

# EVALUATION OF A MICROCOLONY DETECTION METHOD AND PHAGE ASSAY FOR RAPID DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* IN SPUTUM SAMPLES

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**Abstract.** Early and rapid diagnosis of tuberculosis is necessary for both treatment and control of the disease. This study evaluated two microcolony observation techniques based on liquid and solid media and a mycobacteriophage assay, to evaluate their effectiveness in the diagnosis of pulmonary TB compared with a standard culture (BACTEC 460 and LJ medium). Middlebrook7H9 (M7H9) broth based on microcolony determination detected 57/61 positives cultures (n=200) with a sensitivity of 93.4% and a specificity of 87.1%. M7H11 agar detected 57/62 positive cultures (n=198) with a sensitivity of 91.9% and a specificity of 89.7%. The mycobacteriophage assay detected 98/143 (68.5%) of positive samples. The time to positivity was 48 hours in the mycobacteriophage assay versus 7 days in both the M7H9 broth and M7H11 agar. The costs in comparison with the culture (BACTEC 460 and LJ) were 33% and 48% for the microcolony and mycobacteriophage methods, respectively. Microcolony methods were rapid and cost effective compared to standard cultures. The mycobacteriophage assay, despite its lower sensitivity, has a short turn around time, and may be recommended as a screening test in countries with a low prevalence of tuberculosis.

## INTRODUCTION

Tuberculosis (TB) is a major cause of morbidity and mortality throughout the world (Andersen *et al*, 2000). Under expanded DOTS program coverage, cure rates for diagnosed TB cases are 90%. However, inadequate case detection remains a major limiting factor in TB control (Perkins and Kriski, 2002). Rapid diagnosis of TB remains difficult. Isolation of *Mycobacterium tuberculosis* by culture is considered to be the gold standard. Traditional solid medium culture methods [*eg*, culture on Löwenstein Jensen (LJ slants)] have sensitivities around 76.9% (Badak *et al*, 1996), while sensitivities of automated liquid systems are

higher (84.4%) (Stager *et al*, 1991; Badak *et al*, 1996; Pfyffer *et al*, 1997). Current guidelines recommend at least one solid medium should be inoculated along with an automated liquid system. The combined sensitivity of the solid and liquid culture is 92.1%, which is higher than either single method (Stager *et al*, 1991; Wilson *et al*, 1995). Major limitations of the current culture techniques include high cost and prolonged result time.

Other diagnostic modalities used in the diagnosis of tuberculosis include molecular and serological methods. Molecular methods are expensive and their field validity has yet to be established (Savic *et al*, 1992; Pfyffer *et al*, 1994; Pfyffer, 1999). Serological methods are reported to have low sensitivities (60-75%) and specificities (85-99%) (Ulrich *et al*, 1998; Poltumarthy *et al*, 2000; Bellete *et al*, 2002). There is an urgent need to develop rapid, low cost alternative diagnostic tests with improved

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sensitivity and specificity. The microcolony method for culture and identification of *Mycobacterium* was first reported in 1970 (Runyon, 1970). This method has been recently proposed as an inexpensive alternative for the rapid detection of *Mycobacterium tuberculosis* (Welch *et al*, 1993; Caviedes *et al*, 2000; Flouney and Twilley, 2001). Microcolony detection depends upon identification of the characteristic strings and tangles of *M. tuberculosis* (in either broth or on agar) using simple light microscopy. Use of light microscopy allows earlier detection of growth as oppose to standard egg-based culture methods.

The ability of mycobacteriophages to multiply in *Mycobacterium* is the basis of yet another recently developed diagnostic assay (Albert *et al*, 2002). Mycobacteriophages multiply within *M. tuberculosis* and are detected as plaques using separate indicator for *Mycobacterium* species. In this study we compared the microcolony and mycobacteriophage assays with the standard culture, evaluating their sensitivity, specificity, rapidity, and cost-effectiveness.

## MATERIALS AND METHODS

Sputum samples submitted to the microbiology laboratory of a tertiary care hospital in Karachi were used for this study. All samples were from clinically suspected cases of pulmonary tuberculosis. Microcolony detection (broth and agar) and mycobacteriophage assay were each evaluated against the standard culture on Löwenstein Jensen medium (Oxoid) and BACTEC460. A minimum of 80 sputum samples were needed with an anticipated routine culture positive rate of 20/100 with a 95% confidence interval and a standard error of 5%. Sample sizes used for the three methods were as follows: microscopic observation of M7H9 broth in 217, microcolony detection on M7H11 agar in 224, and mycobacteriophage assay in 500, with a total of 941 samples.

