

## Comparative Evaluation of Microscopy and Culture Methods in the Diagnosis of Pulmonary Tuberculosis in HIV Infected Patients

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**Tuberculosis is a serious infectious disease with considerable public health problem due to its person to person transmission, morbidity and mortality. To evaluate the performance of different laboratory methods such as direct microscopy, concentration methods and culture in the diagnosis of pulmonary tuberculosis in HIV infected patients. Sputum samples were collected using standard procedure. For each sputum sample, four smears were prepared, one direct smear, second after processing by Petroff's method, third by NALC-NaOH method and fourth after processing by Sodium hypochlorite method. Ziehl Neelsen (ZN) staining was done on these smears using standard techniques. Deposit obtained after processing by Petroff's method and NALC-NaOH method were inoculated on to Lowenstein Jensen (LJ) medium and Middlebrook's 7H11 agar medium. A total of 75 HIV infected patients presenting with symptoms of pulmonary tuberculosis were included in the study. 11 (14.6%) were direct sputum smear positive and 13 (17.3%) were AFB positive after concentration and culture positive. Out of 139 sputum samples tested, 22 (15.8%) were positive in direct smear. 25 samples, (18%) were positive for AFB after concentration and culture. We conclude that concentration methods are sensitive and reliable in the diagnosis of pulmonary TB in HIV infected patients. Sodium hypochlorite and Petroff's concentration methods are simple, cost effective and reliable provided centrifugation is done at 3800xg. Culture methods are sensitive and specific but take time for growth of *M. tuberculosis* (4-6 weeks).**

**Keywords:** *M. tuberculosis*, Petroff's method, NALC-NaOH method, Sodium hypochlorite method, Lowenstein Jensen medium, Middlebrook's 7H11 agar medium.

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Tuberculosis (TB) is a serious infectious disease with considerable public health problem due to its person to person transmission, morbidity and mortality. The morbidity and mortality caused by TB is immense, with 8.8 million cases with nearly 2 million deaths estimated to have occurred in 2003 alone.<sup>1,2</sup> The Human Immunodeficiency Virus (HIV) epidemic has had a huge impact on rise in incidence of TB in the worst affected countries.

TB is a major cause of death among HIV infected people amounting for atleast 11% of Acquired Immunodeficiency Syndrome (AIDS) deaths worldwide. Early diagnosis followed by prompt treatment is essential to prevent the morbidity and mortality caused by TB. This is most important with patients having TB in HIV infected. The relative significance of various laboratory methods may differ especially in HIV infected patients with TB. Therefore studies are needed to determine useful techniques to diagnose TB in HIV infected patients.

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## MATERIAL AND METHODS

The present study was carried out between July 2005 and June 2007 in the Department of Microbiology, of a tertiary health care centre in Mangalore. To evaluate the performance of different laboratory methods such as direct microscopy, concentration methods and culture in the diagnosis of pulmonary tuberculosis in HIV infected patients. The study population included HIV infected patients of all age group with symptoms consistent with pulmonary tuberculosis. Informed consent was obtained from all patients and the study was approved by the Institutional Ethics Committee. HIV infection was diagnosed by ELISA/Rapid/Simple tests strategy for diagnosis as recommended by WHO and National AIDS Control Organization, India.<sup>3,4</sup> Inclusion criteria were HIV seropositivity, informed consent and clinical suspicion of pulmonary TB. Exclusion criteria were treatment for TB within the previous 3 months or initiation of TB treatment before sample collection. Detailed clinical and physical examination was done including radiological examination for pulmonary infiltrate. Sputum samples were collected using standard procedure.<sup>5</sup> Sputum samples were collected in sterile disposable leak proof container with a screw-capped lid and processed within 2 to 3 hrs of collection, whenever immediate processing was not possible, the samples were refrigerated at 4°C and processed the next day.

### Processing of samples

All samples were processed inside Biological safety cabinet (BSC) by wearing laboratory coats, gloves and facemasks.<sup>5,6</sup> For each sputum sample, four smears were prepared, one direct smear, second after processing by Petroff's method, third by N-acetyl L-cysteine sodium hydroxide (NALC-NaOH) method and fourth after processing by Sodium hypochlorite (Bleach) method. Ziehl Neelsen (ZN) staining was done on these smears using standard techniques.<sup>5,6</sup> Deposit obtained after processing by Petroff's method and NALC-NaOH method were inoculated on to Lowenstein Jensen (LJ) medium and Middlebrook's 7H11 agar medium.

