

## Safe susceptibility testing of *Mycobacterium tuberculosis* by flow cytometry with the fluorescent nucleic acid stain SYTO 16

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The time needed to obtain susceptibility results of *Mycobacterium tuberculosis* using classical methodologies is still too long, and flow cytometry is a promising technique in the setting of the clinical laboratory, giving fast results. A safe, reliable and rapid method to study susceptibility to streptomycin, isoniazide, rifampicin and ethambutol is described. Isolates of mycobacteria, grown for 72 h in the absence or presence of antimycobacterial drugs in the mycobacteria growth indicator tube (MGIT), were heat-killed, stained with SYTO 16 (a nucleic acid fluorescent stain that only penetrates cells with severe lesion of the membrane) and then analysed by flow cytometry. Sixteen strains with different susceptibility patterns were tested and an excellent correlation with the BACTEC MGIT 960 protocol for susceptibility was shown. In contrast to resistant strains, sensitive strains lose their cellular integrity after incubation with antimycobacterial drugs, allowing SYTO 16 to penetrate the cells. Comparing the intensity of fluorescence of *Mycobacterium* cells incubated with antimycobacterial drugs with that of drug-free cells, after staining with SYTO 16, it was possible to distinguish between sensitive, intermediate and resistant phenotypes. Other cytometric assays have been described for mycobacteria susceptibility testing but these have lower accuracy and safety. The described flow cytometric assay is a simple, fast, safe and accurate way to determine susceptibility of *M. tuberculosis*.

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### INTRODUCTION

In the last decade, the re-emergence of tuberculosis and the increase in resistance of *Mycobacterium tuberculosis* to antimicrobial agents (Cohn *et al.*, 1997; Raviglione *et al.*, 1995; World Health Organization, 1997) has emphasized the need for methods for quick detection of the agent and for fast determination of the susceptibility pattern. Antimicrobial susceptibility testing for tuberculosis takes weeks and delays therapy, which may further compromise the patient's health and increase the disease incidence and transmission to close contacts. Efforts to control tuberculosis are directly related to the slow growth of the organisms, and the time required for identification and susceptibility testing, which usually takes between 2 and 8 weeks. For many years the agar proportion method has been considered the standard method in the United States (Canetti *et al.*, 1963). A number of methods have been proposed that greatly decrease the time needed to

obtain results. However, automated device susceptibility testing, such as the radiometric BACTEC 460TB, the most frequently used, or the BACTEC MGIT 960, still require a minimum of 4–12 days before results become available. Luciferase reporter mycobacteriophages have been recently described as a rapid method for identification and susceptibility testing of the *M. tuberculosis* complex (Banaiee *et al.*, 2003), giving a turnaround time nearly three times faster than the BACTEC system (mean value of 5 days), but it is a complex procedure and provides discrepant results with ethambutol.

We have previously developed applications of flow cytometry to clinical microbiology, namely to mycology, which allowed us to obtain quick information regarding susceptibility of *Candida* to classical antifungals (Pina-Vaz *et al.*, 2001a, b). Moreover, it was possible to clarify the mechanism of action of some compounds with antifungal activity that were not considered classical antifungals (Pina-Vaz *et al.*, 2000a, b, 2004b) and to detect the expression of efflux pumps (Pina-Vaz *et al.*, 2001b), which represent the most important

Abbreviations: MGIT, mycobacteria growth indicator tube; PI, propidium iodide; SIRE, streptomycin, isoniazid, rifampicin and ethambutol.

mechanism of resistance. We have also assayed, with success, the use of laser scanner cytometry (solid-phase cytometry) to detect mycobacteria directly in clinical samples (Pina-Vaz *et al.*, 2004a). A rapid (24 h) method using a flow cytometric approach was described by Norden *et al.* (1995) and further tested by others (Bownds *et al.*, 1996; Kirk *et al.*, 1998), the biosafety issue remaining an important drawback to its uptake. Later on, Moore *et al.* (1999) inactivated the cells with paraformaldehyde before flow cytometric analysis of unstained particles, resulting in a rather low accuracy. In the present study, we describe a cytometric protocol to assess the susceptibility of *M. tuberculosis* to the four frontline antimicrobials streptomycin, isoniazid, rifampicin and ethambutol, and compared it with the BACTEC MGIT 960. The described assay is a quick, safe and accurate method, as heat-inactivated mycobacteria cells are analysed following staining with SYTO 16, a nucleic acid stain, which distinguishes them from debris.

