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Luciferase reporter phage phAE85 for rapid detection of rifampicin resistance in clinical isolates of *Mycobacterium tuberculosis*

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ABSTRACT

Objective: To evaluate luciferase reporter phage (LRP) phAE85 in rapid detection of rifampicin resistance in a region where TB is endemic. **Methods:** One hundred and ninety primary isolates on Lowenstein-Jensen medium were tested. Middlebrook 7H9 complete medium with and without rifampicin at 2 μ g/mL was inoculated with standard inoculum from suspensions of the clinical isolate. After incubation for 72 h, LRP was added. Following 4 h of further incubation, light output from both control and test was measured as relative light units. Strains exhibiting a reduction of less than 50% relative light units in the drug containing vial compared to control were classified as resistant. Results were compared with the conventional minimum inhibitory concentration method (MIC) of drug susceptibility testing. **Results:** The two methods showed high level of agreement of 97% (*CI* 0.94, 0.99) and *P* value was 0.000 1. The sensitivity and specificity of LRP assay for detection of rifampicin resistance were 91% (*CI* 0.75, 0.98) and 99% (*CI* 0.95, 1.00) respectively. Time to detection of resistance by LRP assay was 3 d in comparison with 28 d by the minimum inhibitory concentration method. **Conclusions:** LRP assay with phAE85 is 99% specific, 91% sensitive and is highly reproducible. Thus the assay offers a simple procedure for drug sensitivity testing, within the scope of semi-automation.

1. Introduction

In most developing countries, tuberculosis (TB) is the most common cause of death among opportunistic infections associated with HIV infection[1]. Emergence of multi drug resistant (MDR) strains that are resistant to the two primary anti-tubercular drugs, isoniazid and rifampicin (RMP), have been reported worldwide[2]. Treatment of TB without information on the drug susceptibility pattern of the clinical isolate increases the risk of treatment failure and the spread of resistant strains. MDR-TB cannot be treated with first line drugs and its management using second line drugs requires proper organization[2]. Extensively drug resistant strains develop due to delay in diagnosis and mismanagement of MDR infections[3]. These strains lead to increased mortality and nosocomial out breaks[4]. Detection of resistance to RMP serves as a surrogate marker for diagnosis of MDR-TB[5].

Rapid liquid based systems like the non radiometric, fully automated MGIT960 systems are expensive and require specific bio-safety facilities, despite the low time to detection[6]. Genotypic nucleic acid amplification tests (NAAT), both commercial and in-house, are reported to have widespread inconsistencies[7]. Most forms of NAAT are more reliable with smear positive cases with high specificity and positive predictive value and hence can be used for ruling-in TB[8]. The more recent Genotype MTBDRplus (Hain Life Sciences, Nehren, Germany) has excellent sensitivity and specificity for detecting RMP resistance[9]. Despite the advantages, the method is complex, expensive and requires specific facilities in terms of infra structure[10].

Inexpensive direct sensitivity tests, based on direct inoculation of sputum deposit on the drug-containing Lowenstein-Jensen (LJ) slopes[11], give the susceptibility pattern of the isolates by 4 weeks, but they require a reasonable number of bacilli in the sample. Other less expensive drug susceptibility tests (DST) use redox indicator dyes[12] or measure levels of adenosine triphosphate (ATP)[13]

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for quantifying mycobacterial growth. The results of the ATP bioluminescence assay did not compare well with that of conventional drug sensitivity test[14]. However, TB endemic regions where the laboratory resources are limited still depend on the standard DST using LJ medium that requires 4-6 weeks for the results to become available.

The need of the hour is a rapid, reliable yet cost effective test that can substitute the conventional DST and can be useful for better management and treatment for patients with TB especially in program conditions. Reporter phages that detect viable microorganisms offer a specific tool for DST[15]. The present study was to evaluate luciferase reporter phage (LRP) construct phAE85 in rapid detection of RMP resistance in a region where TB is endemic.

