Phage lysin as a substitute for antibiotics to detect *Mycobacterium tuberculosis* from sputum samples with the BACTEC MGIT 960 system

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Abstract

Phage lysin was evaluated as a substitute for antibiotics in sputum samples processed by a modified Petroff's method for the detection of *Mycobacterium tuberculosis* with the MGIT 960 system. One hundred and fifty sputum samples were processed, inoculated onto two slopes of Lowenstein–Jensen medium, and divided in to two aliquots of 0.5 mL each. One aliquot was added to 7 mL of MGIT medium containing polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin (PANTA) (MGIT-PANTA) and the other was added to 7 mL of MGIT medium containing 0.8 mL of lysin (MGIT-Lysin). The samples were randomized and incubated at 37°C in the MGIT 960 system. The sensitivity and specificity of MGIT-Lysin were 97% and 88%, respectively, as compared with MGIT-PANTA. The average times to detection with MGIT-Lysin and MGIT-PANTA were 9.3 and 8.6 days, respectively. The rate of contamination with MGIT-PANTA and MGIT-Lysin were 16% and 7.3%, respectively. Phage lysin can be substituted for antibiotics in processed sputum samples for the detection of *M. tuberculosis*.

Keywords: Diagnosis, MGIT 960, normal flora, phagebiotics, phage lysin

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Introduction

Primary isolation of *Mycobacterium tuberculosis* from sputum samples, on either solid or liquid media, and identification of the drug sensitivity pattern is essential for determination of the disease status of a patient. Overgrowth of normal flora escaping the action of the sputum-processing agent is a major problem that affects the sensitivity of any rapid assay [1]. Combinations of antibiotics, in the form of either polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin (PANTA) or polymyxin B, amphotericin B, carbenicillin and trimethoprim (PACT), and other compounds are incorporated into the media used for the primary isolation of tubercle bacilli to minimize the risk of non-mycobacterial contamination. However, it is recommended that the exposure of primary isolates to these antibiotics should be limited, as these agents affect the long-term growth of mycobacteria [2].

The use of cocktails of phages as phagebiotics substituting for antibiotics to control the overgrowth of normal flora in processed sputum samples has been demonstrated [2]. This constitutes a novel, non-chemical and bio-friendly approach to tackle the problem of non-mycobacterial contaminants. Phage-based enzyme, lysin, was also added to phagebiotics for the effective control of normal flora. The combination of phagebiotics with lysin and lysin alone were found to be efficient and comparable for control of the overgrowth of normal flora without hampering the viability of *M. tuberculosis* [3].

In the present study, phage lysin was evaluated for decontamination of processed sputum samples in comparison with PANTA for the primary isolation of *M. tuberculosis* with the BACTEC MGIT 960 system. In addition, lysin was evaluated in comparison with PANTA for the growth and retrieval of *M. tuberculosis* with Lowenstein–Jensen (LJ) medium.

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Materials and Methods

Preparation of phage lysin

Phage lysin was prepared individually from phages Chedec 11, Chedec 20 and Chedec 21, as described previously [3]. These phage lysins were dissolved in albumin dextrose complex (4 g of D-glucose, 0.85 g of NaCl and 10 g of bovine serum albumin in 100 mL of distilled water) at its final stage of preparation, and pooled prior to use.

Effect of lysin on the growth of *M. tuberculosis*

One hundred and twenty sputum samples were collected from patients attending the Tuberculosis Research Centre, Chennai, and processed by modified Petroff's method. Two LJ slopes were inoculated from the processed deposits and used as controls. The deposits were washed with 0.067 M phosphate-buffered saline at pH 6.8, and divided into two aliquots of 0.5 mL each. To one aliquot, 0.5 mL of PANTA was added by mixing 0.1 mL of PANTA in 0.4 mL of 7H9 medium supplemented with 0.5% glycerol and 10% albumin dextrose complex (G7H9) (7H9-PANTA). To the other aliquot, 0.5 mL of lysin was added by mixing 0.1 mL of pooled lysin in 0.4 mL of G7H9 (7H9-Lysin). The aliquots were randomized and incubated at 37°C for 18–24 h. Two more LJ slopes were inoculated from all of the aliquots and incubated. The LJ slopes were read every week up to 8 weeks. The results were compared after decoding.

Evaluation of lysin for the decontamination of sputum samples

A total of 150 sputum samples were collected from patients attending the Tuberculosis Research Centre, Chennai; smears prepared from these samples were stained by the auromine phenol staining method. The samples were processed by modified Petroff's method. Two LJ slopes were inoculated from the deposits thus obtained, and incubated at 37°C for up to 8 weeks. The deposits were washed with phosphate-buffered saline and divided into two aliquots of 0.5 mL each. One aliquot was added to a MGIT tube containing 7 mL of medium and 0.8 mL of PANTA (MGIT-PANTA). Another aliquot was added to a MGIT tube containing 7 mL of medium and 0.8 mL of pooled lysin (MGIT-Lysin). The tubes were randomized and kept in the MGIT 960 instrument. Further readings and confirmation of the presence of tubercle bacilli were performed according to the manufacturer's protocol. The results were compared after decoding.

