Rapid identification and susceptibility testing of *Mycobacterium tuberculosis* from MGIT cultures with luciferase reporter mycobacteriophages

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In a prospective study conducted in a diagnostic laboratory in Mexico City, luciferase reporter mycobacteriophages (LRPs) were evaluated for their utility and performance in identification and antibiotic-susceptibility testing of *Mycobacterium tuberculosis* complex (MTC) isolates from MGIT-960 cultures. Eighty-four consecutive MGIT cultures recovered from 54 patients were included in this study. The LRPs confirmed mycobacterial growth in 79 (94 %) of 84 MGIT cultures. Failure to confirm growth was due to low inoculum ($n = 1$) or growth with non-tuberculous mycobacteria ($n = 4$). The median time to confirmation of MGIT cultures was 1 day (range 1–55). Confirmed cultures were identified with *p*-nitro- α -acetylaminobenzoyl-L-histidine-L-leucine (NAP), a selective inhibitor of MTC species, and results obtained with LRPs were compared with those obtained by BACTEC-460. The sensitivity and specificity of the LRP NAP test were respectively 97 and 100 %, and the median turnaround time for identification was 3 days with both methods. The accuracy and speed of the LRPs for susceptibility testing with rifampicin, streptomycin, isoniazid and ethambutol were compared with BACTEC-460 and discrepant results were tested by the conventional agar proportion method. In total, 72 MTC cultures were tested. The overall agreement between the LRPs and BACTEC-460 was 98.6 %. Four isolates (5.6 %) were falsely identified as ethambutol-resistant. The median turnaround time for susceptibility testing was 3 days (range 3–57) with the LRPs and 9 days (range 7–29) with BACTEC-460. LRPs offer an accurate and rapid approach for identification and susceptibility testing of *M. tuberculosis* from MGIT-960 cultures.

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INTRODUCTION

The worldwide emergence of drug-resistant tuberculosis has changed views on the way we treat infections caused by *Mycobacterium tuberculosis*. This change reflects our understanding of the failures of standard regimens in patients with drug-resistant infections (Espinal *et al.*, 2000; Becerra *et al.*, 2000). In resource-rich countries, the standard of care has

shifted from standard therapy with first-line drugs to individually tailored regimens based on the susceptibility profile of each isolate (Joint Tuberculosis Committee of the British Thoracic Society, 1998; Tenover *et al.*, 1993). Timely delivery of this information has also proven to be critical under some settings (Turett *et al.*, 1995). Meanwhile, in the developing world, where resources are limited, patients continue to undergo diagnosis with acid-fast smears and are subsequently treated with standard anti-tuberculosis regimens. In these countries, the clinical utility of conventional antibiotic-susceptibility testing (AST) methods has been offset by their long turnaround times, and the utilization of more rapid methods such as BACTEC and MGIT has

Abbreviations: AST, antibiotic-susceptibility testing; LRP, luciferase reporter mycobacteriophage; MTC, *Mycobacterium tuberculosis* complex; NAP, *p*-nitro- α -acetylaminobenzoyl-L-histidine-L-leucine; NTM, non-tuberculous mycobacteria.

not proven to be cost effective (Heifets & Cangelosi, 1999). Thus, there is still a need for an approach that can offer both rapid and affordable susceptibility testing so that appropriate therapy can be initiated in a timely manner.

Luciferase reporter mycobacteriophages (LRPs) have been described previously as a rapid method for identification and susceptibility testing of *M. tuberculosis* complex (MTC) (Jacobs *et al.*, 1993; Riska *et al.*, 1997). This phenotypic approach utilizes genetically engineered reporter phages to detect viable mycobacteria in the presence and absence of antibiotics. Compared with existing methods, susceptibility testing with LRPs is faster and does not utilize radioactive reagents (Jacobs *et al.*, 1993; Riska *et al.*, 1997). In a recent report, we showed that LRPs perform favourably in comparison with existing methods when used for detection, identification and susceptibility testing of MTC isolates from primary sputum cultures (Banaiee *et al.*, 2001). In that study, the greatest time-saving benefit of the LRP system was in performing susceptibility testing, not in primary culture detection. Therefore, in the present study, we examined the utility and performance of LRPs for species identification and susceptibility testing when applied to clinical cultures recovered by an automated culture detection system (MGIT-960). We show that, upon confirming mycobacterial growth in MGIT cultures, the LRPs can identify isolates and perform susceptibility testing rapidly and accurately.

