Evaluation of acridine orange staining for rapid detection of mycobacteria in primary specimen

SANGEETHA NAGARAJAN
Department of Microbiology,
PSG INSTITUTE OF MEDICAL SCIENCE & RESEARCH

Abstract :
Background - Tuberculosis continues to be a major health problem worldwide. The emergence of drug-resistant organisms threatens to make this disease once again incurable. So, timely isolation and identification of M.tuberculosis becomes important. The acid fast bacilli in the sputum can be detected microscopically by Ziehl-Neelsen stain and fluorescent stain.

Objectives - Evaluation and comparison of Phenolic-Acridine orange fluorescent staining method for detection of M.tuberculosis in suspected samples with the traditional Ziehl-Neelsen stain.

Materials and Methods - One hundred sputum samples were collected from suspected cases of pulmonary tuberculosis from both IPOP patients attending PSG hospitals. Samples were decontaminated by using N-acetyl-L-cysteineNaOH method. Smears were prepared and stained by Phenolic-acridine orange fluorescent stain and Ziehl-Neelsen stain. Samples were also inoculated on Lowenstein Jensen medium (gold standard).

Results - Out of 100 samples 20 were positive by fluorescent phenolic-acridine orange stain and 22 were positive by Ziehl-Neelsen stain. Culture was positive on Lowenstein Jensen medium for 31 of the samples.

Conclusion - In fluorescence microscopy smears are examined under 40x objective, so it is less time consuming and the fluorescing bacilli easily identified. The differentiation of the bacilli was better in phenolic-Acridine orange staining as the dull green background enables easy visualization. The fluorescence intensity of the Acridine orange-stained AFB was stable over several days when the stained smears were kept in the dark. Phenolic-Acridine orange can be considered as an alternative fluorescent stain for demonstrating acid fast bacilli in clinical samples.

Keyword : phenolic-Acridine orange, Ziehl-Neelsen stain, M.tuberculosis
Introduction:
Tuberculosis continues to be a major health problem worldwide. In 2008, the World Health Organization (WHO) estimated that one-third of the global population was infected with TB bacteria. With the spread of AIDS, tuberculosis continues to lay waste to large populations. The emergence of drug-resistant organisms threatens to make this disease once again incurable. So, timely isolation and identification of *M. tuberculosis* becomes important. Along with traditional Ziehl-Neelsen (ZN) stain, we can use the fluorescent staining techniques for demonstration of acid fast bacilli. The most important advantage of the fluorescence technique is that slides can be examined at a lower magnification, thus allowing the examination of a much larger area per unit of time. In fluorescence microscopy, the same area that needs examination for 10 minutes with a light microscope can be examined in 2 minutes (1). Thus fluorescent staining method reduces smear turnaround time. Auramine O and Acridine Orange both can be used for fluorescent staining to demonstrate the acid fast bacilli. Smith wick et al (2) and Katila et al (3) compared the Rhodamine – Auramine stain with acridine orange stain for demonstrating the acid fast bacilli. The disadvantage of rhodamine is that it is carcinogenic while Auramine O stain can be stored only for 3 weeks (1). Phenolic-Acridine Orange fluorescent staining of mycobacterium was found to be more sensitive and specific than other fluorescent stains (4). In the present study we evaluated Acridine orange stain and compared it with Ziehl-Neelsen stain and culture on Lowenstein-Jensen medium to detect acid fast bacilli in sputum samples from suspected cases of Tuberculosis.

Materials & methods:
One hundred sputum samples were collected from clinically suspected cases of tuberculosis from both outpatient / Inpatients attending the PSG Hospitals. Sputum samples were decontaminated by using N-acetyl-L –cysteine –NaOH method (5). Fresh digestant was prepared by adding equal amounts of 4% NaOH and 2.9% sodium citrate. 0.5 g NALC powder was added per 100 ml of sodium hydroxide-sodium citrate solution. After the NALC was added, the digestant was used within 24 hours. The collected specimen was added to a 50 ml plastic centrifuge tube, not exceeding 10 ml. NALC solution was added in equal volume to that of the specimen. With the cap tightened the tube (about 5 - 20 seconds /tube) was vortexed and inverted to ensure that the NALC solution contacted all surfaces of tube and cap. The specimen was allowed to stand 15 - 20 minutes. Tube was filled with 50 ml of sterile phosphate buffer solution at pH 6.8. and swirled by hand to mix well. The specimen was concentrated in a centrifuge at a speed of 3,000 x g for 15 minutes. The supernatant fluid was carefully decanted from the pellet. The pellet was resuspended with phosphate buffer (pH 6.8) using a sterile Pasteur pipette to achieve a final volume of 1 to 3 ml.

