Rapid detection of multidrug-resistant *Mycobacterium tuberculosis* using the malachite green decolourisation assay

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Early detection of drug resistance in Mycobacterium tuberculosis *isolates allows for earlier and more effective treatment of patients. The aim of this study was to investigate the performance of the malachite green decolourisation assay (MGDA) in detecting isoniazid (INH) and rifampicin (RIF) resistance in* M. tuberculosis *clinical isolates. Fifty* M. tuberculosis *isolates, including 19 multidrug-resistant, eight INH-resistant and 23 INH and RIF-susceptible samples, were tested. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and agreement of the assay for INH were 92.5%, 91.3%, 92.5%, 91.3% and 92%, respectively. Similarly, the sensitivity, specificity, PPV, NPV and agreement of the assay for RIF were 94.7%, 100%, 100%, 96.8% and 98%, respectively. There was a major discrepancy in the tests of two isolates, as they were sensitive to INH by the MGDA test, but resistant by the reference method. There was a minor discrepancy in the tests of two additional isolates, as they were sensitive to INH by the reference method, but resistant by the MGDA test. The drug susceptibility test results were obtained within eight-nine days. In conclusion, the MGDA test is a reliable and accurate method for the rapid detection of INH and RIF resistance compared with the reference method and the MGDA test additionally requires less time to obtain results.*

Key words: *Mycobacterium tuberculosis* - malachite green decolourisation assay - susceptibility testing - isoniazid - rifampicin

Early detection of drug resistance in *Mycobacterium tuberculosis* isolates allows for appropriate and timely treatment (Martin et al. 2008). There are many well-defined phenotypic methods for testing the drug susceptibility of *M. tuberculosis*. The proportion method, performed on Löwenstein-Jensen and 7H10 or 7H11 agar media, is recommended as a reference method. However, this method requires at least three-six weeks to obtain results (Kent & Kubica 1985). There are also rapid automated systems for drug susceptibility testing. The BACTEC 460TB system (Becton Dickinson Diagnostic Systems, Sparks, MD, USA), which is no longer commercially available, is a semi-automated rapid system that is expensive and contains radioactive material. The BACTEC MGIT 960 system (Becton Dickinson Diagnostic Systems) is a non-radiometric rapid automated system. Finally, molecular methods of susceptibility testing are available, including the expensive commercial Xpert MTB/RIF and Genotype MTBDR*plus* assays (Bwanga et al. 2009, Friedrich et al. 2011, Chang et al. 2012).

New rapid, inexpensive, reliable and reproducible colourimetric methods have been recently developed. In particular, the nitrate reductase assay and resazurin microplate method have been commonly used (Angeby et al. 2002, Coban et al. 2004, Martin et al. 2007, 2011, Palomino et al. 2007, WHO 2010). Farnia et al. (2008) reported that the colourimetric malachite green decolourisation assay (MGDA) could be used for the rapid detection of resistance.

The aim of this study was to investigate the performance of the MGDA test for the detection of isoniazid (INH) and rifampicin (RIF) resistance in *M. tuberculosis* clinical isolates.

MATERIALS AND METHODS

Bacterial isolates - Fifty *M. tuberculosis* isolates were tested in this study. Nineteen isolates were multidrug-resistant (MDR), eight isolates were only resistant to INH and the remaining isolates were sensitive to both INH and RIF. The BACTEC 460TB system was used as the reference method for testing of the MDR isolates and the BACTEC MGIT 960 system was used as the reference method for testing the remaining isolates. The concentrations of INH and RIF for the BACTEC 460 TB/ BACTEC MGIT 960 systems were 0.1/0.1 and 2.0/1.0 µg/mL, respectively.

The H37Rv-ATCC 25618 (susceptible to all drugs), ATCC 35822 (resistant to INH), ATCC 35838 (resistant to RIF) and ATCC 35820 (resistant to STR) strains were used for quality control. Ethical approval was not required for this study.

Preparation of antibiotics and malachite green (MG) - The INH and RIF antibiotics used in this study were purchased from Sigma and the MG dye was from Merck (Germany). INH and MG were dissolved in sterile distilled water and RIF was prepared in methanol. Stock solutions were prepared as 1,000 µg/mL, 1,000 µg/mL and 50 µg/mL for INH, RIF and MG, respectively. All reagents were stored at -20ºC until use.

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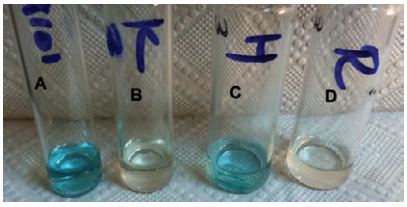
Preparation of bacterial inocula - Bacterial colonies were freshly grown on Löwenstein-Jensen media, transferred into tubes containing physiologic saline and 15-20 glass beads and vortexed for 30 sec. The tubes were kept in a vertical position for 30 min at room temperature to allow for the sedimentation of aerosols and large particles. The turbidity of the supernatant was adjusted to that of the McFarland 1 standard.