METHODS

Specimens. From March to June 2000, 84 consecutive MGIT cultures with mycobacterial growth were evaluated in the laboratory of clinical microbiology of Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City. None of these isolates overlapped with those presented in our earlier study (Banaiee *et al.*, 2001). Specimens were obtained from a total of 54 patients located in Mexico City, Huachuquingo, Puebla, and Orizaba, Veracruz, and consisted of 67 sputa, four spinal fluid samples, four urine samples, four gastrointestinal biopsies, three lymph nodes, one lung tissue sample and one abscess.

LRP. The construction of phAE142, the phage used in this study, was described recently by Bardarov *et al.* (2003). Phage phAE142 was propagated and titrated on lawns of *Mycobacterium smegmatis* mc² 4502 in our laboratory over the course of a week as described previously (Banaiee *et al.*, 2001). High-titre phage stocks ($\sim 10^{11}$ p.f.u. ml⁻¹) were stored at 4 °C for several months.

Antibiotics. Lyophilized antibiotics (Becton Dickinson) were dissolved in sterile water to make the following 200× stock solutions: 40 µg isoniazid (INH) ml⁻¹, 400 µg rifampicin (RMP) ml⁻¹, 80 µg streptomycin (SM) ml⁻¹ and 1000 µg ethambutol (EMB) ml⁻¹. Working stocks (20×) were prepared with sterile water. *p*-Nitro- α -acetylaminobenzylhydroxypropylphenone (NAP) stock solutions (Riska *et al.*, 1997) were diluted with sterile water to prepare 20× (150 µg ml⁻¹) working stocks. All stocks were stored at -40 °C.

Isolation in MGIT-960. All specimens were processed for cultivation in the MGIT-960 system according to the manufacturer's standard protocol (Becton Dickinson Diagnostic Instrument Systems). The MGIT instrument read each tube hourly and triggered an alarm when growth was detected. Positive cultures were confirmed with an acid-fast

stain and cultures free of contaminants were advanced for identification and susceptibility testing.

Growth confirmation by LRP assay. Secondary cultures were created by adding 1.5 ml of MGIT cultures to 1.5 ml Middlebrook 7H9 broth (Difco) supplemented with 1% (v/v) glycerol and 10% (v/v) ADC (albumin, glucose, catalase) (Becton Dickinson). Secondary cultures were incubated at 37 °C and checked for mycobacterial growth on days 1, 3, 5, 7, 11, 15, 19, 23, 27, 41 and 55 post-incubation. The detailed protocol was described previously (Banaiee *et al.*, 2001). Briefly, at each time-point, 50 µl secondary culture was infected with 5 µl LRP and luciferase activity was quantified in a luminometer at 3 h post-infection.

Culture titration. Bacillus concentrations were enumerated before (day zero) and after confirmation of growth by the LRP assay. Cultures were serially diluted and plated onto 7H10 agar supplemented with OADC and PANTA (Becton Dickinson). Plates were incubated at 37 °C and c.f.u. were quantified at 3–6 weeks.

NAP test and susceptibility testing

(i) LRPs. Identification and susceptibility testing were performed simultaneously as described previously (Banaiee *et al.*, 2001). Briefly, 5 µl sterile water or 5 µl each 20× antibiotic were added to 95 µl phage-confirmed secondary cultures and incubated at 37 °C for 40 h. Following phage infection, luciferase activity was quantified at 3 and 6 h and the susceptibility profiles were calculated accordingly.

(ii) Radiometric AST. All positive MGIT cultures underwent presumptive identification with the BACTEC-460 NAP differentiation test (Becton Dickinson Diagnostic Instrument Systems). Subsequently, those isolates identified as MTC were confirmed with the AccuProbe DNA hybridization assay (Gen-Probe) and advanced for susceptibility testing with the BACTEC-460 system. Briefly, 100 µl of each positive MGIT culture was inoculated aseptically into a 12B vial and incubated at 37 °C. At a growth index of ≥ 500 , radiometric AST was performed according to the manufacturer's instructions using standardized antibiotic concentrations (2 µg RMP, 0.1 µg INH, 2 µg SM and 7.5 µg EMB ml⁻¹) and cut-off points as recommended by the manufacturer (Becton Dickinson Diagnostic Instrument Systems). Turnaround time for susceptibility testing was defined as the amount of time it took to complete testing from the time MGIT cultures became available (for testing).