Phenolic-Acridine Orange fluorescent staining procedure Samples were stained by using Phenolic-Acridine Orange fluorescent stain (2) and Ziehl-Neelsen stain. The Acridine Orange (AO) reagent prepared by dissolving 5g of phenol crystals in a solution containing 50ml of distilled water, 25 ml of glycerol and 25ml of 95% ethanol was added. Then 1g of Acridine Orange was added and the mixture was stirred briefly, allowed to settle this solution for overnight, this helped the Acridine Orange to dissolve completely. Acid alcohol destaining –

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counterstaining solution was prepared by mixing 74 ml of 95% ethanol, 26 ml of distilled water, 0.5 ml of concentrated HCL and 0.2 g of methylene blue. The smears were heat fixed on a clean glass slide and covered with Acridine orange reagent for 15 minutes and then rinsed with distilled water. Smears were then covered with destaining – counterstaining solution for 2 minutes and rinsed with distilled water, and allowed to air dry.

Ziehl-Neelsen staining procedure

ZN staining was done according to the standard techniques. The heat fixed smear covered with strong carbol fuschin and heated gently by using spirit lamp until steam rises from the slide for 2 to 3 times. Smears were stained for 5 minutes. The slides were washed gently using distilled water. The smear was covered with 25% sulphric acid for 2-4 minutes. The smear was washed thoroughly using distilled water. If smear was not decolorized properly the step was repeated for another 1-3 minutes. The smear was covered with 0.1% methylene blue for 30 seconds, washed with distilled water and allowed to air dry. Smears were examined under oil immersion power and graded according to RNTCP guidelines (6) AFB positive control smears were included and stained in each batch. The smears were screened for the presence of AFB, Leica fluorescent microscope by using a 20x objective. The presence of bright orange red fluorescing rods against a dull green background was confirmed with a 40x objective. Samples were inoculated on LJ medium and incubated at 37°C for minimum six weeks. Cultures were examined at regular intervals for the growth. Organisms were identified by standard biochemical tests (7). Culture results were taken as gold standard.

Results

In the present study out of 100 samples, 20 samples were positive for acid fast bacilli by Acridine orange fluorescent stain and 22 samples were positive for acid fast bacilli by Ziehl-Neelsen stain. Culture was positive on Lowenstein – Jensen medium for 31 samples. (Table 1) Results of the study revealed that phenolic-acridine orange stain as sensitive as Ziehl–Neelsen stain (Table 2)

Sputum microscopic examination for acid fast bacilli is usually conducted in suspected cases of tuberculosis. In traditional Ziehl-Neelsen stain smears are examined under oil immersion power. In fluorescence microscopy smears are examined under high power magnification, so it is less time consuming and the fluorescing bacilli easily identified. The sensitivity of acridine orange stain was comparable to that of ZN stain in our study. The differentiation of the bacilli was good in phenolic-Acridine orange staining as the dull green background enables easy visualization. Moreover, since methylene blue used in phenolic-acridine orange staining method, partially blocks the fluorescence of the back ground debris, with little yellow green fluorescning background, it is easy to search for bright orange red fluorescing bacilli. In addition, the shelf life of phenolic –acridine orange stain is 6 months (3). The fluorescence intensity of the Acridine orange-stained AFB was stable over several days when the stained smears were kept in the dark (3). Considering all these factors Acridine orange can consider as good fluorescent stain for demonstrating acid fast bacilli in clinical samples.
Table 1

<table>
<thead>
<tr>
<th>Method</th>
<th>Positive(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acridine orange stain</td>
<td>20</td>
</tr>
<tr>
<td>Ziehl–Neelsen stain</td>
<td>22</td>
</tr>
<tr>
<td>Culture on Lowenstein-Jensen medium</td>
<td>31</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Method</th>
<th>Sensitivity compared to culture on Lowenstein-Jensen medium (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acridine orange stain</td>
<td>65</td>
</tr>
<tr>
<td>Ziehl–Neelsen stain</td>
<td>71</td>
</tr>
</tbody>
</table>

Reference:


6 Manual for laboratory technicians Smear Microscopy for Detection of Acid-fast Bacilli – Revised National Tuberculosis Control Programme, Jan 2009:17