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Evaluation of the BACTEC MGIT 960 system and the resazurin microtiter assay for susceptibility testing of *Mycobacterium tuberculosis* to second-line drugs

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ABSTRACT

Drug resistance in tuberculosis is a major threat to public health and control of the disease worldwide. Given the need of a rapid and accurate detection of *Mycobacterium tuberculosis* resistance to second-line drugs, this study evaluated the performance of the BACTEC MGIT 960 for second-line, drug susceptibility testing in comparison with the resazurin microtiter assay (REMA), in order to implement the automated methodology in the diagnostic routine of a reference laboratory. Drug susceptibility testing (DST) for second-line drugs of 151 MDR *M. tuberculosis* clinical isolates was performed by both BACTEC MGIT 960 and REMA, and a panel of 26 *M. tuberculosis* reference isolates from a proficiency test was tested by the BACTEC MGIT 960. DST for second-line drugs by the BACTEC MGIT 960 system was more rapid, highly reproducible and showed 100% of proficiency. After these results, this methodology was successfully implemented in our diagnostic routine for all MDR-TB patients.

1. Introduction

Drug resistance in *Mycobacterium tuberculosis* is a major threat to public health. In 2015, it was estimated that 3.9% of new cases and 21% of previously treated cases of tuberculosis (TB) had multidrug-resistant (MDR) or rifampicin-resistant TB, and 9.5% of people with MDR-TB had extensively drug-resistant TB (XDR-TB) (WHO, 2016). In this context, drug susceptibility testing (DST) against second-line TB drugs is a very important tool for improving the treatment and control of TB worldwide (Lee et al., 2014).

Molecular assays, such as the Genotype MTBDR*sl* (Hain Lifescience, Nehren, Germany), provide second-line DST results in days, but they are not always cost-effective, mainly in low- and middle-income countries, as is the case of Brazil. Moreover, performing molecular assays demands a more complex laboratory infrastructure and trained personnel.

The Instituto Adolfo Lutz (IAL) is the reference TB laboratory in the state of São Paulo, located in the southeast region of Brazil. Every year, São Paulo notifies 17,000 new cases of TB, which represent 20% of the total number of new cases notified in Brazil (Ministério da Saúde, 2017).

In 2008, the WHO stated that few laboratories in the world had the capacity and qualifications required to reliably perform DST for second-line drugs (WHO, 2008). Based on this document, in 2010, Brazil's TB Reference Center, Prof. Hélio Fraga, published a Technical Note recommending the BACTEC MGIT 960 (BD, Maryland, USA) automated system for performing DST to second-line drugs in laboratories that had this methodology standardized and had proven proficiency with a panel of reference strains (Ministério da Saúde, 2010).

Before this recommendation, the IAL used to perform the resazurin microtiter assay (REMA) for second-line drugs only for a few MDR cases and upon medical request. Although the REMA has been largely used for determining minimum inhibitory concentration (MIC) of drugs against many bacterial species, it has low reproducibility (Lee et al., 2014). Furthermore, the REMA is not suitable at a reference laboratory that performs DST of nearly 4000 *M. tuberculosis* isolates per year, as it requires many manipulation steps and represents a biosafety risk to the technician.

In light of this, the aim of our study was to evaluate the BACTEC MGIT 960 system for DST of MDR *M. tuberculosis* clinical isolates against second-line drugs in our routine, and to compare this methodology with the REMA.

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2. Materials and methods

This work was approved by the Scientific Technical Committee of the IAL, São Paulo, Brazil (no 113D/2012).

2.1. Clinical isolates

The IAL routinely performs DST against the first-line drugs (streptomycin, isoniazid, rifampicin and ethambutol) of *M. tuberculosis* isolated from patients from the state of São Paulo, by the automated BACTEC MGIT 960. In order to standardize this methodology for second-line drugs, this study included 151 MDR isolates tested by the IAL in 2008. These isolates were stored at − 70 °C in Sauton medium with 10% of glycerol, according to Giampaglia et al. (2009), and reactivated in Middlebrook 7H9 medium (BD Difco™) supplemented with OADC (BD BBL™), which is known as 7H9-S medium. The cultures were incubated aerobically at 37 °C for 20–30 days.

We also tested, for second-line drugs, 26 *M. tuberculosis* reference strains received from the National TB Reference Center Laboratory in Brazil, Prof. Hélio Fraga, as part of a proficiency test, by the BACTEC MGIT 960, in order to validate this methodology in our routine. These strains were provided by the Supranational Reference TB Laboratory, Dr. Carlos G. Malbrán, from Buenos Aires, Argentina.

2.2. Drugs

Drugs were obtained from Sigma (Sigma Chemical Co, St Louis, MO, USA) in powder form. Stock solutions were prepared according to Clinical and Laboratory Standards Institute (CLSI, 2012) in the following concentrations: 21 mg/ml for capreomycin, 8.4 mg/ml for amikacin, 21 mg/ml for kanamycin and 16.8 mg/ml for ofloxacin, and stored at − 20 °C. Capreomycin, amikacin and kanamycin were dissolved in sterile distilled water, and ofloxacin was dissolved in 0.1 N NaOH with subsequent dilutions in sterile distilled water. Final concentrations used in MGIT were: 2.5 μg/ml for capreomycin, 1 μg/ml for amikacin, 2.5 μg/ml for kanamycin and 2 μg/ml for ofloxacin (Rodrigues et al., 2008).

