A comparative study of ziehl-nelson staining and auramine staining in sputum sample for the diagnosis of pulmonary tuberculosis.

INTRODUCTION
Tuberculosis is worldwide public health problem caused by Mycobacterium tuberculosis. Globally, it is estimated that one-third of population is asymptomatically infected with tuberculosis. Mostly the new cases and death occurs in the developing countries where infection is mainly acquired in the childhood. India alone accounts for one-fourth of the burden globally. There are various methods for bacteriological diagnosis of tuberculosis. Currently, radiometric assay allows detection of Mycobacterium tuberculosis growth and provides antibiotic sensitivity results more rapidly usually within 10 days. However use of the technique is limited because culture medium contains radioactive carbon. Genetic probes are on the other hand quite easy to use and allow identification of culture bacteria in only a few hours by polymerase chain reactions. This method has not become a routine laboratory technique, particularly due to lack of sufficient specificity and sensitivity. Serological tests are currently not reliable enough for the diagnosis of tuberculosis. Microscopic examination and culture are still essential elements of the bacteriological diagnosis of tuberculosis in microscopic examination; the diagnosis of tuberculosis is confirmed on the basis of demonstration of tubercle bacilli in the sputum or any other pathological material. Smear examination is believed to be simple, cheap, quick and practicable and effective case finding method for developing countries. Ziehl-Nelson is the most extensively used procedure for the demonstration of mycobacterium tuberculosis in smear. The requisites for the staining procedures are; basic fuchsin, phenol, absolute alcohol; sulphuric acid and methylene blue. Microscopic examination under oil immersion objective reveals mycobacterium are red bacilli. Fluorescent staining by Auramine is other methods of staining. In this a smear is made from the specimen and stained with fluorescent stain called auramine. The auramine stain enters the wall of Mycobacterium tuberculosis bacillary cell and makes them glow against dark background under UV light. Microscopic examination under low power objective will reveal mycobacteria as glowing yellow white, rice like bacteria in the smear.

The present prospective study was undertaken to see the efficacy of Ziehl-Nelson method versus fluorescent staining in the detection of mycobacterium in sputum sample.

AIMS AND OBJECTIVES
To study the sensitivity and benefits of fluorescent microscopy over conventional ZN staining.

MATERIAL AND METHODS
Total of 300 sputum samples irrespective of all age groups were collected from 150 patients suspected with pulmonary tuberculosis including the immunocompromised patients who came to the outpatient department of PGI Rohtak. A comparative study was done from January – March, 2014 in the hospital. Two sputum samples were collected; one spot and the second one early morning fasting sample. It was collected in a sterile, clean; wide mouth container .Each sample was processed. In Ziehl–Nelsen staining procedure; sputum slide is heat fixed and carbol fuchsin is poured over the entire slide, which is kept for 5 minutes with gentle heating .It is rinsed properly and decolourised with 25% sulphuric acid, kept for 2-3 minutes. Slide is rinsed again and counterstained with 0.1% methylene blue for further 30 seconds. Lastly slide is washed and dried up and seen under 100x magnification of light microscope. In Auramine O staining, flood the slide with Auramine –phenol and keep it for 7-10 minutes. Wash well and decolorize for 2 minutes with acid alcohol, two times. It is rinsed and counterstained with 0.1% potassium permanganate with 30 seconds. Wash it well and observe under 40 x magnifications of fluorescent microscope. The results were graded as per the International Union against Tuberculosis guidelines.

