu *et al.*, 2002, 2003) and 2,3-diphenyl-5-thienyl-(2)-tetrazolium chloride (Lee *et al.*, 2006; Yamane *et al.*, 1996), have been proposed as a method for the detection of drug susceptibility. The yellow dye 2,3,5-triphenyltetrazolium chloride (TTC) is reduced in living cells by dehydrogenase to produce insoluble red TTC formazan crystals (Caviedes *et al.*, 2002; Denizot & Lang, 1986; Thom *et al.*, 1993). The only reported use of TTC has been for the detection of mesophilic anaerobic bacteria in the canning industry (Kvasnikov *et al.*, 1974).

In this study, we have evaluated the possibility of using the TTC colorimetric assay for detecting the susceptibility of *M. tuberculosis* to INH and RIF in liquid medium.

METHODS

***M. tuberculosis* isolates.** The *M. tuberculosis* isolates used in this study were recovered from patients with active clinical infections at the Iranian National Research Institute of Tuberculosis & Lung Disease, Tehran, Iran (2005–2006). These included 11 isolates of *M. tuberculosis* which were resistant to RIF and INH (MDR isolates), and 12 isolates susceptible to RIF and INH (sensitive isolates). Drug susceptibility testing of these isolates was performed using the proportion method with INH (0.2 µg ml⁻¹) and RIF (40 µg ml⁻¹) in

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Abbreviations: INH, isoniazid; MDR, multidrug-resistant; RIF, rifampicin; TTC, 2,3,5-triphenyltetrazolium chloride.

Löwenstein–Jensen medium. Resistance to any of the drugs tested was defined as $\geq 1\%$ growth on drug-containing medium compared with a control medium.

Preparation of inocula. Several loopfuls of growth from 14-day-old bacterial cultures on Löwenstein–Jensen medium were transferred to sterile tubes containing five to ten glass beads and 4 ml Middlebrook 7H9GC broth (Difco) supplemented with 10% oleic acid-albumin-glucose-catalase. The contents of the tubes were homogenized by vigorous agitation in a vortex mixer for 2–3 min. Tubes were allowed to stand for 20 min to allow larger particles to settle and the turbidity of the supernatant was adjusted to McFarland no. 1 standard (approx. 3×10^7 c.f.u. ml⁻¹) and then diluted to 3×10^6 c.f.u. ml⁻¹.

TTC preparation. TTC (Merck) was dissolved in sterile distilled water at a concentration of 5 mg ml⁻¹ at room temperature. The solution was filtered through a 0.22 µm filter and stored at -70 °C until needed.

Susceptibility test using TTC. The test was performed with a critical concentration of 0.2 µg ml⁻¹ for INH and 2.0 µg ml⁻¹ for RIF in 7H9GC broth (Syre *et al.*, 2003). For each isolate, panels of four tubes with 1.65 ml 7H9GC broth were used: one tube with 0.2 µg INH ml⁻¹, one tube with 2.0 µg RIF ml⁻¹ and two tubes without any antibiotic (for growth and contamination control). Each experimental tube received 100 µl of a 3×10^6 c.f.u. ml⁻¹ bacterial suspension (final concentration; 1.5×10^5 c.f.u. ml⁻¹). Next, 250 µl (0.625 mg ml⁻¹) of the TTC solution was added to each tube and the samples were incubated at 37 °C. After 48 h incubation, the tubes were inspected for colour change as indicated by the formation of a red ring at the bottom of the tube. In the absence of a colour change, incubation was continued and the tubes were inspected daily for a change in colour for up to 14 days, at which point the results were recorded and interpreted.

Culture tubes containing 7H9GC and TTC without drugs inoculated with each isolate were used as controls for assessing bacterial growth based on colour change. An uninoculated culture incubated under the same growth conditions served as a control for contamination, indicated by an absence of colour change.

Interpretation of results. An isolate was considered resistant to a drug when a colour change appeared in the drug-containing tube and growth control tube but not in the contamination control tube. Conversely, isolates were considered sensitive to a drug when no colour change appeared in the drug-containing and contamination control tubes, yet was observed in the growth control tube.

RESULTS AND DISCUSSION

In this study, among 12 bacterial isolates which were susceptible to INH by the proportion method, 11 were confirmed as susceptible and one as resistant using the TTC

method. The 11 INH-resistant isolates that were identified using the proportion method were also determined to be resistant using the TTC method. When the isolates were assessed for their ability to grow in the presence of RIF, there was complete agreement between the two methods for all 11 resistant isolates and for 11 of 12 susceptible isolates (Table 1).

The sensitivity (i.e. the ability to detect true drug resistance) and specificity (i.e. the ability to detect true drug susceptibility) of the TTC colorimetric method at critical drug concentrations were 100% and 92%, respectively, for both INH and RIF. The agreement observed between our colorimetric method using TTC and the proportion method suggests that this method represents an appropriate alternative for drug susceptibility testing. This finding is supported by previous studies. For example, Syre *et al.* (2003) suggested colorimetric nitrate reductase-based antibiotic susceptibility as a new method with 100% sensitivity and 95% specificity for INH, and 94% sensitivity and 100% specificity for RIF. Franzblau *et al.* (1998) reported a 93.6% consensus between the microplate Alamar blue assay and Bactec assay as a standard method. In a multicentre evaluation, Martin *et al.* (2005) evaluated MTT and resazurin assays for testing the susceptibility of *M. tuberculosis* to first-line antituberculosis drugs and obtained excellent results for RIF and INH with levels of specificity and sensitivity of between 96% and 99%, similar to our results.

The colorimetric TTC test results were available on the second day of incubation for two isolates (8.7%) out of the 23 tested in this study, on the fifth day for 18 isolates (78.3%) and on the sixth day for the remaining three isolates (13%) (Table 1). The results for detecting drug susceptibility were available after a mean of 4.9 days of incubation, similar to the 5 days required for the nitrate reduction assay (Syre *et al.*, 2003), but earlier than the 8 days necessary for the Alamar blue assay (Franzblau *et al.*, 1998). Therefore, the inexpensive and easy-to-use tetrazolium indicator method described in this study can replace Alamar blue and other rapid colorimetric methods, thereby yielding a more cost-effective and time-efficient method than those previously developed.

In conclusion, the shorter turn-around time, simple technique and cost-effectiveness make the TTC colorimetric

Table 1. Comparison of the proportion and TTC susceptibility methods and interpretation time for results in the TTC assay for detection of MDR *M. tuberculosis*

Susceptibility status of the isolates	No. of isolates by proportion method	No. of isolates by TTC method	Time of interpretation of result in TTC method		
			2 days	5 days	6 days
Sensitive isolate	12	11	2	8	1
MDR isolate	11	12	0	10	2

method a promising alternative for drug susceptibility testing of *M. tuberculosis* isolates in developing countries, where tuberculosis is an important and often fatal disease. Although the colorimetric method was tested here with only RIF and INH, the simplicity of this method may make it possible for testing first-, second- and third-line antimycobacterial agents on a routine basis, thereby allowing the rapid detection of MDR isolates of *M. tuberculosis* while at the same time providing valuable susceptibility data.

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