Fluorescent microscopy and Ziehl–Neelsen staining of bronchoalveolar lavage, bronchial washings, bronchoscopic brushing and post bronchoscopic sputum along with cytological examination in cases of suspected tuberculosis

Vijay Kumar Bodal, Manjit S. Bal, Sunita Bhagat, Jai Kishan, Deepika, Rupinder K. Brar
Department of Pathology, TB and Chest Diseases Government Medical College, Patiala, Punjab, India

Address for correspondence:
Dr. Vijay Kumar Bodal, Department of Pathology, Government Medical College, Patiala, Punjab, India. E-mail: vijay_bodal@yahoo.com

ABSTRACT

Objectives: Ever since the discovery of Mycobacterium tuberculosis in 1882, many diagnostic methods have been developed. However “The gold standard” for the diagnosis of tuberculosis (TB) is still the demonstration of acid fast Bacilli (AFB) by microscopic examination of smear or bacteriological confirmation by culture method. Materials and Methods: In suspected 75 patients with active pulmonary TB, the materials obtained bronchoscopically, were bronchoalveolar lavage (BAL), bronchial brushings, bronchial washings and post bronchoscopic sputum. Four smears were made from each of the specimen. Fluorescent Staining, Ziehl–Neelsen (ZN), Pap and May Grunwald–Giemsa (MGG) stains were carried out for cytological examination.

Results: Fluorescent stain yielded maximum AFB positivity in all the methods, that is 36 (48%) in post fibre-optic bronchoscopy (FOB) sputum and 19 (25.33%) by fluorescence microscopy in both bronchial brushings and bronchial washings. Maximum yield of AFB with ZN staining 12 (16%) was equal to the post FOB sputum and bronchial brushings samples. It was followed by 6 cases (8%) in BAL and 4 (5.3%) in bronchial washings. The cytological examination was suggestive of TB in only 8 (10.66%) cases in bronchial washings and 6 (8%) cases in post FOB collection. It was equal in BAL and Bronchial brushings each that is 5 (6.67%). Conclusion: Bronchoscopy is a useful diagnostic tool and fluorescent microscopy is more sensitive than ZN and cytology. On X-ray examination, other diseases like malignancy or fungus can also mimic TB. So apart from ZN staining or fluorescence microscopy, Pap and MGG stain will be worthwhile to identify other microorganisms.

KEY WORDS: Bronchial washings, fluorescent stain, Mycobacterium tuberculosis

INTRODUCTION

Many diagnostic methods have been developed ever since after discovery of Mycobacterium tuberculosis (MTB) by Robert Koch in March 24, 1882.[1] Despite that, “The gold standard” for the diagnosis of tuberculosis (TB) is still a bacteriologic confirmation by culture method and acid-fast Bacilli (AFB) smear microscopy. In developing countries, TB laboratories services can conduct sputum smear microscopy at provincial and district hospitals.[2] The number of Bacilli in the smear corresponds fairly closely to the concentration of Bacilli in the sputum. The probability of not finding AFB decreases steadily as the concentration of Bacilli per ml of sputum increases, when the concentration exceeds 1 × 10⁵ organism per ml of sputum, the probability of finding Bacilli approaches 96.2%.[3]
Ziehl–Neelsen (ZN) staining technique will overcome both the hurdles, that is, Procuring the material in patient who are not able to produce sputum and long-time taken by culture. With this aim, the present study was conducted.

MATERIALS AND METHODS

This prospective study was conducted on the 75 cases of sputum for AFB negative suspected pulmonary TB at our institution during the period from 2008 to 2010 after the local research, and ethical committee approval was granted. Informed consent was obtained from all the patients. In present study bronchoalveolar lavage (BAL), bronchial washings, bronchosopic brushings and post bronchosopic sputum were included in the study to evaluate FM and ZN staining along with cytological examination in these suspected cases of TB. Four smears were made from each of specimen of BAL, bronchial brushings, bronchial washings, and post bronchosopic sputum. Staining by fluorescent dye, that is, Auramine (Figure 1) and ZN technique (Figure 2) for *MTB* Bacilli were done. Papanicolaou and May Grunwald-Giemsa stain (Figure 3) for cytological examination were also carried out. Clinical suspicion was based on history of patient, clinical examination and routine investigations like hematological and X-ray examination. These results were presented in frequency table and categorically compared with using Chi-square test with the alternative of Fisher's test.