Statistical analysis

The test of significance was calculated with Fisher's exact test, using SPSS software version 14.

Results

Effect of lysin on the growth of *M. tuberculosis*

The sensitivity and specificity of 7H9-PANTA were 91% and 94%, respectively, and the sensitivity and specificity of 7H9-Lysin were 92% and 91%, respectively, as compared with modified Petroff's culture as the reference standard (Table 1a,b). The sensitivity and specificity of 7H9-Lysin were 98% and 91%, respectively, as compared with 7H9-PANTA. The agreement between the methods was 94% (Table 1c). None of these samples was found to be contaminated when treated with either lysin or PANTA and incubated overnight at 37°C. Only three samples were contaminated by conventional culture, and were excluded from the comparison.

Evaluation of lysin for decontamination of sputum samples

Of the 150 sputum samples collected, 103 were positive (69%) and 47 were negative (31%) by smear with the auromine phenol staining method. Modified Petroff's culture showed growth of *M. tuberculosis* in 83 (55.3%) samples, and

TABLE 1. Effect of polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin (PANTA) and phage lysin on the growth of *Mycobacterium tuberculosis*

a

		Petroff's culture		
		Pos	Neg	Total
7H9-PANTA	Pos	48	4	52
	Neg	5	60	65
	Total	53	64	117

Sensitivity, 91%; specificity, 94%; agreement, 92%; PPV, 92%; NPV, 92%

b

		Petroff's culture		
		Pos	Neg	Total
7H9-Lysin	Pos	49	6	55
	Neg	4	58	62
	Total	53	64	117

Sensitivity, 92%; specificity, 91%; agreement, 91%; PPV, 89%; NPV, 94%

c

		7H9-PANTA		
		Pos	Neg	Total
7H9-Lysin	Pos	51	6	57
	Neg	1	62	63
	Total	52	68	120

Sensitivity, 98%; specificity, 91%; agreement, 94%; PPV, 89%; NPV, 98%

Pos, positive; Neg, negative; PPV, positive predictive value; NPV, negative predictive value.
Sputum samples were processed with a modified Petroff method, and two Lowenstein–Jensen (LJ) slopes were inoculated (Petroff's culture). The sputum deposits were divided into two: to one aliquot, Middlebrook 7H9 medium containing PANTA (7H9-PANTA) was added, and this was used as a control. To the other aliquot, 0.5 mL of Middlebrook 7H9 medium containing phage lysin (7H9-Lysin) was added; this was incubated overnight and inoculated onto two LJ slopes.

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TABLE 2. Comparison of MGIT-PANTA and MGIT-Lysin for the detection of *Mycobacterium tuberculosis* with the MGIT 960 system

a

		Petroff's culture		
		Pos	Neg	Total
MGIT-PANTA	Pos	79	12	91
	Neg	0	35	35
	Total	79	47	126

Sensitivity, 100%; specificity, 74%; agreement, 90%; PPV, 87%; NPV, 100%

b

		Petroff's culture		
		Pos	Neg	Total
MGIT-Lysin	Pos	84	15	99
	Neg	0	40	40
	Total	84	55	139

Sensitivity, 100%; specificity, 73%; agreement, 89%; PPV, 85%; NPV, 100%

c

		MGIT-PANTA		
		Pos	Neg	Total
MGIT-Lysin	Pos	88	4	92
	Neg	3	28	31
	Total	91	32	123

Sensitivity, 97%; specificity, 88%; agreement, 94%; PPV, 96%; NPV, 90%

PANTA, polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin; Pos, positive; Neg, negative; PPV, positive predictive value; NPV, negative predictive value.
Sputum samples were processed with a modified Petroff method, and two Lowenstein–Jensen slopes were inoculated (Petroff's culture). The sputum deposits were divided into two: one aliquot was added to MGIT tubes containing PANTA (MGIT-PANTA) and used as a control. The other aliquot was added to MGIT tubes containing phage lysin (MGIT-Lysin) and incubated in the MGIT system.

no growth in 66 (44%) samples; the remaining sample was contaminated (0.7%). The sensitivity of MGIT-PANTA and MGIT-Lysin was 100%, and the specificities were 74% and 73%, respectively, as compared with conventional culture (Table 2a,b). The sensitivity and specificity of MGIT-Lysin were 97% and 88%, respectively, as compared with MGIT-PANTA as the reference standard. The agreement between the methods was 94% (Table 2c). The average times to detection for MGIT-Lysin and MGIT-PANTA were 9.3 and 8.6 days, respectively, as compared with conventional culture (Table 3). The rates of contamination were 16% with MGIT-PANTA and 7.3% with MGIT-Lysin. The samples that showed contamination with either of the methods were excluded from the comparison.