## METHODS

**Strains.** Sixteen clinical strains of *M. tuberculosis* showing a wide array of susceptibility patterns were included in the study (Table 1). *M. tuberculosis* strain 27294 (H37Rv) belonging to the American Type Culture Collection (ATCC), susceptible to all standard antituberculosis agents, was also included as a quality control in each assay.

**Incubation with the antimicrobial drugs.** The strains were cultured according to the standard procedures of the BD BACTEC MGIT 960 (Becton Dickinson), using mycobacteria growth indicator tubes (MGITs), in duplicate. The antimicrobials tested were streptomycin (1 and 4  $\mu\text{g ml}^{-1}$ ), isoniazid (0.1 and 0.4  $\mu\text{g}^{-1}$ ), rifampicin (1  $\mu\text{g}^{-1}$ ) and ethambutol (5 and 7.5  $\mu\text{g}^{-1}$ ), usually designated SIRE. From the two sets of MGITs [each set including a drug-free growth control (GC) and the tubes with the different above-mentioned drug concentrations], one set was incubated just for 72 h at 37 °C, and the other set was incubated in the BACTEC 960 apparatus until the end of the susceptibility testing, according to the standard protocol of this method.

**Determination of susceptibility using BACTEC 960.** In this equipment the fluorescence due to growing of bacteria in the MGIT is continuously monitored (Ardito *et al.*, 2001). The fluorescent compound is sensitive to the presence of oxygen dissolved in the broth. Initially little fluorescence can be detected but actively growing and respiring micro-organisms consume the oxygen, which allows the compound to emit fluorescence. The relative growth ratio between the drug-containing tubes and drug-free GC tubes was determined by the system's software algorithm. The instrument performed the final interpretation and reported the susceptibility pattern automatically as susceptible or resistant. Whenever two concentrations of each drug are used the level of resistance can be determined. Strains resistant at the critical (lower) concentration but susceptible when incubated with the higher concentration are considered to have low-level resistance (except for rifampicin because only one concentration is used).

**Inactivation of the bacterial cells.** Both following 72 h incubation and at the end of the susceptibility testing performed with BACTEC 960 the corresponding set of MGITs was autoclaved (121 °C, 20 min) to kill the bacterial cells. Classical viability studies were performed to ensure the effectiveness of this procedure. Briefly, 10  $\mu\text{l}$  samples from serial 10-fold dilutions of each tube prepared in 7H9 broth were used to determine the number of c.f.u.  $\text{ml}^{-1}$  on 7H9 medium; the plates were incubated at 37 °C for 3 weeks before reading.

**Optimization of the staining.** Heat-killed cells of *Mycobacterium* were incubated with increasing concentrations of two different stains for nucleic acids: propidium iodide (PI; Sigma) (1, 5, 10, 20 and 30  $\mu\text{g ml}^{-1}$ , for 30 and 60 min) and SYTO 16 (Molecular Probes) (5, 10, 15 and 20  $\mu\text{M}$ , for 30 min). The cells were analysed under epifluorescence microscopy with a Leitz Laborlux K (Leica) fitted with a mercury 50 W lamp, a BP 450–490 nm excitation filter and an LP 515 nm emission filter, and under flow cytometry using a Beckman Coulter XL-MCL flow cytometer equipped with a 15 nm argon laser at FL3 (620 nm) – red fluorescence for PI – and at FL1 (525 nm) – green fluorescence for SYTO 16.

**Determination of susceptibility using flow cytometry.** To optimize the cytometric protocol, both the scatter and the intensity of fluorescence of dead *M. tuberculosis* cells were determined after staining with different concentrations of PI and SYTO 16. Flow cytometric analysis was performed on fixed cells (heat-killed), treated and non-treated with SIRE, both after 72 h incubation and at the end of the BACTEC analysis, after staining with 10  $\mu\text{M}$  SYTO 16 for 30 min. Samples were analysed by flow cytometry for a fixed time (60 s) and with a fixed speed of aspiration (low) immediately after vortexing the suspension for 20 s to avoid clumping. The number of fluorescent organisms  $\text{ml}^{-1}$  was obtained following the flow cytometric analysis, and was dependent upon the establishment of gates to eliminate electronic noise and background particles from the medium. An isolate was considered sensitive whenever the number of fluorescent particles in the drug-containing medium was reduced in comparison to the GC (ratio < 1), resistant if no reduction was observed (ratio  $\geq 1$ ), and a low-level resistant strain whenever the strain behaved as resistant with the lower concentration of the drug but as susceptible in the presence of the higher concentration (ratio  $\geq 1$  with the low concentration and ratio < 1 with the high concentration), except for rifampicin (one single concentration tested).

