Diagnostic luciferase reporter phage assay for active and non-replicating persistors to detect tubercle bacilli from sputum samples

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Abstract

Diagnosis of latent tuberculosis infection is a myth for want of a simple, direct tool. Simulation of hypoxic environment was done to create a novel hypothetical model for persistence using processed sputum samples. The adaptation of tubercle bacilli to hypoxic environment seems to be influenced by pre-existing clinical status of the patients at the time of sputum collection, resulting in varied growth pattern. Bacilli from 36 samples did not get adapted to latency of which 15 samples were from patients in whom the disease was well established and the tubercle bacilli in them probably did not experience any stress whatsoever. Similarly, 10 of the 37 samples showing the presence of cultivable cells in both aerobic and anaerobic conditions were from patients who had relapsed. The bacilli in these samples had been probably experiencing stress and thus were ready to adapt to the hypoxic environment. Diagnostic luciferase reporter phage assay for non-replicating persistors (DLRPA-NRP) identified 30 additional positives which failed to grow on Lowenstein–Jensen medium. Presence of viable bacilli in these samples was confirmed by reverse transcriptase-PCR (RT-PCR) for 16S rRNA indicating either the improved sensitivity of the assay to detect actively growing bacilli or its ability to detect non-replicating persistors. The utility of LRP assay to detect both dormant and active tubercle bacilli was explored in this work and was optimized using lysis inhibition to diagnose tuberculosis with rapidity, improved sensitivity and specificity. DLRPA-NRP, a rapid growth based assay is thus developed to detect both dormant and actively growing tubercle bacilli.

Keywords: Diagnostic luciferase reporter phage assay for non-replicating persistors, dormancy, luciferase, mycobacteriophage, *Mycobacterium tuberculosis*

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Introduction

It has been estimated that about 9.2 million new cases and 1.7 million deaths are attributed to tuberculosis in 2006 [1]. Despite increasing knowledge about the pathogenicity and epidemiology of the disease, several problems still remain in the detection, prevention and treatment of *Mycobacterium tuberculosis*-mediated infection [2]. The nature of the tubercle bacillus that persists during apparently adequate chemotherapy or in the interval between infection and reactivation of disease remains a mystery [3]. Calmette, Much and Khomenko, asreported by Grange, believed that there were virulent filterable forms of the tubercle bacilli [3]. But they were not reported in suspensions from different postulated *in vitro* or *in vivo* models for dormancy. This widens the gap between knowledge of the true nature of latent bacilli and of bacilli seen in several reported stimulatory models of persistent tubercle bacilli. Several *in vitro* and *in vivo* systems have been developed to mimic aspects of latent infection [4]. Deb *et al.* [5] developed an *in vitro* multiple stress dormancy model and reported that it efficiently generated *M. tuberculosis* cells meeting all the criteria of dormancy. Wayne's popular model is based on the assumption that *M. tuberculosis* encounters hypoxic conditions during its residence within granulomatous lesions in the host [6]. It was presumed that the bacilli in the sputum specimens were ideal for the simulation of a dormancy model compared with primary cultures because they would bear the remnants of the stress other than the hypoxic environment. Hence, for the first time, sputum specimens processed by a modified Petroff's method were directly taken into a state of slow

oxygen withdrawal by Wayne's method to develop non-replicating persistors in the present work. In addition, we hypothesized and aimed at elucidating the influence of the pre-existing clinical conditions as a defining factor enabling the tubercle bacilli to enter latency.

Growth-based assays are biomarkers that provide conclusive evidence of the presence of viable tubercle bacilli. However, no such methodology is available to measure viable non-replicating persistors of tubercle bacilli. The ability of *M. tuberculosis* to enter a phenotypically drug-resistant non-replicating dormant state during latent infection is a major impediment because the available drugs cannot kill these latent bacilli and there are no growth-based tests to measure dormant bacilli.

