INT J TUBERC LUNG DIS 10(7):808–811

Evaluation of the resazurin microtiter assay for rapid detection of ofloxacin resistance in *M. tuberculosis*

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SUMMARY

OBJECTIVE: To evaluate the performance of the colorimetric resazurin microtiter assay (REMA) method for the detection of ofloxacin resistance.

METHODS: A panel of 120 multidrug-resistant *Mycobacterium tuberculosis* strains was tested blindly by the REMA method and compared with the results obtained using the BACTEC 460 method.

RESULT: A very good correlation was observed between the two methods.

CONCLUSION: The REMA method is simple, rapid and can be an inexpensive alternative procedure for the rapid detection of anti-tuberculosis drug resistance in laboratories with limited resources.

KEY WORDS: *M. tuberculosis*; MIC evaluation; resazurin; second-line drug; quinolone

TUBERCULOSIS (TB) is an infectious disease with high morbidity and mortality worldwide. According to the latest World Health Organization (WHO) report, there were 8.8 million new TB cases in 2002 and more than 2 million deaths were attributed to the disease.[1] TB control has become a problem, particularly for patients infected with *Mycobacterium tuberculosis* strains resistant to at least isoniazid (INH) and rifampicin (RMP), termed multidrug-resistant tuberculosis (MDR-TB).[2] The treatment of MDR-TB is much more difficult and expensive, and the mortality rate is particularly high in developing countries. MDR-TB patients do not respond to treatment with the first-line drugs INH, RMP, ethambutol (EMB), pyrazinamide (Z) and streptomycin (SM).[3–6] Consequently, the treatment of MDR-TB involves the use of reserve drugs called 'second-line drugs'.[7] Several studies have shown that MDR-TB can be cured by a combination of second-line drugs under DOTS-Plus, the treatment strategy proposed by the WHO to address the management of MDR-TB within good control programmes.[8–12]

The fluoroquinolones (FQs) are anti-microbial agents with good in vitro and in vivo activity against *M. tuberculosis*.[13–15] The use of these drugs as second-line anti-tuberculosis agents is recommended for treating MDR-TB.[7,16] As with other antimicrobial agents, the use of FQs can generate resistant mutants.[13,15] To preserve the efficacy of ofloxacin (OFX) in the treatment of TB, laboratories supporting TB services in areas with a high prevalence of MDR-TB must therefore be able to provide prompt and reliable drug susceptibility testing (DST) for OFX and other drugs used in the management of patients.[17] However, it is well known that the proportion method on Löwenstein-Jensen (LJ) medium or Middlebrook 7H10 agar is very slow, requiring several weeks to give results.[18,19]

In the last few years, new tools have been proposed for the rapid detection of *M. tuberculosis* drug resistance. Among these, the radiometric BACTEC 460-TB system (Becton Dickinson Diagnostic Instrument Systems, Sparks, MD, USA)[20] requires expensive automated equipment and the use of radioactivity, which is not appropriate for low-income countries. The Mycobacterial Growth Indicator Tube (MGIT, Becton Dickinson) and molecular tools such as the INNO-LiPA Rif.TB line probe assay (Innogenetics, Ghent, Belgium) have been applied extensively. However, they are expensive and impractical for routine use.[20] Recently, Palomino et al. standardised and evaluated the resazurin microtiter assay method (REMA), a microtiter plate method that uses the reduction of resazurin, and demonstrated very good correlation between results by this method and the proportion method.[19]

In this study, we evaluated the REMA colorimetric method for the detection of OFX resistance of *M.*

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Article submitted 10 November 2005. Final version accepted 22 February 2006.

tuberculosis strains and compared the results with those of BACTEC 460 TB.

MATERIALS AND METHODS

Bacterial isolates

One hundred and twenty MDR-TB isolates originating from Rwanda, Benin and Bangladesh were studied. All isolates had known susceptibility profiles against the first-line drugs RMP, INH, EMB and SM. *M. tuberculosis* H37 Rv (ATCC 27294) was used as the susceptible control. All strains were freshly subcultured on LJ medium before use and were tested using the BACTEC 460 and REMA methods.

Drugs

OFX was obtained from Sigma-Aldrich (Bornem, Belgium). The stock solution was prepared in advance at a concentration of 1 mg/ml in 0.1 N Na0H, filter sterilised and kept at −20°C for no more than 1 month.

Drug susceptibility testing

DST was performed in 7H12 with the recommended critical concentration of OFX 2 μg/ml using the radiometric method BACTEC 460-TB.[21,22]

Colorimetric reagents

A 0.02% stock solution of resazurin sodium salt powder (Acros Organics NV, Geel, Belgium) was prepared in distilled water, filter sterilised and kept at 4°C.