WHO TABLE
Table 1: Grading chart (WHO, IUATLD, 2007) for fluorescent microscopy

<table>
<thead>
<tr>
<th>Grades</th>
<th>AFB per HPF</th>
<th>Fluorescence (x1000 magnification)</th>
<th>Fluorescence (x200-250 magnification)</th>
<th>Fluorescence (x400 magnification)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>Zero AFB / 100HPF</td>
<td>Zero AFB / 1 length</td>
<td>Zero AFB / 1 length</td>
<td></td>
</tr>
<tr>
<td>Scanty</td>
<td>1-9 AFB / 100HPF</td>
<td>1-29 AFB / 1 length</td>
<td>1-19 AFB/1length</td>
<td></td>
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<tr>
<td>+</td>
<td>10-99 AFB / 100HPF</td>
<td>30-299 AFB / 1 length</td>
<td>20-199 AFB / 1 length</td>
<td></td>
</tr>
<tr>
<td>2+</td>
<td>1-10 AFB / 1 HPF</td>
<td>10-100 AFB / 1 length on average</td>
<td>5-50 AFB / 1 length on average</td>
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</tr>
<tr>
<td>3+</td>
<td>&gt;10 AFB / 1 HPF</td>
<td>&gt;100 AFB / 1 field on average</td>
<td>&gt;50 AFB / 1 field on average</td>
<td></td>
</tr>
</tbody>
</table>

RESULTS
Out of 300 sputum samples of 150 patients, 32(10.66%) sputum samples were positive for Ziehl-Nelson and 55(18.33%) sputum samples were positive for Auramine O.

Auramine O stained sputum smear under fluorescent microscope

Fig 2. Ziehl-Nelson stained sputum smear under light microscope. Bacilli appear as pink colored rod shape organism
Discussion
In developing countries, microscopy of the specimen is by far the fastest, cheapest, and most reliable method for the detection of AFB. However, fluorescent staining has been added in Revised National Tuberculosis Control Program (RNCP) because of more sensitive and rapid results and can be used in field areas. In a study done by Ben et al. in 2008, showed that out of 221 sputum samples, 33 (14.9%) samples were positive with Auramine staining and 24 (10.85%) samples were positive with Ziehl-Nelson staining. It demonstrated superior diagnostic results by fluorescent microscopy when compared with conventional light microscopy. Laifangbam et al. in 2009 revealed a study on 102 suspected patients, where in 44.1% patients were positive with Ziehl Nelson staining and 71.6% patients were positive for Auramine O staining. It was found by this study that Auramine O staining was superior to Ziehl-Nelson staining.

In the present study, we conclude that the Auramine O staining is superior to that of the conventionally used ZN stain. It states that out of 300 sputum samples, 32 (10.66%) samples were ZN positive and 55 (18.33%) samples were Auramine O positive. The diagnostic accuracy of fluorescent microscopy was found much more superior and much more sensitive than the conventional light microscopy.

The results of present study indicate that the Auramine staining of sputum smears is a more sensitive method of sputum microscopy for demonstration of AFB in sputum specimen, compared to Ziehl-Nelson staining. The use of Fluorescent Microscopy greatly improves the diagnostic value of sputum smear especially in patients with low density of bacilli that are likely to be missed on Ziehl Neelson stained smears. The method is economical in both time and expense and recommended for laboratories handling large number of sputum specimens.

Conclusion
Sputum examination for the tubercle bacilli is usually conducted for patients clinically and or radiologically suspected of pulmonary tuberculosis. However, the standard method of sputum examination, that is, ZN staining is not sensitive enough and a large number of the suspected cases miss diagnosis. Fluorescent stain is a more efficient over ZN stain in detecting Tubercle bacilli in sputum. Since screening is done under low power of magnification (40X), fluorescence has been found to be less time consuming compared to ZN method (100X) in the diagnosis of tuberculosis. Hence, it has been advocated to be methods of choice where the large numbers of sputum smears are to be examined. The fluorescing bacilli are easily identifiable and cause less eye-strain.

Table 2: Comparison of ZN and Auramine O Staining Reports

<table>
<thead>
<tr>
<th>Staining method</th>
<th>Positive smears</th>
<th>Negative smears</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ziehl Nelson staining</td>
<td>32</td>
<td>268</td>
</tr>
<tr>
<td>Fluorescent staining</td>
<td>55</td>
<td>245</td>
</tr>
</tbody>
</table>

References
3. WHO (2004), Weekly Epidemiological Record, 23 Jan 2004, No.4