Luciferase reporter phage (LRP) assay with first-generation reporter phages needed at least 10^4 organisms/mL to result in readable relative light units (RLU) as measured by luminometer [7]. Rapid drug sensitivity testing was established for isoniazid and rifampicin using primary cultures [8]. The need to improve the sensitivity of this assay and to detect both dormant and actively growing bacilli was realized. Subsequently, TM4-based reporter phages expressing luciferase gene driven by promoters of mycobacterial persistence genes were found to exhibit enhanced photon production in both dormant and actively growing *M. tuberculosis* cultures [9]. In the present work we evaluated their potential as a diagnostic tool for active and persistent tuberculosis using sputum specimens.

Materials and Methods

Bacterial strains, LRP constructs and chemicals

Clinical *M. tuberculosis* isolates (two anti-tuberculosis-drug-sensitive and one multi-drug-resistant strain) were grown at 37°C in Middlebrook 7H9 media (Difco, Franklin Lakes, NJ, USA) supplemented with 5% glycerol and 10% albumin dextrose complex (G7H9). A total of five LRP constructs, namely phAE129, phAETRC16, phAETRC21; phAETRC201 and phAETRC202 [9], were used in this work. They were propagated in *Mycobacteirum smegmatis* mc^2155 using Luria–Bertani medium.

Lysis inhibition

Super-infection with a second phage before the progeny release of the first phage, delays the process of host cell lysis, resulting in increased light output. This phenomenon is referred to as lysis inhibition [10]. Using the phage constructs phAE129, phAETRC16, phAETRC21, phAETRC201 and phAETRC202, a total of 25 combinations were tested for the maximum RLU output with three clinical strains of *M. tuberculosis*. Suspensions of *M. tuberculosis* were made in G7H9 using growth on fresh Lowenstein–Jensen (LJ) slopes. Suspensions of 1×10^7 cells/mL were mixed with equal volumes of 1×10^8 PFU/mL of phage to obtain a multiplicity of infection of 10. Phage–bacteria mixture was incubated at 37°C for 2 h before infection with second reporter phage and incubated further at 37°C for 4 h. Photons released were measured using a tube luminometer (Monolight 2010) after adding 0.33 mM D-luciferin (R&D Systems, Minneapolis, MN, USA) in 0.05 M sodium citrate buffer at pH 4.5.

Evaluation of LRP constructs in aerobic and anaerobic models using sputum samples

Sputum samples from new and old patients suffering from tuberculosis admitted in a prospective study at the Tuberculosis Research Centre, Chennai were randomly selected. Most of the patients, excepting their contacts, had regular follow up and their clinical details were made available for analysis and recorded. A total of 95 sputum samples were processed by modified Petroff's method and each deposit was inoculated onto two LJ slopes. The remaining portion of each deposit was added to 1.4 mL G7H9 along with methylene blue (1.5 mg/L), vancomycin (10 mg/L) and phagebiotics [11] to control the overgrowth of normal flora (Middlebrook 7H9 medium with Albumin Dextrose Catalase with Methylene blue, Vancomycin, Phagebiotics (MADC MVP)) and divided into two parts of 600 μL each. The first part was added to a sterile Bijou bottle with 2 mL MADC MVP and incubated in a shaker incubator. The second part was added to a cryovial with 0.9 mL MADC MVP and sealed. The vial was kept in a shaker incubator for 2 weeks till the blue colour disappeared. Diagnostic luciferase reporter phage assay for non-replicating persistors (DLRPA-NRP) was performed at 3 and 9 days of incubation for the samples grown aerobically. On the 3rd and 9th days, four 250-μL aliquots were diluted ten-fold up to N4 dilution using four different reporter phage combinations. The combinations used for the first set of 27 smear-positive sputum deposits were phAETRC201 followed by phAETRC16, phAETRC201 followed by phAE129, phAETRC16 followed by phAE129 (Table 1), and phAETRC 21 alone.

The photons released were measured as RLU using a luminometer (Monolight 2010). The DLRPA-NRP was performed using the samples in the cryovials for Wayne model. Based on these results, the remaining 68 sputum samples were tested with phAETRC201 followed by phAETRC16, and with phAETRC21 alone.

