REPORT

New methods for the detection of resistance in Mycobacterium tuberculosis

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ABSTRACT

The detection of drug resistance in tuberculosis takes several weeks with conventional methods, and there is a high cost associated with the use of faster methods like commercial kits or molecular techniques. This work evaluates the nitrate reductase method (NRM), the MTT colorimetric assay and resazurin (RES) as alternative assays for the rapid detection of resistance to first line antituberculosis drugs, using the method of the proportions (MP) as a reference. The inhibitory concentrations obtained for the colorimetric assays were 0.25 μg/mL or higher for isoniazide (INH), 1 μg/mL or higher for streptomycin (STR), 4 μg/mL or higher for ethambutol (ETH), and 0.25 μg/mL or higher for rifampicin (RIF). The sensitivity of the assays was higher than 90.0% for all the drugs; the specificity was higher than 88.5% for INH, STR and RIF but lower (higher than 57.8%) for ETH. The NRM showed good correlation for the 4 drugs (sensitivity and specificity higher than 93.7%), and the concordance was 88.2%, 90.7% and 98.2% for RES, MTT and NRM, respectively. This study shows a high level of agreement between the new methods and the MP for the detection of resistance to INH and RIF, and therefore proves their validity as alternatives that can be easily implemented at low-budgeted laboratories.

*I*ntroduction

The conventional methods for the detection of resistance to antituberculosis drugs are based on the growth of mycobacteria in culture media containing them. This implies that obtaining a result with these methods usually takes several weeks. The use of faster commercial systems or molecular techniques, on the other hand, often requires a previous investment in technology and equipment that is beyond the reach of most labs located in poor countries, which are precisely those most affected by tuberculosis (TB) and the appearance of multidrug resistance (MDR).

There is a lot of recently published research concerned with the development of fast and low-cost methodology for the detection of resistance to antimycobacterial drugs, such as colorimetric methods that employ oxidation-reduction status indicators. For example, in 1998 the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was described for this purpose, and, more recently, the application of resazurin (RES) for studies of susceptibility to first and second line antibiotics has been reported [1-3].

Other procedures that have yielded very encouraging results are the nitrate reductase method (NRM) for studies of susceptibility to first line drugs, as well as the phage amplification technique for the detection of susceptibility to rifampicin (RIF) [4, 5].

The phenomenon of mycobacterial resistance to antituberculosis drugs does not constitute a significant health problem in Cuba. However, there is a need for diagnostic tools that provide reliable susceptibility results due to the rising incidence, on a worldwide scale, of MDR. The goal of this study, on a first stage, is to use the nitrate reductase method and the MTT and resazurin (RES) colorimetric assays for the detection of resistance to RIF (which constitutes a strong indicator of MDR) in order to later evaluate these techniques for the detection of resistance to other first line antituberculosis drugs.

The results of this research were awarded in 2004 the National Prize from the National Academy of Sciences of Cuba, under the title "Low circulation of multidrug resistant *Mycobacterium tuberculosis* strains in Cuba. New methods for the detection of resistance".

*M*aterials and methods

Strains

One-hundred and twenty strains of *M. tuberculosis* were studied, belonging to the collection of the National Tuberculosis and Mycobacteria Reference and Research Laboratory from IPK. Twenty strains were used for the evaluation of resistance to RIF, and the remainder was challenged with the 4 first line antituberculosis drugs. The reference *M. tuberculosis* strains H37Rv (ATCC 27294, sensitive to isoniazide (INH), streptomycin (STR), ethambutol (ETH) and rifampicin (RIF)), and ATCC 35822, ATCC 35820, ATCC 35837 and ATCC 35838 (resistant to INH, STR, ETH and RIF, respectively) were used.

Antituberculosis drugs

A solution at 1 000 μg/mL in sterile distilled water was prepared for INH, STR and ETH. For RIF, a 1:2 methanol-sterile distilled water mixture was used as the solvent.

MTT

A solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide at a concentration of 5 mg/mL

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was prepared in PBS, filtered and stored at 4 ºC protected from light. The solubilization of formazan (reduced MTT crystals) was accomplished with a 1:1 mixture of 20% dodecyl sodium sulphate and 50% N,N-dimethylformamide.

Resazurin

A 0.01% solution of RES was prepared in sterile distilled water, filtered and stored at 4 ºC, protected from light.