**Statistical analysis.** Analysis of variance (one-way ANOVA) was performed to compare differences between cellular growth in GC and drug-containing tubes.

**Detection of contamination.** The BACTEC 960 system does not detect the specific growth of *Mycobacterium*. Any other micro-organism able to grow as a contaminant in the MGIT will give a false positive result. Although the manufacturer's standard protocol does not recommend a systematic control of contamination, at our laboratory we routinely perform a Kinyoun stained smear to exclude bacterial or fungal contamination, particularly when the BACTEC system reports a resistant strain. In the present study, and in a more expedite procedure, 200  $\mu\text{l}$  from each MGIT was collected, stained with PI following heat inactivation, and analysed under flow cytometry to exclude the presence of red fluorescent cells. As mycobacteria do not stain with PI, such an occurrence would clearly indicate microbial contamination. Whenever contamination was detected, susceptibility testing was repeated.

## RESULTS

Heat-killed mycobacteria cells (confirmed by viability tests) did not stain with PI, even at high concentrations of this fluorescent probe. Conversely, SYTO 16 was shown to be an excellent probe for staining mycobacteria, 10  $\mu\text{M}$  for 30 min being enough to cause dead mycobacterial cells to fluoresce. The susceptibility patterns to SIRE of the different tested strains of *M. tuberculosis*, determined with the BACTEC 960 system, are shown in Table 1. There was an excellent correlation between the BACTEC results obtained after 12–15 days and the flow cytometry results obtained following 72 h incubation (Table 1). According to the flow

**Table 1.** Results of susceptibility testing of 16 strains of *Mycobacterium tuberculosis* and of the quality control strain ATCC 27294 (H37Rv) to streptomycin, isoniazid, rifampicin and ethambutol (concentrations given in  $\mu\text{g ml}^{-1}$ ), determined with the BACTEC 960 system and by flow cytometry

The phenotype (Phen.) with the BACTEC system was based on the growth in MGITs of cells treated with different antimycobacterials and growth in the drug-free tube: sensitive (S) if it was S to both concentrations, resistant (R) if it was R to to both concentrations, and low-level resistant (l-l R) if it was R to the lower concentration and S to the higher. The phenotype by flow cytometry was defined by the ratio between the number of fluorescent cells on drug-containing medium versus the number of fluorescent cells on drug-free medium: a ratio  $< 1$  represents an S phenotype,  $\geq 1$  represents a R phenotype, and  $\geq 1$  with the lower concentration of the drug and  $< 1$  with the higher concentration corresponds to a l-l R phenotype.