Reverse transcription-PCR of 16S rRNA

Sputum deposits were stored at −80°C after a portion of the same was used for inoculation into solid media and DLRPA-NRP. Sputum deposits, which were negative for growth by the conventional method and positive by DLRPA-

TABLE 1. Luciferase reporter phage assay using 2.5 mL medium using different reporter phage combination

Phage combination	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
phAETRC16/phAE129	68	20.6	32.5	53.8
phAETRC201/phAETRC16	75	15.4	57.7	28.6
phAE129/phAE129	50	56.6	43.5	62.6

NPV, negative predictive value; PPV, positive predictive value.

NRP, were chosen. Reverse transcription (RT-) PCR assay was performed with these deposits after extracting RNA using the acid–guanidinium thiocyanate–phenol–chloroform method [12]. DNA contamination was removed by DNAasel treatment, heat-inactivated at 70°C for 5 min and subsequently RNA was extracted with phenol–chloroform. Complementary DNA was synthesized from extracted RNA and 16S rRNA was amplified using a commercially available RT-PCR kit (Bangalore Genei, India). The primers used for this purpose were forward, 5′-GTGGCGAACGGGTGAGTAAC-3′ and reverse, 5′-GAGTCTGGGCCGTATCTCAG-3′

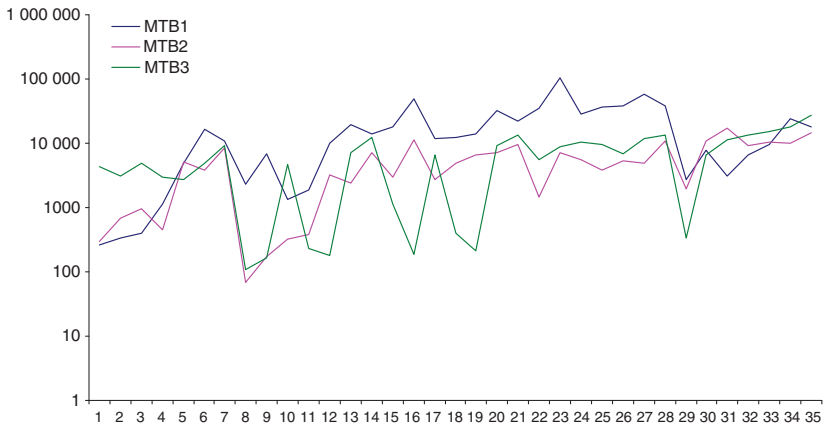
Results

Lysis inhibition

The LRP constructs phAETRC21 and phAETRC201 gave maximum RLU output with both sensitive and resistant strains of *M. tuberculosis*. The procedure using lysis inhibition yielded maximum RLU with the multi-drug-resistant isolate using phAETRC201 followed by any other construct for second infection (Fig. 1). However, with a drug-susceptible strain of *M. tuberculosis*, phAETRC201 followed by phAETRC202 gave the maximum light output. Hence, phAETRC201 followed by phAETRC202 was optimal in both multi-drug-resistant and drug-sensitive strains (Fig. 1).

Evaluation of LRP constructs in aerobic and anaerobic models using sputum samples

With the optimized assay format using four combinations of the phage constructs, the assay was evaluated in 27 smear-positive sputum samples in comparison with conventional culture. All the 27 smear-positive samples were detected by DLRPA-NRP whereas one of them did not grow on LJ medium. The two assay formats using phAETRC201 followed by phAETRC16 and phAETRC21 alone were able to detect 26 out of 27 smear-positive samples. Hence these two formats were chosen for further use and another set of samples, including 41 smear-negative samples and 27 smear-positive samples, was tested. In all, in 95 samples both these assay formats were able to diagnose 73 as positives, out of which only 49 were positive by a conventional culture method (Table 2). DLRPA-NRP had identified 30 new positives that failed to grow on the LJ