2.3. Susceptibility testing by the BACTEC MGIT 960

DST for second-line drugs was performed according to the protocol described by Adami et al. (2017). For each isolate, six MGIT tubes were used: one as the growth control, one tube for each drug and one tube containing a final concentration of 500 μg/ml of p-nitrobenzoic acid (PNB), in order to differentiate *M. tuberculosis* from non-tuberculous mycobacteria. Briefly, the growth of the isolates reactivated in MGIT was adjusted to the 1.0 MacFarland standard. Then, a 1:5 dilution was prepared with sterile distilled water, from which 0.5 ml was inoculated into the drug- and PNB-containing tubes. As of the 1:5 dilution, a 1:100 suspension was prepared, and then 0.5 ml of this suspension was inoculated into the growth control tube. For each batch of tests, the reference strain H37Rv, which is maintained at our strain collection at − 70 °C and is susceptible to all drugs tested, was reactivated in Löwenstein-Jensen and included. All bacterial suspensions used in DST were checked for purity on BHI agar (Oxoid). Incubation of tests into the MGIT instrument was performed according to Rüsch-Gerdes et al. (2006). The inoculated tubes (growth control and tubes with drugs) were placed in a DST set carrier with five positions and entered into the instrument as 'unknown drugs'. When the growth control reached a growth unit (GU) of 400, the instrument flagged the test set as complete. The complete tests were removed from the instrument and the results were read on EpiCenter platform, version 6.20A (BD, Maryland, USA). If the GU of the drug-containing tubes was > 100 when the GU of the growth control was 400, the results were defined as resistant. If the GU values of the drug-containing tubes were ≤ 100, the results were considered susceptible. Tubes containing PNB were incubated separately and finalized manually after three days of DST completion.

2.4. Susceptibility testing by the resazurin microtiter assay (REMA)

Second-line drugs were tested according to Palomino et al. (2002). Serial dilutions of each drug were prepared directly in sterile 96-well flat-bottom microtiter plates in a final volume of 100 μl of 7H9-S medium. Drug concentrations used were 10 to 0.3 μg/ml for capreomycin; 8 to 0.25 μg/ml for amikacin; 20 to 0.6 μg/ml for kanamycin; and 8 to 0.25 μg/ml for ofloxacin. For inoculum preparation, the mycobacterial growth in MGIT was adjusted to the 1.0 MacFarland standard. This suspension was diluted 1:20 in 7H9-S medium and then 100 μl was used as the inoculum. Growth controls containing no drug and sterility controls without inoculation were also included. The plates were covered, sealed and incubated at 37 °C for 10 days. After this period, 30 μl of a resazurin solution at 0.01% was added to each well. The plates were then incubated overnight and assessed for color development. Alteration from blue to pink indicates reduction of resazurin and therefore, bacterial growth. The MIC was defined as the lowest drug concentration that inhibited bacterial growth, in comparison to the growth control well. Breakpoints for each drug were the same as used for DST by the BACTEC MGIT 960.

The final results of susceptibility or resistance were considered according to the MIC. When the MIC was lower than the breakpoint for a drug, the isolate was considered susceptible; when the MIC was higher than the breakpoint, the isolate was considered resistant.

The REMA was considered concordant with the BACTEC MGIT 960 when the final result remained susceptible or resistant, independently of the MIC.

Regarding reproducibility, when the MIC ranged until two dilutions, we considered the REMA technique as reproducible. When the variation was equal or above three dilutions, the technique was considered as non-reproducible.

2.5. Data analysis

Results were analyzed using Fleiss' kappa (κ) and agreement (Landis and Koch, 1977) for each tested drug. κ-Values were interpreted as follows: 0.21–0.40, fair agreement; 0.41–0.60, moderate agreement; 0.61–0.80, substantial agreement; and 0.81–1.00, nearly perfect agreement.

3. Results

Among the 151 MDR clinical isolates tested against second-line drugs, 18 (11.9%) were XDR by the BACTEC MGIT 960, while only 3 (2%) of them were XDR by the REMA also. Both methodologies were repeated for the 15 discordant isolates and the results remained the same.

Comparing both methodologies, 122 (80.8%) isolates exhibited concordant results (Table 1) and 29 (19.2%) isolates exhibited discordant results (Table 2). Of the 122 isolates with concordant results, 115 (94.3%) were susceptible to all the drugs tested, while 7 (5.7%)

Table 1
Concordant results between the BACTEC MGIT 960 and the resazurin microtiter assay.

Results	No of isolates (%)
Susceptible to all drugs	115 (94.3)
Resistant to OFX	3 (2.5)
Resistant to AMK + KAN	1 (0.8)
Resistant to AMK + KAN + OFX	1 (0.8)
/18//Resistant to all drugs	2 (1.6)
Total	122 (100)

CAP = capreomycin; AMK = amikacin; KAN = kanamycin; OFX = ofloxacin.