Strain	BACTEC 960											Flow cytometry/SYTO 16										
	Streptomycin			Isoniazid			Rifampin		Ethambutol			Streptomycin			Isoniazid			Rifampin		Ethambutol		
	1	4	Phen.	0.1	0.4	Phen.	1	Phen.	5	7.5	Phen.	1	4	Phen.	0.1	0.4	Phen.	1	Phen.	5	7.5	Phen.
M1	S	S	S	R	S	l-l R	R	R	S	S	S	< 1	< 1	S	$\geq 1$	< 1	l-l R	$\geq 1$	R	< 1	< 1	S
M2	S	S	S	S	S	S	S	S	S	S	S	< 1	< 1	S	< 1	< 1	S	< 1	S	< 1	< 1	S
M3	R	S	l-l R	S	S	S	S	S	R	S	l-l R	$\geq 1$	< 1	l-l R	< 1	< 1	S	< 1	S	$\geq 1$	< 1	l-l R
M4	R	S	l-l R	R	R	R	S	S	S	S	S	$\geq 1$	< 1	l-l R	$\geq 1$	$\geq 1$	R	< 1	S	< 1	< 1	S
M5	R	S	l-l R	S	S	S	S	S	S	S	S	$\geq 1$	< 1	l-l R	< 1	< 1	S	< 1	S	< 1	< 1	S
M6	R	S	l-l R	R	R	R	R	R	S	S	S	$\geq 1$	< 1	l-l R	$\geq 1$	$\geq 1$	R	$\geq 1$	R	< 1	< 1	S
M7	S	S	S	R	R	R	R	R	R	R	R	< 1	< 1	S	$\geq 1$	$\geq 1$	R	$\geq 1$	R	$\geq 1$	$\geq 1$	R
M8	R	R	R	R	R	R	R	R	R	R	R	$\geq 1$	$\geq 1$	R	$\geq 1$	$\geq 1$	R	$\geq 1$	R	$\geq 1$	$\geq 1$	R
M9	R	R	R	R	R	R	R	R	R	R	R	$\geq 1$	$\geq 1$	R	$\geq 1$	$\geq 1$	R	$\geq 1$	R	$\geq 1$	$\geq 1$	R
M10	S	S	S	S	S	S	S	S	S	S	S	< 1	< 1	S	< 1	< 1	S	< 1	S	< 1	< 1	S
M11	R	S	l-l R	S	S	S	S	S	R	S	l-l R	$\geq 1$	< 1	l-l R	< 1	< 1	S	< 1	S	$\geq 1$	< 1	l-l R
M12	S	S	S	S	S	S	S	S	S	S	S	< 1	< 1	S	< 1	< 1	S	< 1	S	< 1	< 1	S
M13	S	S	S	S	S	S	S	S	S	S	S	< 1	< 1	S	< 1	< 1	S	< 1	S	< 1	< 1	S
M14	S	S	S	S	S	S	S	S	S	S	S	< 1	< 1	S	< 1	< 1	S	< 1	S	< 1	< 1	S
M15	R	R	R	R	S	l-l R	R	R	R	R	R	$\geq 1$	$\geq 1$	R	$\geq 1$	< 1	l-l R	$\geq 1$	R	$\geq 1$	$\geq 1$	R
M16	R	R	R	R	R	R	R	R	R	R	R	$\geq 1$	$\geq 1$	R	$\geq 1$	< 1	R	$\geq 1$	R	$\geq 1$	$\geq 1$	R
H37Rv	S	S	S	S	S	S	S	S	S	S	S	< 1	< 1	S	< 1	< 1	S	< 1	S	< 1	< 1	S

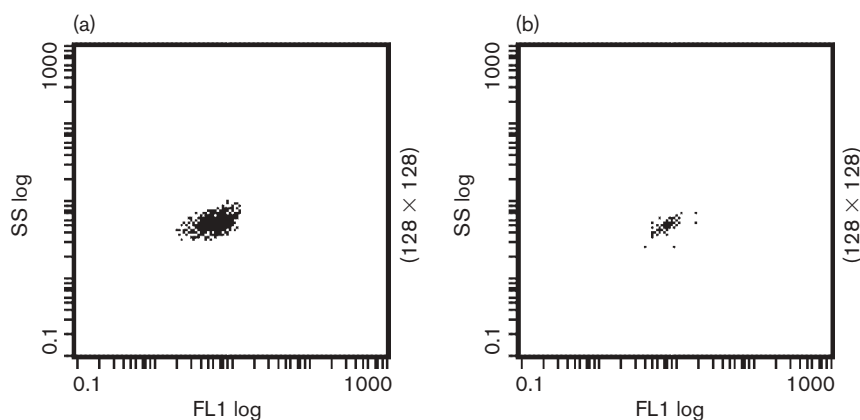
cytometry results, a sensitive phenotype showed a significant reduction ( $P < 0.001$ ) of the fluorescent events in drug-containing tubes, in comparison with the GC (Fig. 1); a low-level resistant phenotype was considered when no reduction of the fluorescent events happened in the presence of the lower concentration of the drug but there was reduction with the higher concentration (except for rifampicin, as a single concentration was tested); a resistant phenotype experienced no reduction of the fluorescent events. These analysis criteria were valid for all the tested drugs.

The flow cytometry protocol detected contamination in two cases, showing PI-positive cells. The corresponding samples on the BACTEC protocol yielded resistant phenotypes after 3 and 4 days incubation. Gram staining of the growth showed Gram-negative bacteria in one case and fungi in the other. The susceptibility tests were repeated after decontamination of the initial MGIT, without further problems.