RESULTS

A total of 75 patients who’s diagnosis of suspected pulmonary TB was made on clinical examination, routine hematological investigations and X-ray examination. There were 48 (64%) male patients as compared to 27 (36%) female patients with a male to female ratio of 1.78:1. Among these maximum number of cases 26 (34.67%) were seen in the 20–39 years age group [Table 1].

The most common presenting symptom was cough, that is, 63 (84%) followed by fever 53 (72.25%) and weight loss 41 (55.7%) in both males and females [Table 2].

18 (24%) patients had a history of treatment with ATT in the past of whom 14 (77.7%) were reconfirmed to be tubercular with either of the selected methods. Among the 75 patients, there were 4 (5.3%) patients with HIV infection, 75% of HIV + were AFB positive by FM in BAL specimens. FM yielded maximum number of AFB positive in all types of the specimen as compared to the ZN and cytology. Fluorescent microscopy yielded maximum AFB positive cases, that is, 38 (50.7%) cases in BAL samples followed by 36 (48%) cases among post fiber-optic bronchoscopy (FOB) sputum samples. ZN was seen maximum AFB positive with bronchial brushing and post FOB sputum samples, that is, 12 (16%) cases each. Cytology was only suggestive of TB in 8 (10.66%) cases in bronchial washings.

In bronchial washings 75% and BAL specimen 66.66% of the ZN AFB positive slides were of 1 + grade whereas 52.6% of FM AFB + was scanty [Table 3].

Of 38 AFB + cases by FM in BAL were reconfirmed to be AFB + in 29 cases by FM in post FOB sputum samples. Of the 12 AFB + with ZN in bronchial brushings were also found to be AFB + in 9 cases by FM in post FOB sputum samples. Similarly
of the 19 AFB + with FM in bronchial brushings were found AFB + in 13 cases by BAL. Of the 5 cases suggestive of TB in bronchial brushings cytology was reconfirmed AFB + 4 by FM in post FOB sputum samples. Post FOB sputum 12 AFB + cases by ZN were reconfirmed in 11 cases by FM. Post FOB sputum 36 AFB + cases by FM were reconfirmed in 29 by FM in BAL [Table 4].

DISCUSSION

Tuberculosis remained one of the deadliest diseases worldwide and number one killer in developing countries. The situation is worse in India and affects the young and productive population. Male to female ratio of 1.78:1 in this study is well related to higher male: female ratio with 1.48:1 in a study done by Shrestha et al. it implies that OPD attendance is more in male than female patients.

Out of the total 75 cases, maximum number of cases 26 (34.67%) were seen in the 20–39 years age group and out of that 16 (61.55%) were males and 10 (38.5%) were females. Present study data is comparative with a study conducted by Sawy et al. and Prasanthi and Kumari.

The commonest presenting symptom among the 75 suspected cases of TB was cough, that is, 63 (84%) followed by (70.6%) cases with fever and 22 (29.3%) cases with hemoptysis. Sawy et al. in 2004 found that 75.4% patients with hemoptysis in his study out of which 73.3% turned out to be TB positive with smear plus culture.

Among the HIV-positive patients maximum yield of AFB positivity were 3 (75%) by FM in BAL specimens and ZN yielded 1 (25%) of 4 cases. This present study was very much comparable with the study conducted by Prasanthi and Kumari.

In this present study, the highest rate of AFB positivity was among the smears stained with auramine (FM) in all types of the specimen in comparison to the ZN and cytology. This is in concordance with various different studies. The high rate of positivity is due to that FM uses an acid-fast fluorochrome dye (e.g., Aura mine O or auramine-rodhamine) with an intense light source such as a halogen or high-pressure mercury vapour lamp [Figure 1]. By comparison, conventional microscopy uses the carbol-fuchsin, ZN or Kinyoun acid-fast stains, and can be used with a conventional artificial light source or reflected sunlight. The conventional microscope uses visible light (400–700 nm) to illuminate and produce a magnified image of a sample [Figure 2]. A fluorescent microscope, on the other hand, uses a much higher intensity light source that excites a fluorescent species in a sample of interest. This fluorescent species in turn emits a lower energy light of a longer wavelength that produces the magnified image instead of the original light source. The number of organisms observed is 3.65 times than with the ZN stain.