2. Materials and methods

2.1. Drugs

The antibiotic RMP (Sigma, MO) was initially dissolved in dimethyl formamide (Merck) and diluted in distilled water to obtain a concentration of 1 mg/mL and filtered through 0.45 μm membrane filter. Aliquots were stored at -80 ℃ till use.

2.2. LRP

LRP phAE85 was used in the study. The construct was propagated and titrated on lawns of *Mycobacterium tuberculosis* (*M. tuberculosis*) mc2155[16] as previously described[17].

2.3. Cultures used

Primary isolates of *M. tuberculosis* grown on LJ medium were selected as soon as growth was observed. One hundred ninety cultures were included in the study.

2.4. DST

Conventional minimum inhibitory concentration (MIC) method was performed using standard protocol[18].

2.5. DST by LRP method

The *M. tuberculosis* isolates were coded for LRP assay. Suspension of growth was prepared in sterile Middlebrook 7H9 medium and the turbidity was adjusted to #2 McFarland's standard. About 100 μL of the suspension was added separately to two vials containing 350 μL of 7H9 medium (control) and 350 μL of 7H9 medium containing RMP at a final concentration of 2 μg/mL (test).

The cultures were incubated at 37 ℃ for 72 h after which 50 μL of phAE85 was added at a multiplicity of infection of 10. After a further incubation of 3-4 h, 100 μL of the cell phage suspension was mixed with an equal volume of 0.33 mmol/L D-luciferin (Sigma, MO) in 0.05 mol/L sodium citrate buffer at pH 4.5 in a cuvette. Light output was measured immediately in a luminometer (Monolight® Model 2010 Analytical Luminescence Laboratory, Ann Arbour, MI) as relative light units (RLU) at 10 s integration. Duplicate readings were taken for all the vials. Categorization of cultures was done based on standardization experiments. Cultures showing more than 50% reduction in RLU in 'test' compared to 'control' were classified as sensitive and less than 50% reduction were considered as resistant.

Whenever the mean RLU of the controls was less than 1 000, the experiment was repeated. Susceptibility patterns of the strains were compared with conventional assay results. Cultures of *M. tuberculosis* H37Rv and duplicates of 10% cultures were doubly coded and repeated as internal quality controls.

2.6. Statistical analysis

Results of conventional LJ and LRP assay were analyzed using SPSS software version 14.0. Statistical tests included *Chi* square, Kappa, and Fisher exact test. The test was based on 95% confidence interval.

3. Results

3.1. Phage propagation and titre

LRP phAE85 was propagated in bulk and stored at 4 °C. A titre of 5×10^{8} pfu/mL was used throughout the assay.

3.2. Phage infection and light production

All 190 strains of *M. tuberculosis* were found to be susceptible to infection by the LRP construct phAE85 and all of them yielded measurable RLUs using the luminometer. Less than 10% of cultures yielded <1 000 RLU with the control and the assay was repeated for all of them using heavy inoculum.

3.3. Conventional LJ

Among 190 cultures used, 34 strains were reported as resistant and the remaining 154 strains were reported as susceptible by conventional LJ method.

3.4. DST by LRP

Among the 190 cultures tested, 34 strains were reported as resistant to the drug by the conventional method. Among them, 31 (91%) were identified as resistant by the LRP assay. Among the remaining 156 susceptible cultures, LRP assay identified 154 strains as susceptible and 2 as resistant (99%) (Table 1).

Table 1
Susceptibility to rifampicin by conventional minimum inhibitory concentration (MIC) and luciferase reporter phage (LRP) assay.

LRP	Conventional MIC		
	Resistant	Sensitive	Total
Resistant	31	2	33
Sensitive	3	154	157
Total	34	156	190

Comparison between the test and the conventional LJ medium showed high level of agreement of 97% (*CI* 0.94, 0.99) and *P* value was 0.000 1. The sensitivity and specificity of LRP assay for detection of RMP resistance were 91% (*CI* 0.75, 0.98) and 99% (*CI* 0.95, 1.00) respectively. The positive and negative predictive values of the assay were 94% (*CI* 0.78, 0.99) and 98% (*CI* 0.94, 1.00) respectively. Internal quality control cultures yielded 100% reproducible results. Time to detection of resistance was 3 d using the primary isolate in comparison with 28 d for the MIC method.