Statistical analysis. The sensitivity, specificity and accuracy of the susceptibility testing and NAP test were calculated. Differences in proportions were evaluated by χ^2 test.

RESULTS AND DISCUSSION

Growth confirmation

In order to determine whether there was sufficient inoculum in the MGIT cultures to perform the phage-based NAP test and susceptibility testing, the LRP assay was performed on 84 consecutive MGIT cultures recovered from 54 patients. Of the 84 cultures tested, mycobacterial growth was confirmed in 79, giving an overall confirmation rate of 94%. The LRPs confirmed growth in 72 of 73 MTC cultures and in 7 of 11 non-tuberculous mycobacteria (NTM) cultures, giving respective confirmation rates of 98.6 and 63.6%. The identities of the five unconfirmed cultures included one MTC, three *Mycobacterium avium* complex and one *Mycobacterium kansasii*. The failure to detect the first was due to low bacterial load, as demonstrated by c.f.u. counts on day zero

(< 10 000 ml⁻¹). Growth of this isolate was readily detected when a larger inoculum was used. The failure to confirm four NTM cultures was not unexpected, since resistance to the TM4 phage has been described previously for some 20 NTM species (Riska *et al.*, 1997; Timme & Brennan, 1984). From the public health perspective, LRPs are still suitable for diagnostic purposes given that MTC organisms are by far the most important clinical mycobacterial isolates; the only ones that warrant AST and also the only ones for which rapid institution of therapy has been shown to make a difference (Turett *et al.*, 1995).

The median time to confirmation of MGIT cultures was 1 day (range 1–55), and 82.3 % of the cultures were confirmed within 5 days (Fig. 1). There were 12 cultures for which growth confirmation was delayed beyond 7 days. In 10 of these, late confirmation was shown to be due to low starting MTC bacterial load ($n = 3$; 11, 19 and 19 days), contamination with non-mycobacterial organisms ($n = 4$; 11, 19, 27 and 55 days) or growth with NTM species ($n = 3$; 19, 27 and 41 days). For the remaining two cultures, the cause was not investigated. Titration results for the three MTC cultures with low starting inoculum showed that all three had fewer than 10 000 c.f.u. ml⁻¹ on day zero compared with a mean of 760 000 c.f.u. ml⁻¹ ($n = 36$) for cultures that were confirmed on day 1. Titration results for the two cultures confirmed on day 19 showed that they had risen to 200 000 and 350 000 c.f.u. ml⁻¹, respectively. Contamination of the four late-confirmed cultures with bacteria and fungi points out a deficiency in our protocol that can be overcome by adding PANTA antibiotic supplement to the secondary cultures.

NAP testing

The LRP NAP test was performed on the 79 confirmed MGIT cultures and results were compared with those obtained by BACTEC-460. Overall agreement between the LRPs and BACTEC-460 was found in 77 of 79 tests (97.5 %). Seventy of 72 MTC cultures (97.2 %) were identified correctly. When

Fig. 1. Time-course of confirmation of MGIT cultures. Growth in MGIT cultures was confirmed with the LRPs and times were recorded ($n = 79$).

the two misidentified cultures were screened for contaminants, both of them were shown to harbour non-mycobacterial organisms. False-resistant NAP results due to microbial contaminants have been reported previously (Laszlo & Siddiqi, 1984). To avoid this problem, we propose that cultures identified as NTM should be screened for contaminants.

Sensitivity of the LRP NAP test, the ability to detect MTC cultures, was 97.2 %. Specificity of the LRP NAP test, the ability to detect NTM isolates, was 100 %. There was no statistically significant difference between the results obtained with LRPs and BACTEC-460 ($P = 0.439$). The median turnaround time for identification of MGIT cultures was 3 days (range 3–57) with the LRPs and 3 days (range 1–5) with BACTEC-460. Similar turnaround times have been reported for BACTEC by Heifets & Cangelosi (1999). Although NAP was used to demonstrate the feasibility of selective inhibitors in the LRP assay, other compounds such as hydroxylamine hydrochloride or *p*-nitrobenzoate could be used in place of NAP (Gross & Hawkins, 1985). Like NAP, these compounds are also highly specific inhibitors of MTC species but, unlike NAP, they are both commercially available and affordable (Gross & Hawkins, 1985).