TABLE 3. Average time to detection (days) for MGIT-PANTA and MGIT-Lysin

	Smear		Culture	
Grade	PANTA	Lysin	PANTA	Lysin
Negative	11.5 (3–38)	12.4 (3-39)	12.8 (2–39)	15.8 (4–27)
Colonies	NA	NA	10.0 (2–19)	10.4 (2–14)
1+	9.1 (3–29)	9.8 (2– 27)	9.3 (1–17)	9.0 (1–22)
2+	6.5 (2–12)	6.6 (3–14)	6.0 (2–12)	6.0 (1–12)
3+	5.9 (1–8)	6.2 (3–9)	5.0 (2–7)	5.4 (2–10)
Average	8.3	8.8	8.6	9.3

PANTA, polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin; NA, not applicable.
Ranges are given in parentheses.

Among 66 culture-negative samples, 14 were positive (21%) by MGIT-PANTA, with a mean time to detection (TTD) of 12.8 days. Of the remaining 52 samples, 35 were negative (53%) and 17 were contaminated (26%). Of the samples treated with phage lysin (MGIT-Lysin), 17 were positive (26%), with a mean TTD of 15.8 days, 40 were negative (61%), and only nine were contaminated (13%). Among 83 culture-positive samples, 14 grew few colonies, and 22, 33 and 14 resulted in growth graded as 1+, 2+ and 3+, respectively, on LJ medium. When PANTA was used (MGIT-PANTA) for the detection of tubercle bacilli, 77 samples were positive and the remaining six samples were contaminated. Similarly, when phage lysin was used (MGIT-Lysin), 82 samples were positive and only one sample was contaminated. None of the samples positive on LJ medium was missed by both MGIT-PANTA and MGIT-Lysin, resulting in a sensitivity of 100%. The sample that was contaminated by LJ was also contaminated by both MGIT-PANTA and MGIT-Lysin (Table 4).

Statistical analysis

Sputum samples processed and treated with phage lysin for the detection of *M. tuberculosis* with the MGIT 960 system

TABLE 4. Overall performance of MGIT-PANTA and MGIT-Lysin for the detection of *Mycobacterium tuberculosis* with the MGIT 960 system

	MGIT-PANTA			MGIT-Lysin		
Culture grade (no. of samples)	Positives (TTD) in days	Negatives	Contamination	Positives (TTD) in days	Negatives	Contamination
Negatives (66)	14 (12.8)	35	17	17 (15.8)	40	9
Colonies (14)	11 (10.0)	0	3	14 (10.4)	0	0
1+ (22)	21 (9.3)	0	1	22 (9.0)	0	0
2+ (33)	31 (6.0)	0	2	32 (6.0)	0	1
3+ (14)	14 (5.0)	0	0	14 (5.4)	0	0
Contamination (1)	0	0	1	0	0	1
Total (150)	91	35	24	99	40	11
	150			150		

PANTA, polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin; TTD, time to detection.

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showed significance as compared with samples treated with PANTA. Fisher's exact test showed a value of 0.000.

Discussion

The mechanical pressure and the chemical pressure induced when sputum specimens are processed with either a strong alkali such as 4% NaOH (Petroff's method) or a combination of N-acetyl-L-cysteine (NALC) and 2% NaOH (NALC-NaOH method) leave the tubercle bacilli in the sputum unsuitable for any rapid assay, especially for phage-based assays [2]. Sputum specimens have inherent features that hamper the detection of viable tubercle bacilli, resulting in difficulties in rapid tuberculosis diagnosis [4]. The main problem is caused by the normal flora present in the sputum specimens, and problems are also caused by mucous strands, enzymes and other inhibitory factors, and the remains of chemicals used in sputum processing. The normal flora escaping the action of sputum processing chemicals tend to overgrow and mask the growth of tubercle bacilli, leading to poor sensitivity in detecting *M. tuberculosis*. Contamination in liquid-based detection systems by particular bacteria or fungi reflects the presence of these microorganisms in the specimens received for culture and the ability of these organisms to survive decontamination processes [5]. The most common organisms causing contamination are from respiratory sources (*Bacillus*, *Staphylococcus*, *Micrococcus*, *Pseudomonas*, coagulase-negative staphylococci and *Corynebacterium*). In some studies, the presence of yeast and moulds has also been reported.