Nitrate reductase method

The NRM was performed following the methodology described by Ängeby *et al*., 2002 [4]. Löwenstein-Jensen (LJ) medium was used, with a KNO_3 concentration of 1 000 µg/mL. The same critical concentration employed in the method of the proportions (MP) was used for each drug. The turbidity of the inoculum was adjusted using tube 1 of the McFarland scale as a reference, followed by dilution 1:10 into PBS. The 3 control tubes were inoculated with 200 µL of the diluted bacterial suspension, and the tubes containing the drug were inoculated with 200 µL of the inoculum adjusted with tube 1 of the McFarland scale. An incubation at 37ºC for 7 days was used, after which one of the control tubes was developed by adding 500 µL of a mixture of one part 50% hydrochloric acid, two parts of 0.2% sulfanilamide, and two parts of 0.1% N-1-naphthylethylenediamine dihydrochloride. For those strains in which the control tubes developed a pink color, their drug tubes were also developed; otherwise, the tubes were reincubated to be developed at days 10 and/or 14. A strain was classified as resistant when the intensity of the color in the developed drug tube was more intense than that of the control tube; and classified as sensitive if otherwise.

Colorimetric assays

The colorimetric MTT and RES assays followed the methodology described by Abate *et al.* [1] and Palomino *et al.* [2]. One-hundred µL of Middlebrook 7H9 medium were dispensed into each of the 96 wells of a sterile plate, and double serial dilutions of each drug were performed until the working concentration established for each of them was reached (1-0.0312 µg/mL for INH, 8-0.25 µg/mL for STR, 32-1 µg/mL for ETH and 2-0.0625 µg/mL for RIF). Each well also received 100 µL of the inoculum, consisting of a 1:20 dilution of a bacterial suspension adjusted to the turbidity of tube 1 of the McFarland scale. Growth control wells were also included for each evaluated strain, and the wells on the periphery of the plate received 200 µL of sterile distilled water to compensate for evaporation losses during incubation. The plates were sealed, incubated at 37 ºC, and developed after 7 days by the addition of 10 µL of MTT or 30 µL of RES. A change in coloration from blue to pink or from yellow to violet indicated the reduction of RES or MTT, respectively, indicating cellular viability. The minimum inhibitory concentration was defined as the lowest drug concentration yielding no change in color.

Method of the proportions

The MP followed the methodology described by Canetti *et al*. 1963 [6], using the critical concentrations recommended for each drug: 0.2 µg/mL, 4 µg/mL, 2 µg/mL and 40 µg/mL for INH, STR, ETH and RIF, respectively.

Statistical analysis of the results

The results were processed using the statistical MEDCALC application software, using as a reference the results obtained with the MP. Curves of the receptor operating characteristics (ROC curves) were made for each drug in order to estimate the cut-off points for the colorimetric assays and for the calculation of the figures for sensitivity, specificity and the coefficient of concordance.

Results

The use of the colorimetric assays and the NRM on the 20 studied strains allowed the verification of the usefulness of these techniques for the fast detection of mycobacterial resistance to RIF. The concordance obtained when comparing the results with those of the reference method was 100% [7].

Based on the study of 100 strains of *M. tuberculosis*, the established cut-off points for the RES and MTT assays were higher than 0.25 µg/mL, higher than 1 µg/mL, higher than 4 µg/mL and higher than 0.25 µg/mL for INH, STR, ETH and RIF, respectively. The differences for the area under the curve were not statistically significant between both methods, with $p > 0.05$ and a 95% confidence interval.

Table 1 shows the statistical parameters obtained for each drug with the colorimetric assays and the NRM. The colorimetric assays had high sensitivity and specificity values for INH and RIF, but had low specificity for STR and ETH due to a high number of false positives. In the case of the NRM these parameters were higher than 93.7% for all the drugs, and both had a value of 100% for RIF. The global concordance reached in the study was 88.2%, 90.7% and 98.2% for the RES, MTT and NRM assays, respectively [8].

Discussion

A varied number of methods are currently available for the evaluation of the susceptibility of *M. tuberculosis* to antimycobacterial drugs, but none is perfect and their results have not yet convinced the clinicians on the need for effective treatments that can curb the spread of resistant strains [9]. Conventional techniques based on the culture of mycobacteria are available since years ago and are used in most laboratories, specially the MP, described by Canetti *et al*. in 1963 [6], that still constitutes the current reference standard for studies of susceptibility in TB. These techniques are highly laborious and have the disadvantage of requiring

Table 1. Statistical parameters obtained from the study of 100 *M. tuberculosis* strains.

Anti-TB Drugs	Sensitivity (%)			Specificity (%)			Concordance (%)		
	RES	MTT	MNR	RES	MTT	MNR	RES	MTT	MNR
INH	100	100	95.6	96.4	96.4	100	98.0	98.0	98.0
STR	93.8	91.7	93.7	88.5	88.5	98.0	91.0	90.0	96.0
ETH	94.1	94.1	100	57.8	71.1	97.8	64.0	75.0	99.0
RIF	100	100	100	98.4	100	100	99.0	100	100

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incubations of 4 to 6 weeks for interpreting the results, due to the slow growth rate of *M. tuberculosis*.