## DISCUSSION

Multidrug-resistant *Mycobacterium* strains have emerged during the last decade, a fact that makes the rapid detection of such isolates a critical topic for effective treatment (Cohn *et al.*, 1997; Tenover *et al.*, 1993). Resistance to SIRE, the four primary drugs, also makes tuberculosis difficult to treat and eradicate (Pablos-Mendez *et al.*, 1998), thus representing a serious public health problem. To combat multidrug-resistant tuberculosis, the National MDR TB Task Force recommends antimicrobial susceptibility testing of all the initial and follow-up isolates of *M. tuberculosis* from each individual patient. With the use of MGIT technology, namely with the BACTEC MGIT 960 system, a considerable advance was achieved regarding susceptibility testing of mycobacteria, as it showed good correlation with the BACTEC 460TB, an accepted reference method (Ardito *et al.*, 2001; Rüscher-Gerdes *et al.*, 1999). According to this method, 4–14 days (median of 7.3 days) are needed to obtain results (Ardito *et al.*, 2001), in addition to the considerable time previously needed for the isolation of the strain. Although considerably shorter when compared with the previous reference methods like the proportional method, it still remains a lengthy procedure, both for the patient and the community. A great

effort has been made regarding the detection of genetic resistance, but only in the particular cases of isoniazid and rifampicin was the prediction of resistance possible (Torres *et al.*, 2000). Furthermore, the complexity of the methodology and the possible involvement of other genes make its value still a matter of debate (Khisimuzi *et al.*, 1996). Banaiee *et al.* (2003) have compared susceptibility of *M. tuberculosis* using luciferase reporter mycobacteriophages and MGIT BACTEC 460. Despite the few discrepant results, the turnaround time varied between 3 and 57 days, 77.8% of the tests being completed in 5 days. Another study previously addressed susceptibility testing of *Mycobacterium* using flow cytometry. It was based upon the ability of viable *M. tuberculosis* organisms to hydrolyse fluorescein diacetate, providing results soon after 24 h incubation with the drugs (Norden *et al.*, 1995; Kirk *et al.*, 1998). This assay had the advantage of not requiring the active multiplication of mycobacteria, but had additionally the great inconvenience of aerosolization of viable *M. tuberculosis*, which may occur at several points throughout the procedure. A safer cytometric assay was developed by Moore *et al.* (1999). After 72 h incubation with the drugs, the authors fixed the cells with paraformaldehyde and the number of organisms  $\text{ml}^{-1}$  was determined by flow cytometry. This assay is based upon the counting of unstained particles, which, by being of very little size ( $1-4 \times 0.3-0.6 \mu\text{m}$ ), might consequently be confused with debris or any other particles. We preferred to base our cytometric study on counting dead stained cells for a fixed time, and using a fixed aspiration speed: dead cells to make the procedure safe, and stained cells to avoid counting errors. The experiments were repeated at least twice, with highly reproducible results. We have studied two fluorescent probes that stain nucleic acids of permeabilized dead cells. PI is a stain that penetrates most micro-organisms with severe lesion of the cytoplasmic membrane (Boye *et al.*, 1983; Pina-Vaz *et al.*, 2000a, b, 2001a, 2004b) that had never been previously used with mycobacteria. We have demonstrated that the *Mycobacterium* cell wall is impermeable to PI, probably due to its complex lipid content. We took advantage of this fact, using PI to evaluate microbial contamination. The detection of red-stained cells on the cytometric analysis of samples from each MGIT represents other micro-organisms growing as contaminants, meaning that the



**Fig. 1.** Intensity of fluorescence at 525 nm (FL1 log) versus sideward angle light scatter (SS log), displayed as a contour plot profile of a typical sensitive strain of *Mycobacterium tuberculosis*. (a) Control cells not treated with antimycobacterial drugs; (b) cells treated with  $1 \mu\text{g}$  streptomycin  $\text{ml}^{-1}$  for 72 h at  $37^\circ\text{C}$ . In both cases the cells were heat-killed and stained with SYTO 16. The reduction of the number of fluorescent events is evident on treated cells.

susceptibility test is not valid. The fluorescent probe SYTO 16 is a non-symmetric cyanine with three positive charges that, when linked to nucleic acids, increases the fluorescence about 500 times, colouring the dead cells bright green (Roth *et al.*, 1997). SYTO 16 was formerly used to study the growth cycle of *Mycobacterium avium* (Ibrahim *et al.*, 1997). After developing an optimization protocol, we showed that SYTO 16 is a useful probe for studying the susceptibility of *M. tuberculosis* to SIRE. Following 72 h incubation, this probe allowed a clear distinction between the different susceptibility phenotypes to SIRE. Flow cytometry, a technology that has already changed completely fields like haematology and cellular analysis, may additionally bring significant improvements in clinical microbiology, as found in the present case. Its clinical adoption will most probably be a considerable benefit for both patients and the community in general.

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