### Direct microscopic examination

#### Acid-fast staining procedure

On a new slide, purulent part of the

sputum was taken and a thin uniform smears were prepared using each sample, air dried, heat fixed for and stained by Ziehl Neelsen staining method.<sup>5</sup>

#### Ziehl Neelsen (ZN) staining method

The smear was flooded with carbol-fuchsin, was heated intermittently 3-4 times till the fumes appear for a period of 5 min, then was washed with tap water, and decolourised with 25% sulfuric acid for 2 mins, was then washed with tap water and counter stain with 0.1% methylene blue for 30 sec, and was washed with tap water and allowed to air dry and examined under the microscope objective (1000x). The observation and grading were done as per RNTCP guidelines.<sup>7</sup>

#### Concentration methods

##### Petroff's method

Sputum samples were decontaminated and concentrated using Petroff's method.<sup>5,8</sup> In a 15 ml screw capped tube sputum was mixed with equal amount of 4% sodium hydroxide. This mixture was incubated at 37°C with intermittent shaking for 20 min. It was then centrifuged at 3800xg for 15 min. The supernatant was discarded and to the sediment 1 drop of 1% phenol red indicator was added which showed pink colour, to this 8% hydrochloric acid was added drop by drop till the colour changes to yellow. Sediment thus obtained was used for smear and culture.

##### N-acetyl L-cysteine sodium hydroxide (NALC-NaOH) method

Sputum samples were decontaminated and concentrated using NALC-NaOH method.<sup>5,6,10</sup> In a 15 ml screw capped tube sputum was mixed with equal volume of NALC-NaOH digestant. Allowed the tubes to stand at room temperature for 20 min. Mixture was diluted with sterile distilled water, and centrifuged at 3800xg for 15 min. The supernatant was discarded and the sediment was resuspended in 1-2ml of sterile water and was used for smear, culture and PCR.

##### Sodium hypochlorite (Bleach) method

Sputum samples were concentrated using sodium hypochlorite method.<sup>11</sup> In a 15 ml screw capped tube, sputum was mixed with equal amount of 5% sodium hypochlorite. Solution and was mixed well and left at room temperature for 15 min with intermittent shaking. Then 8 ml of distilled water was added after mixing, centrifuged at 3800xg for 15 min. The supernatant was discarded and the sediment was mixed with remaining fluid

and smears were prepared by applying a drop of the resuspended sediment with a sterile pipette to a slide. The slides were air-dried, heat fixed and stained by the ZN staining method and observed under the microscope (1000 x).

### Culture

#### Lowenstein Jensen (LJ) medium

Sputum samples decontaminated by Petroff's method and NALC-NaOH was inoculated on the slant of the LJ medium (Hi Media, Mumbai) and incubated aerobically at 37°C for 4 to 8 weeks. The inoculated LJ media were observed after five days of incubation and then once in every week for mycobacterial growth up to 8 weeks.<sup>8,9</sup> A standard procedure was used for the identification of the *M. tuberculosis*.<sup>9,10</sup> The test included time required for growth, colony morphology, AFB smear, niacin test. Culture that showed no growth after 8 week was reported as negative.

#### Middlebrook's medium

Sputum samples decontaminated by Petroff's method and NALC-NaOH was inoculated on Middlebrook's 7H11 agar medium (Hi Media, Mumbai) and incubated aerobically at 37°C for 4 to 8 weeks, was observed for growth once every week upto 8 weeks.<sup>6,8</sup> Culture that showed no growth after 8 week was reported as negative.

#### Identification of *M. tuberculosis*

*M. tuberculosis* was identified based on time required for growth, colony morphology and niacin test.<sup>9,10</sup>

## RESULTS

A total of 75 HIV infected patients presenting with symptoms of pulmonary tuberculosis were included in the study. Sputum samples were collected from these patients. 11 (14.6%) were direct sputum smear positive and 13 (17.3%) were AFB positive after concentration (Table 1).