The BACTEC system for direct susceptibility testing in clinical laboratories did not find practical application, for four main reasons: 'unknown effect of five antibiotics (PANTA) added to prevent the growth of contaminants, on the activity of the tested agent' is one reason among the four [6]. There have been reports of PANTA added to suppress the growth of contaminating bacteria in the MGIT 960 system having some inhibitory effect on some mycobacteria other than *M. tuberculosis* complex [7]. This inhibition varies from species to species and between isolates within a species. PANTA has been replaced by a combination of vancomycin, amphotericin B and nalidixic acid to minimize the rate of contamination in the MGIT 960 system without reducing mycobacterial recovery. However, vancomycin itself has a bactericidal effect on some mycobacteria, such as *Mycobacterium fortuitum*, *Mycobacterium senegalense*, *Mycobacterium septicum* and unidentified strains of mycobacteria [8].

Inhibitory effects of PANTA on the growth of *Mycobacterium genavense* and *Mycobacterium kansasii* in BACTEC primary cultures have been reported. Preliminary experiments showed that polymyxin B and azlocillin exerted some growth inhibition on *M. genavense* [9], and nalidixic acid had an inhibitory effect on the growth of *M. kansasii* [10]. In the selective antibiotic combination PACT, carbenicillin has been reported to have potentially inhibitory effects on mycobacteria [11]. Furthermore, it has also been reported that PACT used in the MB Redox tube method reduces mycobacterial growth in specimens collected from sterile body sites [12]. As an alternative to combinations of antibiotics, phage lysin was used in the present study to control the overgrowth of normal flora without compromising the viability of *M. tuberculosis*.

Inclusion of a liquid medium for primary isolation and identification of *M. tuberculosis* has been recommended [13]. Conventional solid culture with either LJ medium or 7H11 agar medium is the reference standard. In the 1990s, the radiometric semi-automated BACTEC 460TB system was the liquid-based detection method that was most widely used for mycobacteria, and was universally accepted as the reference standard for liquid medium [14]. Owing to the presence of radiolabelled carbon in the medium and semi-automation, the BACTEC 460 system was replaced by the non-radiometric, fully automated MGIT 960 system. The MGIT 960 system has been evaluated and is considered to be a valuable alternative to the BACTEC 460 system [15]. However, a higher rate of contamination was reported for the MGIT 960 system than for the BACTEC 460 system [16]. The higher contamination rate was attributed to the presence of rich protein i.e. albumin dextrose catalase in MGIT 960 medium [14], the percentage of NaOH used to decontaminate sputum samples [17], and climatic conditions and delays in transport of samples to the laboratory [18]. The high contamination rate with the MGIT 960 system resulted in a lower recovery rate of *M. tuberculosis* [19].

The average TTD for the MGIT 960 system was reported to range between 8.5 and 13.3 days. In this study, the average TTDs for both MGIT-Lysin (9 days) and MGIT-PANTA (8 days) were found to be low (Table 3). In 2009, Rodrigues *et al.* [16] reported TTDs of 7, 8, 11 and 16 days for samples with 3+, 2+, 1+ and negative smear grades, respectively. In the present study, average TTDs were 5, 6, 9 and 11 days for the detection of *M. tuberculosis* from samples with smear grades of 3+, 2+, 1+ and negative, respectively.

A slight increase was noted in the average TTD for MGIT-Lysin as compared with MGIT-PANTA. The difference was found to be not significant by *t*-test (p 0.84). An in-house preparation of albumin dextrose complex was used for MGIT-Lysin, whereas a growth supplement containing oleic acid was used for MGIT-PANTA. The difference in growth supplement may be attributed to the increased

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average TTD for MGIT-Lysin. Of the samples treated with MGIT-PANTA and MGIT-Lysin, 24 (16%) and 11 (7.3%), respectively, showed overgrowth of normal flora, and eight samples showed growth with both. Three samples that resulted in overgrowth of normal flora only in MGIT-Lysin were negative in MGIT-PANTA, and among 16 samples that resulted in overgrowth of normal flora only in MGIT-PANTA, six samples were positive and the rest were negative in MGIT-Lysin. Phage lysin used to control overgrowth of normal flora in processed sputum samples significantly increased the rate of recovery of *M. tuberculosis* and decreased the rate of contamination as compared with the use of a combination of antibiotics, i.e. PANTA, without hampering the viability of *M. tuberculosis*.

The approach of utilizing bacteriophages and their lysin to control the overgrowth of normal flora in processed sputum samples have been filed for patenting through the Intellectual Property Rights Unit of the Indian Council of Medical Research (File No. 2819/DEL/2008). Sequencing of bacteriophages Chedec 11, Chedec 20 and Chedec 21 has been initiated. Furthermore, on the basis of the genome sequence, phage lysins of these phages will be identified, characterized and purified to favour the bulk production of lysins by genetic engineering. Accordingly, the cost-effectiveness of lysin preparation will be calculated in comparison with PANTA, and a multicentre evaluation will be performed to assess the efficacy of phage lysins in decontaminating processed sputum samples.

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Transparency Declaration

No conflicts of interests declared.

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