Sputum samples were decontaminated with N-acetyl-L-cysteine (NALC) sodium hydroxide and concentrated by centrifugation (3,000g) for 30 minutes (Nolte and Metchock, 1999). The sediment was used for AFB microscopy, cultured in a BACTEC 12B vial (Becton Dickinson) and on LJ slant (Oxoid) in addition to one of the three methods under evaluation.

Smears for microscopy were stained using auramine. Smears positive on fluorescent microscopy were further confirmed by staining with Kinyoun modification of Ziehl-Neelsen stain (Nolte and Metchock, 1999). LJ slant was inoculated with approximately 0.3 ml of decontaminated specimen. Readings were taken once a week, and any growth was stained by the Kinyoun method, to test for acid-fast bacilli. LJ slants were held for eight weeks before being discarded as negative for AFB.

Each 12B vial was inoculated with 0.5 ml of decontaminated sputum. The growth index of the inoculated 12B vials was checked two times a week for the first two weeks, and weekly thereafter for another four weeks (total 6 weeks). A growth index of 10 or more was considered positive, acid-fast bacilli were checked by Kinyoun staining.

Final confirmation of isolates growing on LJ slant and in BACTEC 12B vials as *Mycobacterium tuberculosis* complex was done by the NAP test (p-Nitro- $\alpha$ -acetylamino- $\beta$ -hydroxypropiophenone) (BACTEC). Decontamination of the contaminated BACTEC 12B vial was done according to manufacturer's recommendations (Becton Dickinson).

### Microcolony detection (M7H9 broth)

Eight hundred microliters of sample, after decontamination/concentration, was inoculated into 7.2 ml of Middlebrook 7H9 broth (Difco) containing 5.9 g/l of Middlebrook7H9 broth base, 0.3% glycerol, 1.25 g/l bacto casitone (Difco), 10% OADC, and 160  $\mu$ l of PANTA antimicrobial supplement stock (20  $\mu$ l/

ml). The sputum-medium mixture was distributed into 6 wells of a sterile 24-well plate at 1.2 ml per well. Broth controls were included in each assay to check for cross contamination. Each run also included a susceptible *M. tuberculosis* control H37Rv at 1/50 dilution of  $1 \times 10^8$  CFU/ml<sup>-1</sup>. This control was examined daily for growth for 6 to 8 days. Plates were sealed with polyethylene tape and incubated at 37°C for up to 40 days. Each well was examined on a daily basis for growth of colonies using an inverted light microscope at 40x magnification. Each well was examined for approximately 30 to 45 seconds. Any suspected microcolonies were stained by the Kinyoun method. The final identification of isolates was done by the NAP test.

#### Microcolony detection (M7H11 agar)

Middlebrook 7H11 agar medium (Difco) was inoculated with 100 µl of the decontaminated samples. The plates were sealed and incubated for 6 weeks at 37°C in 5% CO<sub>2</sub>. Plates were examined under a 40x objective using an inverted microscope for growth, once during the first week, then 2-3 times a week for up to six weeks. Each batch included positive controls, an internal *M. tuberculosis* strain and H37Rv inoculated onto separate 7H11 plates. Any suspected microcolonies were stained using the Kinyoun method.

#### Mycobacteriophage assay

The methodology used was in accordance with the Fast Plaque TB Test (Biotec laboratories, UK). Fifteen milliliters of supplemented M7H9 broth was added to 1 ml of decontaminated sample. The mixture was then centrifuged (3,000g for 20 minutes). The supernatant was discarded and the pellet was resuspended in 1 ml supplemented M7H9 broth and transferred to a reaction vessel tube for overnight (15-18 hours) incubation at 37°C. Following incubation, 100 µl of solution, containing mycobacteriophages was added to each sample tube, incubated at 37°C for 1

hour, then 100 µl of virucidal agent were added. The contents of the reaction tubes were thoroughly mixed by rolling and inverting the tube, to ensure contact of the virucidal agent with the entire inner surface of the tube to aid in the inactivation of any exogenous phages. Samples were incubated at room temperature for 5 minutes. The virucidal activity was then neutralized using 5 ml of supplemented M7H9 broth and 1 ml of indicator *M. smegmatis* cells. The entire contents of the reaction tube were added to 5 ml M7H9 agar base (kept at 50-60°C in a water bath) and poured into a sterile Petri dish. Once set, the Petri dish was inverted and incubated at 37°C for 18-24 hours. The number of plaques (zone of clearing) in the lawn of host cells was recorded. Each batch also included a negative and positive control. The negative control composed of only M7H9 agar base should have <10 plaques. The positive control, consisting of *M. smegmatis* infected with phage and treated with a virucide, should have resulted in 20-300 plaques. The results were considered valid only if the positive and negative control values were within the specified range. A positive result revealed at least 20 plaques.