Of 75 HIV infected patients with symptoms of pulmonary TB, 11 (14.6%) were direct smear positive and 13 (17.4%) were culture positive (Table 2).

Out of 139 sputum samples tested, 22 (15.8%) were positive in direct smear. Twenty-five samples, (18%) were positive for AFB after concentration (Table 3).

Out of 139 sputum samples tested, 22

(15.9%) were positive in direct smear and 25 (18%) were culture positive (Table 4).

Out of 139 sputum samples tested, 22 (15.9%) were positive in direct smear and culture method, 3 (2.2%) were direct smear negative and culture positive, 114 (82%) were negative in direct smear and culture method (Table 5). Therefore, the sensitivity and specificity of direct microscopic examination were 88% and 100% respectively. The positive and negative predictive values of direct smear were 100% and 97.4% respectively.

Out of 139 sputum samples tested, 25 (18%) were positive in concentration and culture method and 114 (82%) were negative in direct smear and culture method (Table 6). These results indicate that compared to culture (gold standard) concentration method has specificity and sensitivity of 100%.

## DISCUSSION

Current TB control efforts are based on early diagnosis of cases followed by prompt, adequate treatment. In countries with high prevalence of TB and HIV, better tests and more efficient diagnostic methods are needed. In the present study we observed that, out of 75 HIV infected patients 17.3% were proved to have TB. This is consistent with a result of a previous study on TB in HIV infected patients.<sup>12</sup> In HIV infected patients TB is a common opportunistic infection, affecting every organ in the body. As estimated one in three people with AIDS will die of TB. TB may occur relatively early in the course of HIV infection. HIV fuels the tuberculosis epidemic in several ways. HIV promotes progression to active TB both in people with recently acquired and with late *M. tuberculosis* infections. HIV is the most powerful known risk factor for reactivation of latent tuberculosis infection to active disease. HIV infected people are more susceptible to TB infection when they are exposed to *M. tuberculosis*. In the present study, Out of 139 sputum samples tested from 75 patients (64 patient gave two sputum samples, 11 patients gave one sputum sample), 15.8% were positive in direct smear and 18% were positive for AFB after concentration. This is consistent with the results of a previous study where centrifugation combined with any chemical method was used.<sup>13</sup>

Sputum microscopy is the most important test for the diagnosis of pulmonary tuberculosis in low-income and middle-income countries, where 95% of tuberculosis cases and 98% of deaths occur.<sup>14</sup> In these countries, most laboratories use smears of unconcentrated sputum (direct smears) with Ziehl Neelsen staining. Direct microscopy is fast, simple, inexpensive, widely applicable, and highly specific for *M. tuberculosis* in tuberculosis-endemic countries. In addition, microscopy identifies the most infectious patients.<sup>15-17</sup> Smear negative tuberculosis is disproportionately higher in HIV-positive than in HIV-negative individuals, and has been linked to poor treatment outcomes, including death, especially in areas devastated by the HIV epidemic.<sup>17-21</sup> Microscopy does not, by definition, identify smear-negative pulmonary tuberculosis. Clearly, improvement

in the sensitivity of microscopy would be of great potential value. Reports describing newer sputum processing methods with chemical processing and sputum concentration to improve the sensitivity of microscopy.<sup>11</sup> In the present study, among concentration methods, Petroff's, N-acetyl L-cysteine sodium hydroxide and sodium hypochlorite methods, more bacilli were observed in smears prepared after sodium hypochlorite concentration. There is paucity of reports on sodium hypochlorite concentration in TB in HIV infected patients. An advantage of sodium hypochlorite concentration is the reduction in time spent on sputum examination. As a potent disinfectant, sodium hypochlorite also has advantage of limiting the risk of laboratory infection.<sup>11</sup> Chemicals, such as sodium hydroxide (NaOH) and a solution of N-acetyl L-cysteine

**Table 1.** Comparison of direct smear and concentration methods in diagnosis of pulmonary TB in HIV infected patients

Result	Directsmear	Concentration methods		
		Petroff's method	N-acetyl L-cysteine sodium hydroxidemethod Number (%) of cases	Sodium hypochlorite method
Positive	11 (14.6)	13 (17.3)	13 (17.3)	13 (17.3)
Negative	64 (85.4)	62 (82.7)	62 (82.7)	62 (82.7)
Total	75	75	75	75

