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A high throughput methodology for susceptibility testing of *Mycobacterium tuberculosis* isolates

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ABSTRACT

MICs for eleven anti-TB drugs with *M. tuberculosis* isolates were obtained by means of agar dilution with multi-point inoculation. The results were compared with classic agar dilution and the MTT assay. The multi-point inoculation method was reproducible with all drugs and correlated with classic agar dilution and MTT assay. This methodology can be used for routine breakpoint drug susceptibility testing (DST) and for MIC determination.

1. Introduction

Tuberculosis (TB) is the leading cause of death by an infectious agent killing 1.8 million worldwide in 2015 (World Health Organization, 2016). Developing countries bear the heaviest burden with Asia and Africa accounting for 61% and 26% respectively of new cases annually (World Health Organization, 2016).

Factors associated with drug resistant TB are HIV infection, non-adherence to control programs and delay or absence of laboratory diagnosis (Kohli et al., 2016; Tadesse et al., 2016). Primary resistance, defined as acquisition of an already resistant strain, is rapidly emerging (O'Donnell et al., 2010). In under-resourced countries, the diagnosis of TB relies solely on microscopy while drug-susceptibility testing (DST) is not done. With classic DST the results become available with considerable delay. To control drug resistant TB, faster and inexpensive methods are needed (Kohli et al., 2016).

The gold-standard DST methods include culture-based methods like the proportion method, resistance ratio and absolute concentration method (Kim, 2005). These are labour intensive and rely on visualizing growth of *M. tuberculosis* which takes 3–6 weeks (Van Klingeren et al., 2007). The more recently developed colorimetric assays include the Microplate Alamar Blue Assay (MABA), the Resazurin Microplate Assay (REMA), the 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and the Nitrate Reductase Assay (Collins and Franzblau, 1997; Kohli et al., 2016). The Microscopic Observation Drug Susceptibility (MODS) assay is a rapid and cheap technique. However, it needs experienced and trained staff to conduct (Moore et al., 2006). These colorimetric assays and MODS are rapid assays but they pose a biohazard risk due to the formation of aerosols (Kohli et al., 2016). The MGIT 960, the BacT/ALERT 3D and ESP culture system II are automated liquid culture systems that use expensive equipment, which makes these unsuitable for under-resourced countries (Kohli et al., 2016). Genotypic DSTs such as the GeneXpert® system and line probe assays are also expensive, detect only identified resistance conferring mutations, require expertly trained staff and detect both viable and dead *M. turberculosis* (Watterson et al., 1998; Boehme et al., 2010).

Agar dilution with multi-point inoculation is a phenotypic, culture-based method that is able to test a large number of isolates at a time, using less materials and inexpensive equipment (Faiers et al., 1991). We report on the reproducibility of agar dilution with multi-point inoculation for DST of *M. tuberculosis* and compare this method with agar dilution on quadrant plates and MTT.

2. Methods

Thirty *M. tuberculosis* isolates with known resistance profiles were used. The resistance conferring genes of these isolates have been sequenced previously (Dookie et al., 2014). Two strains were fully susceptible, 13 MDR and 14 XDR. Multidrug resistant isolates (MDR) are resistant to the first-line drugs isoniazid and rifampicin, extensively drug resistant (XDR) isolates are MDR isolates with additional resistance to the fluoro-quinolones and to at least one of the injectables used for treatment of MDR-TB (kanamycin, amikacin or capreomycin). H37Rv was included as susceptible control. Organisms were inoculated in 10 mL Middlebrook 7H9 broth containing 10% oleic acid-albumin-dextrose-catalase (OADC), 0.2% glycerol and 0.05% Tween 80. Broths

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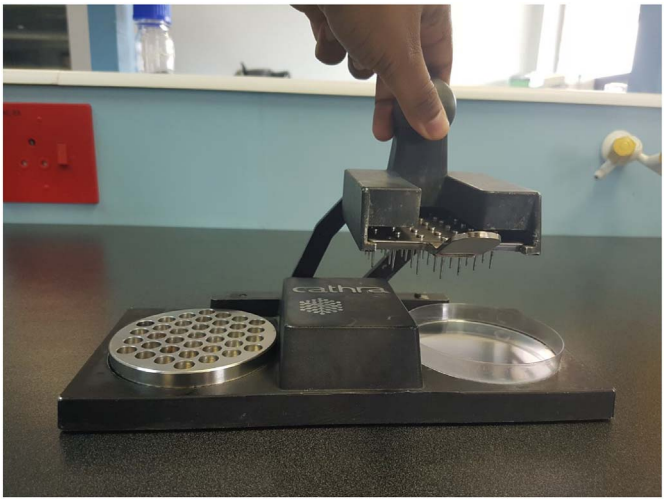

Fig. 1. Multipoint inoculator.

were incubated at 37 °C until an OD_{600nm} reading of 0.7–1 was reached. Each culture was sonicated at 5 A for 20 s and left undisturbed at room temperature for 15 to 20 min for clumps to settle. The top-layer was siphoned off and diluted till OD_{600nm} reading of 0.7. Agar dilution with multi-point inoculation was used to determine the MIC of isolates for 11 different antimicrobial drugs. Two-fold dilutions of the drugs were made in sterile OADC (10% v/v) enriched Middlebrook 7H10 agar supplemented with casitone (10% w/v) and glycerol (0.2% v/v). The concentrations ranged from 128 mg/L to 0.125 mg/L for all 11 drugs. The agar containing the drug dilutions were poured into 90 mm Petri dishes and allowed to solidify.

The strains were inoculated onto the plates using a multipoint inoculator with pins delivering 1 μL, containing approximately 10 cfu, per spot. The plates were incubated at 37 °C for 21 days.