To determine the sensitivities of the various methods, we considered true positive samples as positive by one or more standard culture methods. The p-value was calculated using Mac Nemar's  $\chi^2$  test. A p-value of less than 0.05 was considered significant.

Contamination (bacterial/fungal) was found in 18/941(1.9%) BACTEC 12B vials, and was reprocessed after decontamination per the manufacturer's recommendations (Becton Dickinson). However, 28/941(3%) of LJ slants were excluded from the study due to contamination. For these samples only the BACTEC cultures were followed. Similarly, contamination (bacterial/fungal) was noted in 17/217 (7.8%) samples inoculated in M7H9 broth and 26/224 (11.6%) set up on M7H11 agar, which

were excluded from the study. The mycobacteriophage assay was initially evaluated in 500 samples, of these 30 (6%) had to be excluded from the assay due to unsatisfactory control results. No fungal contamination was noted in any of the mycobacteriophage assay samples, while the bacterial contamination rate was 1.5%. However, since the plaques were readable the results were noted.

## RESULTS

A total of 868 samples were used for final analysis. Of these samples, 200, 198 and 470 samples were tested in M7H9 broth, on M7H11 agar and with the mycobacteriophage assay, respectively, and compared with routine culture (BACTEC/LJ) (Table 1).

Fig 1 is a microphotograph of *M. tuberculosis* complex in M7H9 broth showing the characteristic strings and tangles appearance of microcolonies. Microscopic observation of broth detected 57 out of 61 samples positive by BACTEC/LJ (93.4% sensitivity, 87.1% specificity, 76% positive predictive value (PPV) (Table 1). A further 18 samples showed growth in M7H9, but did not grow in BACTEC/LJ cultures ( $p$ -value=0.004).

Similarly microcolony detection on M7H11 agar (Fig 2) was assessed in 198 samples, with 57/62 true positives (positive on BACTEC/LJ) (91.9% sensitivity, 89.9%

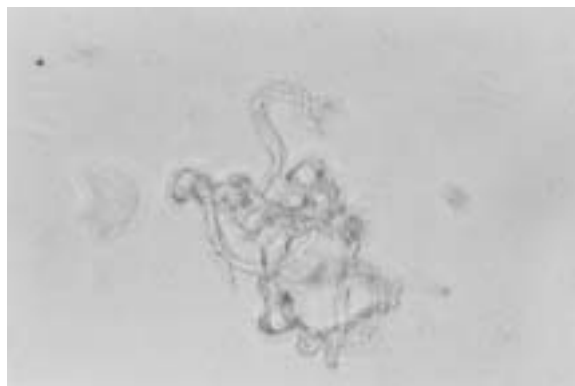


Fig 1—Appearance of *M. tuberculosis* at 400x magnification after 15 days of culture in M7H9 broth. Note the serpentine, ropy aspect of the microcolonies.

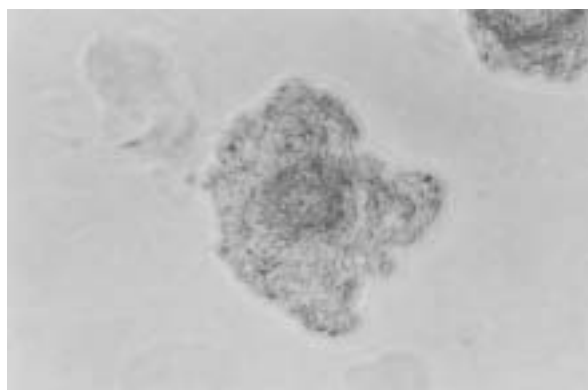


Fig 2—Appearance of *M. tuberculosis* at 400x magnification after 10 days of culture on M7H11 agar medium.

Table 1

Comparison of microcolony and mycobacteriophage methods results with BACTEC/LJ culture.

Test		BACTEC/LJ culture		% Sensitivity	% Specificity	PPV	NPV
		Positive	Negative				
M7H9 broth (n=200)	Positive	57	18	93.4	87.1	76.0	96.8
	Negative	4	121				
M7H11 agar (n=198)	Positive	57	14	91.9	89.7	80.2	96.0
	Negative	5	122				
Mycobacteriophage assay (n=470)	Positive	98	41	68.5	87.4	70.5	86.4
	Negative	45	286				

Table 2

Comparison of microcolony and mycobacteriophage methods results with AFB smears and culture findings.