**Table 2.** Comparison of direct smear and culture in diagnosis of pulmonary TB in HIV infected patients

Result	Number (%) of cases	
	Direct smear	Culture
Positive	11 (14.6)	13 (17.4)
Negative	64 (85.4)	62 (82.6)
Total	75	75

and sodium hydroxide (NaLC-NaOH) to liquefy sputum, together with centrifugation, are widely used in modern laboratories. A recent study has shown the efficacy of sodium hypochlorite concentration method in improving the sensitivity of smear for AFB.<sup>11</sup> Sodium hypochlorite is mycobactericidal and also kills HIV and thus improves safety and acceptability in laboratories. The disadvantage of sodium hypochlorite is that the sediment obtained after concentration can not be used for culture. The advantage of N-acetyl

**Table 3.** Comparison of direct smear and concentration methods in diagnosis of pulmonary TB in HIV infected patients

Result	Directsmear	Concentration methods		
		Petroff's method	N-acetyl L-cysteine sodium hydroxidemethod Number (%) of samples	Sodium hypochlorite method
Positive	22 (15.8)	25 (18)	25 (18)	25 (18)
Negative	117 (84.2)	114 (92)	114 (92)	114 (92)
Total	139	139	139	139

**Table 4.** Comparison of direct smear and culture methods in diagnosis of pulmonary TB in HIV infected patients

Result	Direct smear	Culture	
		LJ medium	Middlebrook's 7H11 agar medium
Number (%) of samples			
Positive	22 (15.9)	25 (18)	25 (18)
Negative	117 (84.1)	114 (82)	114 (82)
Total	139	139	139

**Table 5.** Performance of direct smear and culture in the diagnosis of pulmonary TB in HIV infected patients

Direct smear	Culture		Total
	Positive	Negative	
Positive	22	0	22
Negative	3	114	117
Total	25	114	139

**Table 6.** Performance of concentration method and culture in the diagnosis of pulmonary TB in HIV infected patients

Concentration	Culture		Total
	Positive	Negative	
Positive	25	0	25
Negative	0	114	114
Total	25	114	139

L-cysteine method and Petroff's method is that the sediment obtained after centrifugation can be used for culture, because the viability of mycobacteria is retained during concentration of specimens using these methods. In the present study, of 139 sputum samples, 25 (18%) showed growth of *M. tuberculosis* on LJ medium with mean recovery time of 4-6 weeks and on Middlebrook's 7H11 medium with mean recovery time of 3-4 weeks. These results indicate that *M. tuberculosis* grows faster on Middlebrook's 7H11 medium. Cultivation of *M. tuberculosis* from clinical samples is the "gold standard" for the diagnosis of active TB. It can detect 10-100 bacilli per ml of sputum in comparison with 5,000-10,000 bacilli per ml needed for microscopy.<sup>23</sup> It also provides material for further identification and drug susceptibility testing. Culture on LJ medium is highly specific but it is laborious and time consuming requiring from 4-8 weeks to obtain the results, needs reasonably sophisticated facilities and technical expertise. Thus its usefulness is restricted, especially in resource-constrained settings that have high HIV infection rates. Sputum culture of HIV-infected patients needed more incubation time than that of patients without HIV infection, which could be due to lower bacillary load seen in the sputum of HIV-infected patients. The specificity of culture is also affected by contamination since manipulations in

the laboratory can result in transfer of bacteria from positive to negative samples. Even in microbiology laboratories with the best anticontamination procedures, 1-4% of positive cultures might be false-positives.<sup>24</sup> When compared to LJ medium, Middlebrook's 7H11 agar medium did show the growth in first 10 days to 2 weeks in HIV negative patients.<sup>25</sup> Therefore, both LJ medium and Middlebrook's 7H11 agar medium should be used to increase the sensitivity of detection.

Based on the results of the present study we can conclude that concentration methods such as Petroff's method, NALC-NaOH and sodium hypochlorite method are sensitive and reliable in the diagnosis of pulmonary TB in HIV infected patients. Sodium hypochlorite and Petroff's concentration methods are simple, cost effective and reliable provided centrifugation is done at 3800xg. Culture methods are sensitive and specific but take time for growth of *M. tuberculosis* (4-6 weeks).

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