In this present study among the total of 75 bronchial washing samples maximum smears 19 (25.33%) positive for AFB was with

**Table 1: Age and sex distribution**

<table>
<thead>
<tr>
<th>Age (in years)</th>
<th>Male (%)</th>
<th>Female (%)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-19</td>
<td>1 (16.7)</td>
<td>5 (83.3)</td>
<td>6 (8)</td>
</tr>
<tr>
<td>20-39</td>
<td>16 (61.55)</td>
<td>10 (38.5)</td>
<td>26 (34.67)</td>
</tr>
<tr>
<td>40-59</td>
<td>17 (73.9)</td>
<td>6 (26.1)</td>
<td>23 (30.67)</td>
</tr>
<tr>
<td>60-80</td>
<td>14 (70)</td>
<td>6 (30)</td>
<td>20 (26.6)</td>
</tr>
<tr>
<td>Total</td>
<td>48 (64)</td>
<td>27 (36)</td>
<td>75 (100)</td>
</tr>
</tbody>
</table>

**Table 2: Common presenting features in suspected cases of tuberculosis**

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Male n (%)</th>
<th>Female n (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cough</td>
<td>40 (83.3)</td>
<td>23 (58.5)</td>
<td>0.905</td>
</tr>
<tr>
<td>Fever</td>
<td>32 (66.7)</td>
<td>21 (77.8)</td>
<td>0.310</td>
</tr>
<tr>
<td>Loss of weight/loss of appetite</td>
<td>25 (52.1)</td>
<td>16 (59.3)</td>
<td>0.549</td>
</tr>
<tr>
<td>Breathlessness</td>
<td>16 (33.3)</td>
<td>12 (44.4)</td>
<td>0.339</td>
</tr>
<tr>
<td>Hemoptysis</td>
<td>15 (31.3)</td>
<td>7 (25.9)</td>
<td>0.626</td>
</tr>
<tr>
<td>Chest pain</td>
<td>11 (22.9)</td>
<td>4 (14.8)</td>
<td>0.399</td>
</tr>
<tr>
<td>Expectoration</td>
<td>6 (12.5)</td>
<td>4 (14.8)</td>
<td>0.943</td>
</tr>
</tbody>
</table>

**Table 3: The positivity rate of TB with ZN, FM, and cytology in different specimens among various associated risk factors**

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>Total no. of cases</th>
<th>Bronchial brushing</th>
<th>Bronchial washing</th>
<th>BAL</th>
<th>Post bronchoscopic sputum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ZN+</td>
<td>FM+</td>
<td>Cytology</td>
<td>ZN+</td>
</tr>
<tr>
<td>Allergic bronchitis</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Old Rx case of TB</td>
<td>15</td>
<td>27.8</td>
<td>22.2</td>
<td>16.7</td>
<td>5.6</td>
</tr>
<tr>
<td>Family H/o TB</td>
<td>3</td>
<td>66.7</td>
<td>33.3</td>
<td>66.7</td>
<td>0.0</td>
</tr>
<tr>
<td>Smoker</td>
<td>2</td>
<td>50.0</td>
<td>0.0</td>
<td>50.0</td>
<td>0.0</td>
</tr>
<tr>
<td>HIV</td>
<td>4</td>
<td>25.0</td>
<td>0.0</td>
<td>25.0</td>
<td>25.0</td>
</tr>
</tbody>
</table>

P<0.05. ZN: Ziehl Neelsen; FM: Fluorescent microscopy; BAL: Bronchoalveolar lavage; TB: Tuberculosis

[Downloaded free from http://www.ijpmonline.org on Friday, January 15, 2016, IP: 115.111.224.207]
auramine stained FM. Only 4 (5.33%) cases out of 75 ZN stained smear was found to be positive for AFB. It was compared with a study conducted by Goyal and Kumar in 2013 which was less as compared to Z-N stain (7.47%) and more than fluorochrome staining and more efficient (14.69%) in AFB detection of AFB from cases of Pulmonary TB.