The recent rise in MDR TB cases worldwide and its implications for the control of this disease, as well as the length of the mycobacterial culture-based conventional methodologies, have motivated the development of faster alternatives for the detection of resistance to antituberculosis drugs. An important group among them is constituted by the phenotypic techniques, based on the detection of early signs of cellular metabolism. Several of the phenotypic techniques, such as the BACTEC radiometric system and the MGIT, are commercially available and have results on par with the MP, but have a high operating cost due to their dependence on a constant supply of culture media or high upfront costs in equipment. Additional costs are incurred in the BACTEC system for the disposal and handling of radioactive waste [10, 11].

The knowledge of the molecular basis of drug resistance in TB, together with the availability of the so-called molecular techniques, has allowed the development of genotypic methods for the detection of drug resistance. Some of the advantages of these methods are the possibility of obtaining the results in 24 hours, and the fact that since they do not rely on growth of *M. tuberculosis* and therefore have much lower biosafety requirements, they might be used in our clinics. Unfortunately, they have met only very limited use due to the costs of the necessary equipment and their laboriousness, that places them beyond the reach of poor countries which are precisely those most affected by the increase in resistance of TB to antituberculosis drugs.

In spite of the fact that the colorimetric techniques constitute an easy to implement alternative that yields reliable results for the detection of resistance to INH and RIF (which constitute mainstays of antituberculosis treatments and define MDR), there is an ongoing work for the evaluation of other techniques, with the goal of devising a method which is, simultaneously, affordable to every lab and reliable for the evaluation of all antituberculosis drugs.

The NRM was reported in 2002 for the detection of resistance to first line antituberculosis drugs, using LJ medium and drugs at the same concentration employed by the MP. The results, obtained over a period of 7 to 14 days, were compared to those obtained with the BACTEC radiometric system. According to Ängeby *et al.* the sensitivity was higher than 95.0% for INH, STR and RIF and equal to 75.0% for ETH, whereas the specificity was higher than 96.0% for INH, ETH y RIF, and equal to 83.0% for STR [4].

In this study, after using the NRM for the determination of resistance to INH, SM, EMB and RMP, high sensitivity (higher than 93.7%) and specificity (higher than 98.0%) values were reached for all four drugs under evaluation, obtaining a global concordance of 98.2%. These values are comparable to those obtained with the MP for all the drugs, which suggests that the use of LJ medium in the NRM and of the same drug concentration as that employed in the MP (the reference technique) are important factors for obtaining the results described above.

The NRM has also been compared to the MP on agar, reaching values of sensitivity and specificity of 100% for INH and RIF [12]. Another alternative for the use of the NRM was described by Syre *et al*., in 2003 [13], using the Middlebrook 7H9 liquid medium. These authors used this assay for the detection of strains resistant to INH and RIF, obtaining sensitivities of 100 and 94.0% and specificities of 95.0 and 100%, respectively in comparison with the BACTEC radiometric system.

In order to substitute the conventional techniques with the new methodologies for the detection of drug resistance, future reproducibility studies and the obtention of acceptable levels of sensitivity and specificity are necessary. Additional research should also be carried out to evaluate their performance under real working conditions. With this goal in mind, international studies have been designed in which an identical set of *M. tuberculosis* strains with known susceptibility profiles is evaluated, following the same methodology, in many different institutions.

The National Tuberculosis and Mycobacteria Reference and Research Laboratory has recently participated on a multi-center international study involving four Latin American countries and coordinated by the Unit for Mycobacteria of the Tropical Medicine Institute "Prince Leopold" from Antwerp, Belgium. This study determined the susceptibility to the four first line antitu-berculosis drugs by the NRM and the MTT and RES colorimetric assays, using the MP in LJ as reference technique. In this study, the colorimetric assays reached sensitivity and specificity values higher than 91.7 and 93.3% for INH and RIF, respectively [14]. Likewise, the NRM reached sensitivity values that ranged from 86.6 to 100% for INH, RIF and ETH, whereas the specificity was 100% for RIF and higher than 94.4% for INH and ETH in all participating laboratories [15].

Taking into account the results of this work, together with the international reports published so far, it can be said that these techniques offer new possibilities: faster, easier execution and reliable results that can be used in the evaluation of second line drugs, as well as for direct diagnosis in our clinical setting. These methods can become an important tool that can be used in poor countries, since the only requirement for their implementation is the presence of basic microbiological equipment.

Acknowledgements

This research was funded by the INCO-Concerted Action project *Improve Diagnosis and drug resistance detection in Latin America*, ICA4-CT-2001-10087.

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