Y-axis	First infection	Second infection	Y-axis	First infection	Second infection	Y-axis	First infection	Second infection	Y-axis	First infection	Second infection	Y-axis	First infection	Second infection
1	phAE129	NIL	**8**	phAETRC16	NIL	**15**	phAETRC21	NIL	**22**	phAETRC201	NIL	**29**	phAETRC202	NIL
2	phAE129*	Nil	**9**	phAETRC16*	NIL	**16**	phAETRC21*	NIL	**23**	phAETRC201*	NIL	**30**	phAETRC202*	NIL
3	phAE129	phAE129	**10**	phAETRC16	phAE129	**17**	phAETRC21	phAE129	**24**	phAETRC201	phAE129	**31**	phAETRC202	phAE129
4	phAE129	phAETRC16	**11**	phAETRC16	phAETRC16	**18**	phAETRC21	phAETRC16	**25**	phAETRC201	phAETRC16	**32**	phAETRC202	phAETRC16
5	phAE129	phAETRC21	**12**	phAETRC16	phAETRC21	**19**	phAETRC21	phAETRC21	**26**	phAETRC201	phAETRC21	**33**	phAETRC202	phAETRC21
6	phAE129	phAETRC201	**13**	phAETRC16	phAETRC201	**20**	phAETRC21	phAETRC201	**27**	phAETRC201	phAETRC201	**34**	phAETRC202	phAETRC201
7	phAE129	phAETRC202	**14**	phAETRC16	phAETRC202	**21**	phAETRC21	phAETRC202	**28**	phAETRC201	phAETRC202	**35**	phAETRC202	phAETRC202

* RLU reading taken after 7 hours

FIG. 1. Evaluation of luciferase reporter phage constructs using lysis inhibition in *Mycobacterium tuberculosis* clinical isolates with the phage constructs phAE129, phAETRC16, phAETRC21, phAETRC201 and phAETRC202 a total of 25 combinations were tested for the maximal log10 relative light units in the *x*-axis.

TABLE 2. Performance of optimized luciferase phage receptor (LRP) assay in comparison with the Lowenstein–Jensen (LJ) -based conventional culture/LJ-based conventional culture plus and reverse transcription–polymerase chain reaction (RT-PCR) method in 95 sputum samples

	Conventional/Conventional culture + RT-PCR		
	Positive	Negative	Total
LRP			
Positive	43/72	30/01	73
Negative	06/06	16/16	22
Total	49/78	46/17	95

Sensitivity 88/92%, Specificity 35/94%, Agreement 62/92%, positive predictive value 59/99%, negative predictive value 76/72%.

medium. Six samples positive by the conventional culture were not detected by LRP assay.

Presence of viable bacilli in these samples was tested by performing RT-PCR for 16S rRNA. Two samples positive by culture were used as positive controls while another two samples negative for both culture and DLRPA-NRP were included as negative controls. The RT-PCR of the two culture-positive samples yielded signals. One of the two negative controls produced amplified product of 16S rRNA. However, the smear status of this sample was 2+ by an auramine phenol staining method for acid-fast bacilli. Sensitivity, specificity and agreement of DLRPA-NRP in diagnosing tubercle bacilli in comparison with the culture using LJ medium as gold standard were 88%, 35% and 62%, respectively, with positive predictive value of 59% and negative predictive value of 76% (Table 2). Samples that were negative by conventional culture and positive by DLRPA-NRP were subjected to RT-PCR. When DLRPA-NRP results were compared with conventional culture and RT-PCR combined, there was an increase in sensitivity, specificity and agreement reaching 92%, 94% and 92%, respectively (Table 2).

Survival and growth of the bacilli in a hypoxic environment was evident in 33 samples because they became positive by DLRPA-NRP and 22 of them grew anaerobically in the solid media. Of these 33 samples adapted to hypoxia, 29 grew on solid media when incubated aerobically (Table 3). In samples incubated aerobically, 23 were positive by DLRPA-NRP that did not grow on LJ medium in both aerobic and anaerobic conditions. Of the 95 samples, six were contaminated when incubated under hypoxic conditions. Among the 89 samples remaining, four samples incubated anaerobically grew on LJ when subcultured but failed to grow when cultured directly on LJ. Two other samples positive by DLRPA-NRP were negative on LJ when incubated in both conditions. These six samples were among additional positives picked up by DLRPA-NRP and gave positive signals for 16S rRNA.