Whereas cytology was suggestive of tubercular in 8 (10.67%) cases out of 75 suspected cases of TB (Figure 3). The study was undertaken by Altaf Bachh et al. on bronchial washings in 60 patients with sputum smear-negative pulmonary TB and the result were positive for 35% (21/60) cases.

In present study BAL samples of 75 cases, FM gave the maximum yield, that is, 38 (50.67%) positive for AFB, followed by 6 (8%) positive for AFB by ZN and cytology suggestive of TB in 5 (6.67%) cases only. Present data of ZN positivity is comparable with 3/40 (7.5%) in a different study conducted in 2005 by Saglam et al. But a study performed by Nikbakhsh et al. in 2013 for 290 specimens of BAL, found that 110 specimens (38%) were positive for AFB. As compared to this our study has more positivity for AFB in BAL specimen smears. The higher yield is said to be due to the large volume of saline used and less use of the anesthetic agent. The efficacy of FM proved to be much higher than conventional light microscopy and comparable to that of culture.

The standard method of sputum examination, that is, ZN staining has a poor overall sensitivity (average <60%) and sensitivity is further decreased in HIV co-infection. The amount of sputum on a slide for smear preparation is about 0.01 ml this is spread over an area of 200 mm² (10 mm × 20 mm). Since the area of an oil immersion field seen in the microscope is about 0.02 mm², 10000 such fields would need to be screened in order to examine the entire smear at a magnification of ×1000, that is, ×100 for the oil immersion objective lens and ×10 and an eyepiece. (The size of field in fluorescence microscopy is about 15 times as large as an objective of ×25 and an eye piece ×10.) By examining one length (20 mm) of a smear, some 100–120 microscopic fields are screened, representing about 1% of the smear. The above calculation is for a smear that is 10 mm ×20 mm; in actual practice smears of 20 mm ×30 mm are used.

Fluorochrome stained “smears are scanned at ×250 to ×450 while fuchsin stained smears of the Z-N method are examined at ×1000. This difference in magnification provides a shorter smear evaluation time for the microscopist. Hence, examination and reporting of smear results are faster with the FM. FM has replaced direct ZN smear microscopy as the standard initial diagnostic test for TB in high-income countries.

Among the total of 75 cases, bronchial brushing was positive for AFB in 19 (25.3%) cases by FM staining, 12 (16%) by ZN staining and cytology suggestive of TB in 5 (6.67%) cases. In our study ZN staining of bronchial brushing 12/75 (16%) is comparable with 35/275 (12.7%) with the study of Willcox et al. In 1988, Chawla et al. concluded that a high yield from brush smear was obtained due to their preparation from caseous material wherever visible in the bronchi. With these results a rapid diagnosis was established. The yield from brush smears was found to be significantly better when compared with bronchial aspirate smears (P < 0.01) and post bronchoscopic sputum smears (P < 0.01).

Bronchial brushings and washings are complementary to sputum cytology in the diagnosis of pulmonary lesions. Among the various bronchoscopic specimens, the yield of BAL for MTB is superior to bronchial washings. Transbronchial biopsy cultures and bronchial brushing cultures providing little, if any additional yield. Flexible bronchoscopy provides an immediate diagnosis in a significant portion of TB suspects which may impact the morbidity and mortality especially in milliary TB where the rapid diagnosis is crucial.

CONCLUSION

It was concluded from the comparative analysis that bronchoscopy is a useful diagnostic tool and FM being the most sensitive than ZN and cytology leading diagnosis of 38/75 (50.67%) in otherwise sputum smears negative hidden TB cases. BAL followed by post FOB sputum was a most effective way of getting a fruitful yield of smear positivity. Augmentation of FM to cytology and ZN can enhance the detection rate of AFB positivity in smear-negative pulmonary TB. Hence in smear-negative pulmonary TB bronchoscopy with FM, ZN and cytology should be done simultaneously to get faster, comparatively inexpensive diagnosis of TB.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

REFERENCES
Bodal, et al.: FM and ZN staining of BAL, BW, BB and PBS along with cytological examination in cases of suspected TB