The resazurin microtiter assay

The REMA plate method was carried out as described by Palomino et al.[19] Briefly, the inoculum was prepared from fresh LJ medium in 7H9-S medium (Middlebrook 7H9 broth containing 0.1% casitone and 0.5% glycerol and oleic acid, albumin, dextrose, and catalase [OADC]; Becton Dickinson), adjusted to a n°1 McFarland tube, and diluted 1:20; 100 μl was used as inoculum. The OFX stock solution was thawed and diluted in 7H9-S medium to four times the highest final concentration tested. Serial two-fold dilutions of OFX were prepared directly in a sterile 96-well flat-bottom microtiter plate (VWR, Merck Eurolab, Leuven, Belgium) using 100 μl of 7H9-S. The concentration tested for OFX ranged from 0.25 to 8.0 μg/ml.

A growth control containing no antibiotic and a sterile control with no inoculum was also included on each plate; 200 μl of sterile water was added to all perimeter wells to avoid evaporation during incubation.

The plate was covered with its lid, placed in the original plastic bag and incubated at 37°C under normal atmosphere. After 7 days of incubation, 30 μl of resazurin solution was added to each well and the plate was reincubated overnight. At day 8 of the assay, the minimum inhibitory concentrations (MICs) of OFX were determined by visual reading indicated by a change in colour from blue (oxidised state) to pink (reduced state), indicating the growth of bacteria. The MIC was defined as the lowest drug concentration inhibiting >99% of the inoculum. A strain was considered resistant to OFX if the MIC was >2 μg/ml.[17]

Table MICs of OFX for 120 *M. tuberculosis* isolates determined by REMA compared to BACTEC 460

	REMA results Number of isolates for which the OFX MIC (μg/ml) was						
BACTEC result (*n* of strains)	⩽0.25	0.25	0.5	1	2	4	8
OFX-resistant (14)						8	6
OFX-susceptible (106)	12	15	72	6	1		

MIC = minimum inhibitory concentration; OFX = ofloxacin; REMA = resazurin microtiter assay.

Data analysis

Data analysis was carried out using MedCalc Software (Mariakerke, Belgium).

RESULTS

The Table shows the results obtained with REMA compared with the BACTEC 460 radiometric method, considered the gold standard. Results were available with REMA after an average 8 days of incubation and after 9–12 days with the BACTEC 460 method. For 120 *M. tuberculosis* isolates susceptible to OFX by the BACTEC 460 method, the total of susceptible strains was 106, as indicated in the Table. Of the 120 strains, 14 were resistant with the BACTEC 460 method, and had a MIC value of 4 or 8 μg/ml using the REMA method. The dot-plot diagram obtained with the MedCalc software (Figure) allowed us to establish

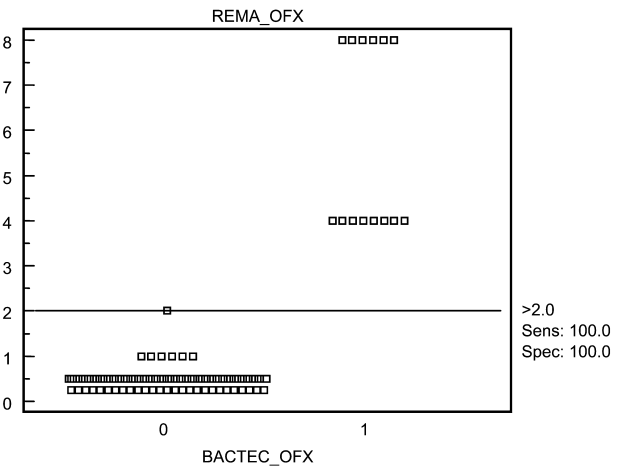

Figure Dot-plot diagram of the susceptibilities of 120 *M. tuberculosis* isolates to OFX by the BACTEC 460 method versus the MIC of the resazurin assay. OFX = ofloxacin; MIC = minimum inhibitory concentration; REMA = resazurin microtiter assay; Sens = sensitivity; Spec = specificity.

a cut-off point that distinguishes susceptible from resistant results. The cut-off represents the 'critical concentration' that defines susceptible and resistant strains based on the best fit of the colorimetric results with the BACTEC 460 method. At 2 μg/ml of OFX, the specificity and sensitivity were both 100%.