TABLE 3. Conventional and luciferase phage receptor (LRP) assay results in sputum deposits grown in aerobic and anaerobic conditions

Aerobic LJ	Aerobic LRP	Wayne LRP	Wayne LJ	Total
P	P	P	P	15
P	P	P	N	9
P	P	N	N	11
P	P	N	P	3
P	N	P	P	4
P	N	N	N	2
P	N	N	P	Nil
P	N	P	N	Nil
N	N	P	P	Nil
N	P	N	P	1
N	P	P	N	2
N	P	P	P	3
N	P	N	N	23
N	N	P	N	Nil
N	N	N	N	16
N	N	N	P	Nil
			Total	89

LJ, Lowenstein–Jensen medium; N, Negative; P, Positive.

Discussion

Subsequent to first phage infection and before cell lysis/progeny release, infection by the second phage leads to lysis inhibition facilitating continuous availability of cellular ATP and luciferase enzyme [10]. Hence, the delay in lysis results in a significant increase in the time of availability of progeny phages inside the cell. Applying this phenomenon, a modified assay format using phAETRC201 followed by phAETRC16 was tested in the present work.

Phagebiotics reported to be non-inhibitory to tubercle bacilli [11] were used in combination with vancomycin and malachite green to control the overgrowth of normal flora in the present experiments.

Using 27 smear-positive sputum samples, phAETRC201 followed by phAETRC16 and phAETRC21 alone detected 26 of the 27 samples. Similarly, both these assay formats failed to diagnose six samples of the 95 tested (Table 2). In four of these samples when incubated anaerobically the constructs could infect the bacilli and produce detectable light.

Solid medium is known to be less favourable than liquid medium for re-growth of dormant bacilli [13]. In 89 sputum samples subjected to hypoxic conditions, the transition was observed in 33 of them as confirmed by DLRPA-NRP whereas 26 samples showed only aerobic growth, reiterating the advantage of using liquid medium for retrieving the latent bacilli. Five out of 33 samples positive by Wayne DLRPA-NRP did not show growth on LJ medium incubated aerobically (Table 3). These samples probably contained only dormant bacteria that are expectorated in sputum specimens as observed by earlier workers [14]. There is a probability that

small number of actively growing bacteria might have been present in these samples in small numbers and so could not be detected by conventional methods. Bacilli from 36 samples did not become adapted to latency, of which 15 samples were from old cases treated for more than a year (Table 3) and five of were from fresh patients with full-blown disease caused by drug-sensitive strains. The majority of these were therefore from patients in whom the disease was established and the tubercle bacilli in them were not experiencing any stress. Probably, they were not prepared to readily become adapted to latency. Although sputum has traditionally been thought to contain actively growing tubercle bacilli, transcript analyses refute the hypothesis [14]. Mycobacteria store triacylglycerols (TGs) under various stress conditions such as hypoxia, exposure to nitric oxide and acidic environments [15]. Extensive accumulation and degradation of TGs were found in the bacilli during entry into and exit from hypoxic dormancy, respectively. The lack of preparedness to enter latency might be associated with the degradation of TGs in the bacilli or lack of accumulation of TGs. Moreover, hypoxia is not the exclusive stress inducing lipid body formation [14] and the absence of other stress factors may probably explain the non-adaptation of tubercle bacilli in such samples to latency.

In 37 of the samples, the presence of cultivable cells was observed in both aerobic and anaerobic conditions (Table 3). Ten of these samples were from patients who had relapsed earlier. The bacilli in these samples had probably experienced stress during the course of the disease and so were ready to adapt to the hypoxic environment. Resuscitation promoting factor might also be playing a vital role in bringing back these dormant bacilli to an actively growing stage. There was probably a lack of resuscitation promoting factor in the earlier group, leading to less preparedness in them to become adapted to latency.