Multipoint inoculators exist as hand operated and as automated devices. We used a hand operated device as shown in Fig. 1. These devices consist of a base with an inoculation arm and a suspension holder. The suspension holder contains a number of wells that varies depending on the make. The inoculation arm has two parts: the movable part and the pin-holder plate. The pin-holder plate has holes equal in number to the number of wells in the suspension holder and these pins are positioned in such a way that each of these sinks into a well of the suspension holder. Each pin has a calibrated flat surface at the bottom and a weight fixed to the top. During inoculation the pin is firmly pressed onto the agar delivering the full volume of the suspension attached to it. The weight has been chosen in such a way that the pressure of the pin does not damage a 1,5 to 2% agar layer. On touch, the pin moves upwards in the hole. Both the detachable pin-holder plate and the suspension holder are autoclaved between batches of isolates to be tested. The inoculation arm is moved from the suspension side with the agar plate to be inoculated and back. When MIC tests are performed, the antibiotic containing plate is replaced with one with the next concentration. No decontamination or sterilization is needed in between. For breakpoint DST, one to three concentrations are used in the same way. Flaming the pins with alcohol in between batches is sufficient during one session.

To validate the results obtained with multi-point inoculation, two established methods for MIC determination were performed with a selection of the strains using three of the antimicrobial drugs: isoniazid, kanamycin and ciprofloxacin. These methods were agar dilution on quadrant plates (QPM: quadrant plate method) and micro-broth dilution with MTT growth detection.

The QPM differs from the multi-point inoculation method in that the surfaces of the agar quadrants were flooded with 100 μL of the bacterial suspension containing approximately 1×10^3 cfu. The plates were incubated at 37 °C for 21 days.

For the MTT, micro-broth dilution was performed in Middlebrook 7H9 broth with 10% OADC, 0.2% glycerol and 0.05% Tween 80 in flat-bottom 96 well plates. Drug concentrations were as in both agar dilution methods. The inoculum was approximately 0.5×10^3 cfu/well. After incubation for 7 days at 37 °C, 20 μL of the MTT solution (5 μL/mg) was added to each well followed by incubation for a further 24 h at 37 °C. Purple precipitates were then observed to indicate growth. A solution containing equal parts of 20% sodium dodecyl sulphate (SDS) and 50% dimethyl formamide (DMF) was added to dissolve the precipitate to facilitate reading with a spectrophotometer (Moodley et al., 2014).

All tests were performed in a class II B2 biosafety cabinet with UPS back up.

Statistical analysis was done using an ordinal regression model (GEE). An intra-class correlation (ICC) of 1 indicates the replicates are identical. An ICC between 0.8 and 1 indicates good intra-class correlation.

3. Results and discussion

Table 1 shows the MICs of the 30 *M. tuberculosis* isolates obtained with agar dilution with multipoint inoculation. The tests were performed three times in triplicate to determine reproducibility. Identical results were found with streptomycin, kanamycin and amikacin (ICC = 1; $p < 0.001$). Poor reproducibility was found with ethambutol (ICC = 0.4; $p = 0.7$). The ICC values for the seven other drugs showed good correlation with ICC values varying between 0.8 and 0.95.

Differences in no. of isolates for isoniazid, rifampicin, ethambutol and clofazimine are due to some isolates being contaminated in one or more of the 9 replicates.

To validate the results, 11 of the isolates were tested in triplicate against isoniazid, kanamycin and ciprofloxacin, comparing the agar dilution with multipoint inoculation with the QPM and MTT test. The results are shown in Table 2. The multipoint inoculation technique showed similar results as those presented in Table 1. ICC values for isoniazid obtained with the QPM were within the satisfactory range ($p = 0.03$) and were good for the other two drugs. However, MICs obtained were 1 to 4 dilutions higher than with the multipoint inoculation method. The MTT test results were highly reproducible for all 3 drugs. The MICs obtained with this method were lower than with the quadrant plate method but higher than with multipoint inoculation.

Multipoint inoculation is a well-established method to inoculate agar plates with multiple bacterial isolates (Steers et al., 1959). The technique is used to determine MICs of fastidious organisms like *Neisseria gonorrhoeae*, *Haemophilus influenzae* and strict anaerobes. Incubation beyond 16 to 24 h diminishes the reliability of disc diffusion susceptibility testing. Since *M. tuberculosis* is a slow growing organism,

Table 1
MICs (mg/L) of 30 *M. tuberculosis* isolates for 11 anti-TB drugs using agar dilution with multipoint inoculation.

	No. of isolates	Median	Range	ICC	95% Cl	*p*-Value
Isoniazid	27	16	8–32	0.8	0.7 to 0.9	< 0.001
Streptomycin	30	16	0.5–128	1	0.9 to 0.9	< 0.001
Rifampicin	29	128	2–128	0.99	0.9 to 0.9	< 0.001
Ethambutol	29	32	4–32	0.4	0.06 to 0.7	0.731
Ethionamide	30	64	2–128	0.9	0.8 to 0.9	< 0.001
Kanamycin	30	4	1–128	1	0.9 to 0.9	< 0.001
Amikacin	30	4	0.5–128	1	0.9 to 0.9	< 0.001
Capreomycin	30	4	0.5–32	0.95	0.9 to 0.9	< 0.001
Ciprofloxacin	30	2	0.125–4	0.9	0.8 to 0.9	< 0.001
Ofloxacin	30	1	0.5–4	0.89	0.8 to 0.9	< 0.001
Clofazimine	27	1	0.125–16	0.8	0.6 to 0.9	< 0.001