DISCUSSION

Traditional DST, such as the proportion method on LJ or agar medium, is time consuming. The BACTEC 460 radiometric system reduces the time to obtaining susceptibility results, but the equipment, tubes and additives are all expensive and generate radioactive waste. The BACTEC, REMA and other DST methods yield quantitative results, such as colony numbers, optical density results and growth indexes. The interpretation of the quantitative results yields a qualitative assessment of 'resistance' or 'susceptibility'.[23] Other commercial tests and molecular tools (INNO-LiPA, Innogenetics) have been developed, but they remain expensive and impractical for routine use.[24] For developing countries, it would be helpful to have a simple, inexpensive test that can rapidly detect resistant *M. tuberculosis* strains. OFX is an FQ that is active in the treatment of MDR-TB, and OFX-containing drug regimens have been proven to be effective and safe for treatment of MDR-TB, even with human immunodeficiency virus (HIV) positive patients.[25] Extensive use of FQs for the treatment of bacterial infections or used in short-course chemotherapy (SCC) might result in primary FQ-resistant TB.[26] In Peru, Armenia and Bangladesh, however, MDR-TB treatment, including the use of OFX, has been implemented to some extent.[27,28]

Up to 10% of the MDR-TB patients in Peru and Armenia harboured OFX-resistant strains. It has recently appeared that resistance to OFX could reach 51.4% among MDR-TB patients in some areas in the Philippines.[16] These authors conclude that FQs are now a significantly less effective alternative for treating MDR-TB cases in their country due to their widespread use in the treatment of TB and possibly in other infections as well. These alarming findings on the prevalence of resistance to second-line drugs, especially among MDR-TB patients, highlight the risk of producing primary resistance to FQs, creating incurable TB strains and thus jeopardising the potential of FQs to become first-line anti-tuberculosis drugs in the DOTS-Plus strategy.

The contribution of TB laboratories worldwide, through rapid and accurate DST, is very important for the management of MDR-TB, especially in low-income countries where most cases of MDR-TB occur.

In this study, the results were obtained after 8 days with the colorimetric method and are in complete agreement with those obtained by the BACTEC radiometric method. The dot-plot showed no significant differences between the methods. In a quantitative test such as REMA, it is also important to establish which of the concentrations involved in the test will separate susceptible from resistant strains. Our proposed cut-off value of 2 μg/ml is in agreement with the MIC proposed by Martin et al.[17] Although 2.0 μg/ml seems a reasonable criterion for OFX drug resistance using REMA and other more conventional methods, this does not necessarily constitute a criterion for clinical resistance.

In summary, this study has shown that the REMA method can be a very useful alternative for the rapid detection of *M. tuberculosis* drug resistance. The microplate format offers many advantages: DSTs of many isolates can be performed at the same time, the method is cost-effective (including the reagents), and it is rapid and gives quantitative (MIC) results. One disadvantage of resazurin assays is the use of liquid media in a microplate form, which might constitute a biohazard as aerosols could be generated.[17] It also requires the extra step of adding the substrate at 7 days of inoculation, increasing the manipulation each time the plate cover is removed.

A larger number of OFX-susceptible and -resistant isolates will be analysed to validate the REMA assay.

Acknowledgements

We thank the Ministry of Health of the Republic of Rwanda, the National TB Programme and the CGRI (Commissariat Général aux Relations Internationales) for financial support. This study was also supported by the European Commission RDP (INCO-DEV Programme), projects ICA4-CT-2001-10087 and by the Damien Foundation (Brussels, Belgium). We thank the staff of LRN (Laboratoire National de Référence, Kigali), and the Mycobacteriology Unit of the Institute of Tropical Medicine for excellent technical assistance.

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RÉSUMÉ

OBJECTIF : Evaluer la performance de la méthode colorimétrique REMA (resazurin microtiter assay) pour la détection de la résistance à l'ofloxacine.
MÉTHODE : Dans un lot de 120 souches multirésistantes de *M. tuberculosis* on a comparé les résultats de la méthode REMA pratiquée à l'aveugle avec ceux de la méthode radiométrique Bactec 460.
RÉSULTAT : Une très bonne corrélation a été observée entre les deux méthodes.
CONCLUSION : La méthode REMA est simple, rapide et pourrait être une alternative peu coûteuse pour la détection de la résistance aux antituberculeux dans les laboratoires de pays à faibles ressources.

RESUMEN

OBJETIVO : Evaluar el rendimiento diagnóstico de la prueba colorimétrica de microvaloración con resazurina (REMA), en la detección de la resistencia a ofloxacina.
MÉTODOS : Estudio con anonimato de una serie de 120 cepas multidrogorresistentes de *Mycobacterium tuberculosis* mediante el método REMA y comparación de los resultados con los obtenidos mediante el sistema BACTEC 460.
RESULTADOS : Se observó una correlación alta entre ambos métodos.
CONCLUSIÓN : La prueba REMA es un método sencillo y rápido y puede constituir otra opción para la detección rápida de resistencia a medicamentos anti-tuberculosos en los laboratorios con recursos limitados.