AFB Smear	Test	BACTEC/LJ culture		% Sensitivity	% Specificity	PPV	NPV	
		Positive	Negative					
Positive	M7H9 broth	Positive	56	2	100	50	96.5	100
		Negative	0	2				
Negative		Positive	1	16	20	88.1	5.9	96.7
		Negative	4	119				
Positive	M7H11 agar	Positive	56	2	98.2	0	96.5	0
		Negative	1	0				
Negative		Positive	1	12	20	91	7.7	96.8
		Negative	4	122				
Positive	Mycobacterio phage	Positive	89	7	74.2	65	92.7	29.5
		Negative	31	13				
Negative		Positive	9	34	39.1	88.9	20.9	95.1
		Negative	14	273				

Table 3

Detection time of MTB by new methods versus standard cultures and AFB microscopy.

Method	Median detection time (range)
AFB Microscopy	2-3 hours
Mycobacteriophage assay	48 hours
Microscopic observation of M7H9 broth	7.8 days (2-24)
Microagar M7H11	Within 7 days
Bactec460	14 days (7-14)
LJ slant	25 days (15-45)

specificity). Fourteen samples yielded growth only in M7H11 agar, not with either BACTEC or LJ cultures (PPV 80.3%, NPV 95.7%) The difference between the two tests was not significant  $p$ -value=0.064) (Table 1).

Both microcolony methods were also analyzed individually for smear positive and negative samples with good sensitivities and PPV for smear positive samples. With smear

negative samples, these methods had a lower sensitivity (20%) and a high NPV (96.7%) (Table 2).

The mycobacteriophage assay was performed on 500 samples. Of these, 30 (6%) had to be excluded from the assay due to unsatisfactory control results. As shown in Table1, this assay detected only 98 (70.5%) of 139 BACTEC/LJ positive samples. Therefore in comparison to the microcolony detection methods, the mycobacteriophage assay had a lower sensitivity (70.5%) and specificity (86.2%) ( $p$ -value =0.746). On the AFB smear positive samples, mycobacteriophage assay had a sensitivity of 74.2% and a specificity of 65%. On smear negative samples, while the sensitivity was low (39.1%), the specificity and negative predictive values were high (88.9% and 95.1%, respectively) (Table 2).

Of the three methods the mycobacteriophage assay had the shortest time to positivity (48 hours). Both microcolony methods (M7H9 broth and M7H11 agar) detected the majority of positive cultures within seven

days of incubation (Table 3). M7H9 broth detected 56% of positive samples within five days, and M7H11 agar detected 57% of positive samples within the first week. The cost of the microcolony methods was about 33% of the combined BACTEC and LJ media cultures, while the cost of the mycobacteriophage assay was half (48%) that of the cost of routine cultures.

## DISCUSSION

This laboratory based study had a 30% (61/200) rate of isolation of *M. tuberculosis* complex from sputum samples by combining the BACTEC and LJ cultures. Most likely the reason for this high rate of positivity by routine culture is that this study was conducted in a tertiary care hospital laboratory, which usually receives specimens from highly suspected cases of tuberculosis from all over the country. In our study, the sensitivity and specificity of microcolony culture in M7H9 broth were 93.4% and 87.1%, respectively. Similar findings were observed in a study conducted in Peru (Caviedes *et al*, 2000). Eighteen samples in our study were positive only on microscopic observation of M7H9 broth, which could be due to cross contamination within the 24-well plates used in the assay. In the future, such cross contamination could be controlled through the use of a single plate per sputum sample or by using well-strips.

Culture on M7H11 agar gave a sensitivity of 91.9% and a specificity of 88.9% compared with routine culture methods (BACTEC and LJ). This shows a good sensitivity of this method even with a small inoculum size (0.1 ml) compare to routine culture. These findings are comparable with another study (Mejia *et al*, 1999) in which sputum samples of clinically suspected cases were used to report sensitivity (78%) and specificity (89.7%) of M7H11 agar versus routine cultures.

In both microcolony methods, a poor yield

for smear negative cases was disappointing, but supported by earlier reports suggesting that M7H11 agar was comparable to LJ for cultures of specimens with numerous to few AFB, but the specimen yield with rare or no AFB is better on LJ medium (Caviedes *et al*, 2000).

Using microscopic observation of broth, the average time to positivity was 7.8 days, compared to 25 and 14 days for LJ and BACTEC, respectively. This is comparable to an earlier report (Caviedes *et al*, 2000) of 9 days (4-31 days) for an average detection time using microscopic observation of broth, compared to 24 days by LJ culture. The use of inverted microscopy further reduces the time to detection of microcolonies, compared to macroscopic detection of growth on LJ media.