Preparation of media - All *M. tuberculosis* isolates were tested in Middlebrook 7H9S broth (containing 0.1% casitone, 0.5% glycerol and 10% OADC). INH and RIF were tested at the critical concentrations of 0.25 µg/mL and 0.5 µg/mL, respectively (Martin et al. 2011). An antibiotic-free growth control tube was also used for each bacterium.

Performing the MGDA test - Three tubes containing INH, RIF or antibiotic-free control growth medium were used for each bacterium. Fifty microlitres of inoculum adjusted to the McFarland 1 standard was added to each tube and the tubes were incubated at 37ºC. Following a seven-day incubation period, 150 µL of MG (50 µg/mL stock solution) was added into each test tube and the tubes were incubated for an additional 24-48 h. Once the MG decolourised in the growth control tube, the test tubes containing antibiotic were evaluated for colour change. If the MG decolourised as a result of bacterial growth in the tubes containing antibiotic, the bacterial sample was reported as resistant (Figure).



A: negative control (not contain drug and bacteria); B: positive growth control; C: isoniazid-susceptible (there is no growth, colour was not decolourised); D: rifampicin-resistant (decolourisation was observed due to bacterial growth).

RESULTS

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and agreement of the MGDA test for INH were 92.5%, 91.3%, 92.5%, 91.3% and 92%, respectively. Two isolates were sensitive to INH by the MGDA test, but resistant by the reference method. Two additional isolates were sensitive to INH by the reference method, but resistant by the MGDA test. For RIF, the sensitivity, specificity, PPV, NPV and agreement of the MGDA test were 94.7%, 100%, 100%, 96.8% and 98%, respectively. One isolate was resistant to RIF by the reference method, but susceptible by the MGDA test (Table). The results were obtained within eight-nine days.

DISCUSSION

Phenotypic methods that are inexpensive, rapid and reliable make susceptibility testing of *M. tuberculosis* easier and more efficient. Studies have shown that colourimetric tests are promising methods for drug susceptibility testing, particularly the resazurin microplate method, the resazurin tube method, the nitrate reductase test, the MTT and the XTT (Angeby et al. 2002, de Logu et al. 2003, Montoro et al. 2005, Coban et al. 2006, Bwanga et al. 2010, Dixit et al. 2012).

MG is a triphenylmethane dye that has antimicrobial effects. MG has been widely used to prevent fungal infections in fish. In contrast to most bacteria and fungi, mycobacteria are resistant to MG. Media for the cultivation of mycobacteria (Löwenstein-Jensen) contain MG to reduce overgrowth by contaminating microorganisms. MG can be reductively decolourised. Mycobacterial resistance to MG may be due to dye reduction and sequestration in the lipid fraction of the cells (Jones & Falkinham 2003).

Gelman et al. (2012) reported that MG interfered with the recovery of mycobacteria on solid culture media following exposure to certain antibiotics, including INH and ethionamide (ETM), that target cell wall biogenesis. This interference did not affect the test results for INH sensitivity because MG was used to determine the viability of bacteria in this test.

Farnia et al. (2008) reported that MGDA could be used for the rapid detection of drug susceptibilities of *M.*

TABLE

Comparing the results of malachite green decolourisation assay (MGDA) with those obtained with reference method

Drug	MGDA	Reference method (n)		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Agreement (%)
		R	S					
INH	R	25	2	92.5	91.3	92.5	91.3	92
	S	2	21					
RIF	R	18	0	94.7	100	100	96.8	98
	S	1	31					

INH: isoniazid; NPV: negative predictive value; PPV: positive predictive value; R: resistant; RIF: rifampicin; S: susceptible.

tuberculosis clinical isolates; the sensitivity, specificity and agreement of the MGDA test were found to be 100% for both RIF and INH and the results were obtained within six-17 days (average of 12 days). In our study, the INH-RIF sensitivities, specificities, PPVs, NPVs and agreements of the MGDA test were 92.5-94.7%, 91.3-100%, 92.5-100%, 91.3-96.8% and 92-98%, respectively and the results were obtained within eight-nine days. There was a major discrepancy in the tests of two isolates that were sensitive to INH by the MGDA test, but resistant by the reference method. In addition, there was a minor discrepancy in the tests of two additional isolates that were sensitive to INH by the reference method, but resistant by the MGDA test.

There is an inconsistency in the first study published by Farnia et al. (2008). The group reported that 50 µL of a 0.02 µg/mL MG stock solution was used for the direct susceptibility test, whereas 50 µL of a 0.02 mg/mL MG stock solution was used for the indirect susceptibility test. Therefore, the concentration of the stock solution used in the study is not clear. Our preliminary studies indicated that using a 0.02 µg/mL stock solution was not suitable for the MGDA test; an MG titration showed that 150 µL of a 150 µg/mL stock solution was suitable for the susceptibility testing using 1 mL test tubes (Figure). Mirabal et al. (2010) reported that MGDA could be used for the rapid detection of pyrazinamide resistance. They used 50 µL of a 50 µg/mL MG solution in their study. No additional studies using MGDA were found in searches of Medline, PubMed, ISI Web, Web of Science and Google Academic databases.

In conclusion, the MGDA test for the rapid detection of INH and RIF resistance is an inexpensive, easy to perform and interpretable method that can be used in laboratories for routine drug susceptibility testing.

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