4. Discussion

Potential of the LRP phAE85 in drug susceptibility assays has already been demonstrated by using microwell plate and by applying photo film based detection in a Bronx box[19]. In the current study, LRP was evaluated using a tube luminometer in a TB endemic area. This instrument yields definitive and quantitative results unlike the Bronx-box which offers semi quantitative results and is dependant on visual interpretation. Such interpretation may yield false results especially in borderline resistant strains. Further, with highly sensitive variations of the luminometer being available including portable models, such an evaluation would aid in establishing a field friendly method for drug susceptibility testing in even remote areas.

The TM4 based phage construct, phAE85, is known to produce 3-10 times higher RLU consistently compared to the first generation reporter phage phAE40 infecting *M. tuberculosis*[20]. The sensitivity and specificity of LRP-DST assay were 92% and 99%. These values were within the range reported by Pai *et al*[21] for LRP-DST using other recombinant phage constructs and methodologies. The agreement with the indirect sensitivity tests was 97% and corresponded closely to that reported for MGIT960 system for determination of resistance to RMP at critical concentration in comparison with conventional resistance ratio method[22]. Discrepant cultures when repeated by both methods yielded no change in the results. Reproducibility of the test was found to be high with internal quality control cultures yielding results 100% similar to original results.

In the current study, LRP-DST yielded five discrepant results out of 190 cultures. False susceptibility was reported in three strains that exhibited low level resistance by the conventional method. Instance of broth based detection systems such as BACTEC 460 and BACTEC MGIT 960 yielding false susceptible results for low level resistant strains have been reported earlier[23]. Two true susceptible strains were reported as false resistant. This discordance is perhaps due to the inherent differences in the methods of inoculation employed by both methods. While LRP assay is performed using sterile aerosol barrier tips, MIC is usually performed using sterile loops where presence of one micro colony of the growth in either could influence the outcome of the test. Such discordance arising due to differences between the methodologies adopted has been reported[24].

Van Deun *et al*. suggested the use of simple screening techniques as no single test is complete and accurate with respect to detection of drug resistant TB[25]. The LRP assay in its present form has low reagent costs and is simple to perform. Given the high specificity of the LRP-DST using phAE85, *i.e*., its high positive predictive value for resistant strains, it could be used to arrive at more appropriate therapies. The assay does not depend on expensive, highly sensitive instruments like BACTEC MGIT 960 or the genotypic methods which require specific infra structure facilities and supplies that limit its application in resource limited settings. Further, WHO recommends use of NAATs only for screening smear positive cases for RMP resistance[26]. Dependence on DST using primary culture still exists in smear negative cases and for treatment monitoring. Rapidity of LRP-DST with a time to detection of 3 d in comparison with 28-42 d for the conventional methods makes it more appealing in program conditions. Possibility of performing the test as an in-house semi automated assay makes it more attractive for medium level laboratories. Multi-centric evaluation of the procedure and feasibility studies for decentralization of the technique may pave the way for introduction of LRP-DST in National Tuberculosis Control Programs as low cost screening technology for smear negative cases as well as for treatment monitoring of MDR TB patients.

The present study uses LRP assay as a rapid drug susceptibility assay for detection of MDR-TB in an endemic setting. LRP assay with phAE85 is 99% specific, 91% sensitive and is highly reproducible. Thus, the assay offers a simple procedure for drug sensitivity testing, within the scope of semi-automation. Wherever it is possible to obtain a culture on solid media, it can be performed with relative accuracy, speed, ease, and low cost.

Conflict of interest statement

We declare we have no conflict of interest.

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