The average detection time for M7H11 microagar was within the 1<sup>st</sup> week. This finding is supported by an earlier study (Welch *et al*, 1993) where the average detection time using thin layer M7H11 was reported as 10 days. The same study further noted that detection time was shorter for smear positive samples.

Of concern was the contamination rate observed with both microcolony detection methods. In this study, the contamination rates for M7H9 broth and M7H11 agar were 7.8% and 11.6%, respectively, which is higher than routine culture methods (BACTEC and LJ). In addition, the contamination rates for these methods were high compared with previously published contamination rates for routine cultures methods, 2.4 to 7.3% for BACTEC and 2.8 to 7.4% for LJ (Wilson *et al*, 1995). This suggests the need for careful handling of these plates during processing and microscopy. For M7H11 agar, the reason for the high contamination rate could be the use of amphotericin B as a sole antibiotic agent. Further studies are needed to evaluate the effect of incorporating other antibiotics, such as polymyxin, carbenicillin and trimethoprim, into M7H11

agar. Previous investigators (Welch *et al*, 1993) reported a contamination rate of 16.5% for M7H11 agar despite the use of both piperacillin and amphotericin B in the media. The high contamination rate of M7H9 broth suggests the antibiotics used in our assays may require adjustment for future use.

For the microcolony methods, mycobacterial growth was observed manually, which resulted in an increased time to diagnosis. In laboratories where the workload is low to moderate, M7H11 agar may be a good addition to routine LJ culture. In addition, this method is inexpensive and fits well in resource limited low technology laboratories where funds may not be available to procure expensive equipment.

Mycobacteriophages have the potential of becoming useful tools in the diagnosis of TB, since they are specific for *Mycobacterium* (Mole and Maskell, 2001). Moreover, phage technology has the considerable advantage of requiring no special equipment other than that already available in a routine culture laboratory. Both the phages and *M. smegmatis* indicator cells can be produced in-house at a low cost, a factor that generally facilitates the transfer of this technology to laboratories in low-income countries. However, since this method includes the culture of *M. tuberculosis*, highly stringent laboratory safety precautions are necessary (Nerney, 1999)

An earlier trial conducted at the South African Institute of Medical Research (SAIMR) in Cape Town, South Africa compared the results from mycobacteriophage assay to the liquid BACTEC culture system. Their data showed the mycobacteriophage assay not only had a specificity of 100% but also an excellent positive predictive value of one, which shows a high level of confidence that a positive result actually represents a true result. In our study, the sensitivity was 70.5% (CI 61.5-74.8%) compared to standard cultures

(BACTEC and LJ). Specificity of the mycobacteriophage assay was 86.2% (83.8-90.2%) with a positive predictive value of 68.5%. Thus indicating that with this assay 70.5% of positive cultures could be detected within two days rather than 4-6 weeks typically required by standard culture. The lower sensitivity in our samples compared to earlier reports (Muzaffar *et al*, 2002) may be due to resistant mycobacteria. Mycobacteria resistant to lysis by mycobacteriophages, growing in spite of the presence of phages have been reported. These bacteria were found to contain viruses, now classified as temperate phages, which did not produce lysis (Nerney, 1999). The mycobacteriophage method has a large negative predictive value in smear negative cases and may thus be of use as a support to exclude active pulmonary tuberculosis in smear negative cases in which there is a clinical suspicion of active disease. The contamination rate was low compared to routine methods. However, a potential limitation of this assay is the technical skill required to perform it. These requirements are likely to be at least as high as those for conventional cultures, which will limit the number of laboratories in which they may be performed (Perkins, 2000). All three methods tested were cost effective compared to the cost of the BACTEC and LJ media cultures.

In conclusion, while the sensitivity for both microscopic observation of M7H9 broth and M7H11 agar was acceptable, the specificity can be further improved by using a single plate or strip for M7H9 broth, which will reduce the chance of cross contamination. The high contamination rate for M7H9 broth raises a concern regarding the antibiotics used. Similarly, M7H11 agar needs the addition of other antibiotics to prevent growth contaminants. Both of these tested methods are rapid and cost effective when compared with BACTEC and LJ cultures. In our view, these methods can be used along with conventional LJ cultures

in laboratories with low to medium workload for rapid presumptive diagnosis. This study was done only on sputum samples. Further study is needed to evaluate other pulmonary and extra-pulmonary samples using these methods, to assess their applicability to low income countries.

The mycobacteriophage method analyzed in this study had a sensitivity and specificity of 70.5% and 86.2%, respectively. Being the second most rapid method after AFB microscopy and having a low contamination rate, this method can be used as a screening test in low prevalence countries where skilled technical staff are available and high cost is not a major issue.

#### ACKNOWLEDGEMENTS

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