Desjardin *et al.*, [16] demonstrated that 16S rRNA remains unaffected and expression of 16S rRNA does not change during different growth conditions. Based on these assets, the presence of 16S rRNA was used to confirm the culture negative and LRP-positive samples. Out of 67 sputum samples that were positive by aerobic DLRPA-NRP, 23 samples did not result in the growth of cultivable cells on LJ medium in both the conditions but were positive by RT-PCR, indicating the advantage of the DLRPA-NRP over conventional methods (Table 3). Mean time to detection was around 7 days using the optimized format of the DLRPA-NRP, which satisfies one of the goals of WHO guidelines [17]. The improved sensitivity and specificity of DLRPA-NRP compared with LJ and RT-PCR, the potential to detect non-cultivable but viable forms, less time to detection, simplicity and cost-effectiveness signify the importance of DLRPA-NRP in providing rapid diagnosis of tuberculosis suitable for the developing world.

Transparency Declaration

None.

References

1. Falagas ME, Kouranos VD, Athanassa Z *et al.* Tuberculosis and malignancy. *QJM* 2010; 103: 461–487.
2. Russell DG, Barry CE 3rd, Flynn JL. Tuberculosis: what we don't know can, and does, hurt us. *Science* 2010; 328: 852–856.
3. Grange JM. The mystery of the mycobacterial 'persistor'. *Tuberc Lung Dis* 1992; 73: 249–251.
4. Zahrt TC. Molecular mechanisms regulating persistent *Mycobacterium tuberculosis* infection. *Microbes Infect* 2003; 5: 159–167.
5. Deb C, Lee CM, Dubey VS *et al.* A novel *in vitro* multiple-stress dormancy model for *Mycobacterium tuberculosis* generates a lipid-loaded, drug-tolerant, dormant pathogen. *PLoS ONE* 2009; 4: e6077.
6. Wayne LG, Lin KY. Glyoxylate metabolism and adaptation of *Mycobacterium tuberculosis* to survival under anaerobic conditions. *Infect Immun* 1982; 37: 1042–1049.
7. Jacobs WR Jr, Barletta RG, Udani R *et al.* Rapid assessment of drug susceptibilities of *Mycobacterium tuberculosis* by means of luciferase reporter phages. *Science* 1993; 260: 819–822.
8. Banaiee N, January V, Barthus C *et al.* Evaluation of a semi-automated reporter phage assay for susceptibility testing of *Mycobacterium tuberculosis* isolates in South Africa. *Tuberculosis (Edinb)* 2008; 88: 64–68.
9. Dusthackeer A, Kumar V, Subbian S *et al.* Construction and evaluation of luciferase reporter phages for the detection of active and non-replicating tubercle bacilli. *J Microbiol Methods* 2008; 73: 18–25.
10. Bode W. Lysis inhibition in *Escherichia coli* infected with bacteriophage T4. *J Virol* 1967; 1: 948–955.
11. Kumar V, Balaji S, Gomathi NS *et al.* Phage cocktail to control the exponential growth of normal flora in processed sputum specimens grown overnight in liquid medium for rapid TB diagnosis. *J Microbiol Methods* 2007; 68: 536–542.
12. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* 1987; 162: 156–159.
13. Biketov S, Potapov V, Ganina E *et al.* The role of resuscitation promoting factors in pathogenesis and reactivation of *Mycobacterium tuberculosis* during intra-peritoneal infection in mice. *BMC Infect Dis* 2007; 7: 146.
14. Garton NJ, Waddell SJ, Sherratt AL *et al.* Cytological and transcript analyses reveal fat and lazy persister-like bacilli in tuberculous sputum. *PLoS Med* 2008; 5: e75.
15. Low KL, Rao PS, Shui G *et al.* Triacylglycerol utilization is required for regrowth of *in vitro* hypoxic nonreplicating *Mycobacterium bovis* Bacillus Calmette–Guérin. *J Bacteriol* 2009; 191: 5037–5043.
16. Desjardin LE, Perkins MD, Teixeira L *et al.* Alkaline decontamination of sputum specimens adversely affects stability of mycobacterial mRNA. *J Clin Microbiol* 1996; 34: 2435–2439.
17. Global tuberculosis control. Surveillance, planning, financing. Report. World Health Organization 2005.