Susceptibility testing

The performance of LRPs in susceptibility testing with RMP, SM, INH and EMB was evaluated and compared with BACTEC-460. The 72 MGIT cultures with MTC growth were tested. The susceptibility pattern of these isolates as determined by LRPs and BACTEC-460 is shown in Table 1. Overall agreement for all four drugs was found in 284 of 288 possible tests (98.6 %). Discrepant results were obtained for four EMB tests.

Of the 72 LRP susceptibility tests performed with RMP, SM and INH, 100 % agreement was found with BACTEC-460 (RMP, 65 susceptible and 7 resistant; SM, 72 susceptible; INH, 63 susceptible and 9 resistant). For EMB, 68 of 72 test results (94.4 %) were in agreement with BACTEC-460. The four isolates with discrepant results were all identified incorrectly as EMB-resistant. Upon retesting with the con-

Table 1. Susceptibility of MTC isolates to four first-line drugs as determined by LRP and BACTEC-460

Drugs were used at the following concentrations (µg ml⁻¹): RMP, 2 (both systems); SM, 0.4 (LRP) and 2 (BACTEC); INH, 0.2 (LRP) and 0.1 (BACTEC); EMB, 5 (LRP) and 7.5 (BACTEC). S, Susceptible; R, resistant.

Drug	Isolates tested	Both S	BACTEC S; LRP R	BACTEC R; LRP S	Both R
RMP	72	65	0	0	7
SM	72	72	0	0	0
INH	72	63	0	0	9
EMB	72	67	4	0	1

ventional agar proportion method (conducted at the National Jewish Medical and Research Center, Denver, CO, USA), the three isolates tested gave results in agreement with BACTEC-460. The false-resistant results obtained with EMB may reflect problems either with the stability of the compound or the concentration ($5 \mu\text{g ml}^{-1}$) used in this study. Recent unpublished data from our laboratory suggest that repeated freeze-thawing might have been the cause. Regardless, there was no statistically significant difference between the two methods for EMB ($P = 0.758$).

We also evaluated the effect of duration of phage infection on the accuracy of AST results. Susceptibility assays were quantified at 3 and 6 h post-infection and results were compared to determine whether the 6 h incubation provided results that were more accurate than the more convenient 3 h incubation. Of 288 susceptibility tests performed, only four disagreements (1.4%) were found between the 3 and 6 h incubations. Furthermore, when the 3 and 6 h results were used to predict the AST patterns for each isolate, the agreement with BACTEC-460 was nearly identical for the two incubation periods (respectively 98.3 and 97.6%).

The sensitivity and specificity of susceptibility testing with the LRPs were determined for each drug. Sensitivity, the ability to detect drug-resistant isolates, was 100% for all four drugs. The specificity, ability to detect susceptible isolates, was 100% for RMP, SM and INH and 94.4% for EMB.

Turnaround times for susceptibility testing were determined for each method. In order to make a fair and accurate comparison with BACTEC-460, growth-confirmation times were included in the turnaround times for the LRPs. For the 72 MTC cultures tested, the median turnaround time was 3 days (range 3–57) with the LRPs and 9 days (range 7–29) with the BACTEC-460 system. With the LRPs, 77.8% of the susceptibility results were completed in 5 days, while, with BACTEC-460, the results started to become available on day 7 (Fig. 2). LRPs have the fastest AST turnaround time

Fig. 2. Time-course of availability of AST results. Susceptibility of MTC cultures to first-line drugs was determined with LRPs (filled bars) and BACTEC-460 (open bars) and turnaround times were recorded ($n = 72$).

amongst known phenotypic methods, being three times faster than BACTEC-460 (Heifets & Cangelosi, 1999) and nearly three times faster than that reported for the manual MGIT AST SIRE system (Rüsch-Gerdes *et al.*, 1999). The rapid AST results obtained with the LRPs are especially attractive because the simplicity and low reagent cost of this method make possible the rapid retesting of questionable results.

In conclusion, we have demonstrated that the LRP technology provides reliable and rapid results when used for identification and drug-susceptibility testing of MTC isolates from MGIT-960 cultures. Future development of the LRP system will focus on optimizing confirmation of MTC organisms from MGIT bottles, better definition of ethambutol susceptibility criteria and replacement of NAP with an alternative agent.

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