A sensitive and specific ES-31 antigen detection based fluorometric assay for confirmation of *Mycobacterium tuberculosis* in cell culture

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Confirmation of presence of *M. tuberculosis* bacilli on microscopic examination is very important in diagnosis of tuberculosis. The present study was undertaken to find the usefulness of mycobacterial ES-31 serine protease as a marker to detect tuberculosis bacilli using fluorescein isothiocyanate conjugated anti-ES-31 serine protease antibody. This immunofluorescence method was compared with Ziehl-Neelsen and auramine-O staining methods for detection of tuberculosis bacilli.

Slides were prepared for each serially diluted tuberculosis *H.37Ra* bacilli (1×10² bacilli/ml to 5 bacilli/ml). Slides for each dilution group were stained by ZN method, auramine-O and immunostaining methods using fluorescein isothiocyanate conjugated anti-ES-31 serine protease antibody. ZN staining method showed efficacy for detection of *M. tuberculosis* *H.37Ra* bacilli up to 1×10² bacilli/ml while auramine-O method showed up to 1×10² bacilli/ml. The presence of bacilli was indicated by green fluorescence on immunostaining using anti-ES-31 antibody conjugate and this method was effective up to 10 bacilli/ml. The slides which were negative for ZN (1×10³ cells/ml) and auramine-O (100 cells/ml) method showed positivity on restaining with immunofluorescent staining method. The results of this preliminary study showed that immunofluorescent staining method using specific anti-ES-31 antibody conjugate was more sensitive for detection of tuberculosis bacilli than ZN and auramine-O methods in samples of laboratory strain. The utility of this method will be studied further in clinical specimens.

**Keywords:** Diagnostic tests, ES-31 antigen, Immunofluorescent staining, *Mycobacterium tuberculosis*

The World Health Organization (WHO) estimated 9.4 million cases of TB globally during 2008⁴. Microscopy is the simplest and most rapid procedure currently available to detect acid fast bacilli in clinical specimen. Direct examination of sputum for acid fast bacilli (AFB) by Ziehl-Neelsen (ZN) stained smears is a standard procedure in the diagnosis of tuberculosis (TB) in high burden countries⁵. ZN is a classical differentiating procedure that uses heat to drive the fuschin stain into the bacilli followed by decolourisation by acid and alcohol. Limit of detection with ZN staining is that it requires atleast 5×10³ bacilli/ml of sputum⁵. Though ZN is simple and less expensive, examination of 100 microscopic fields/slide at high power (at 1000×) is a time consuming tedious job, thus limiting the number of slides that a technician can examine per day⁴. These factors diminish the sensitivity of ZN microscopy, and TB cases may be missed. Most commonly used another alternate staining method is fluorescent microscopy by using auramine-O staining. The contrasting colour of fluorescent microscopy make it easier to detect positive cases at lower magnification screening (at 400×), allowing screening of greater smear area in each field viewed with less time consumption⁴,⁵. Mere presence of acid fast bacilli or positive fluorescence does not confirm the presence of *Mycobacterium tuberculosis* (*M. tb.*) bacilli in any sample.

Mycobacterial ES-31, a 31 kDa serine protease is present on the surface of *M. tb.* *H.37Ra* and *M. tb. H.37Rv*⁶,⁷. Fluorescein isothiocyanate (FITC) conjugated antibody to ES-31 antigen was shown to be useful for differentiation of *M. tb.* bacilli from non-tuberculous mycobacteria and other bacteria⁶,⁷. Hence, in the present study the ability of this immunofluorescent method has been analyzed to detect *M. tb.* bacilli and compared it with that of the ZN and auramine-O methods.

Bacilli used in the present study include *M. tb. H.37Ra* bacilli cultured on L-J medium. Bacilli (2/3⁹ loopful; approximately 4 mg bacillary mass) were taken from culture which contained 4×10⁷ bacilli (bacillary count depending on colony forming unit/ml). This bacillary mass was serially diluted in phosphate buffer saline (PBS, *pH* 7.2) to get the concentrations between 1×10⁷ bacilli/ml and 5 bacilli/ml. Three slides were prepared from each of the serial dilution by speeding 100 µl dilution fluid in central area of slide. One slide was stained by ZN stain using heat driven 1% carbol fuschin followed by...
decolourization by 1% acid-alcohol, another one of same dilution by auramine-O with decolorization by 0.5% acid-alcohol and counterstaining by potassium permanganate and a third by immunofluorescent staining method.

**Immunofluorescent staining**—M. *tb*. H37Ra detergent soluble sonicate (DSS) antigen, was prepared from M. *tb*. H37Ra bacilli. Briefly, bacilli were 5% phenol inactivated in 0.5 M phosphate buffer saline (PBS, pH 7.2) and incubated with sodium dodecyl sulphate (SDS) extraction buffer. The supernatant was dialysed against 0.01 M PBS, pH 7.2 and used as an antigen source. Anti-DSS IgG antibodies were raised in goat by immunizing intramuscularly with 500 µg protein/mL DSS antigen with 1 ml Freund’s incomplete adjuvant on days 0, 20, 33 and 45. Immune sera were collected on days 32, 44, 57, 60 and thereafter fortnightly and anti-SDS IgG was isolated by 33% saturation with ammonium sulphate under ice, followed by diethyl aminoethyl-cellulose ion exchange column chromatography as described earlier. Anti-ES-31 antibody was isolated from anti-DSS IgG by affinity chromatography using ES-31 antigen coupled Sepharose-4B column. FITC labeled antibodies were prepared to localize ES-31 antigen. Briefly, 2.5 mg anti-ES-31 antibody was diluted to 1 ml with 0.145 M NaCl solution. FITC (12.5 µg/mg protein; pH 9.5) was added and the mixture incubated at 25°C for 45 min. Conjugate mixture was passed through Sephadex G-25 column and eluted with 0.01 M PBS (pH 7.2) at a flow rate of about 30 ml/min. First eluted yellow coloured fractions were collected which contained anti-ES-31 antibody conjugated with FITC. FITC conjugate was concentrated by ultra-filtration and stored at 4°C. The detection bacilli in slides of each serial dilution were performed using FITC labeled specific antibodies. Briefly, a slide was incubated with 100 µg FITC conjugated antibody in humidified chamber for 1 h at 37°C followed by three washing with 0.05 M PBS and mounted using mounting medium (50% glycerol, 50% PBS and 0.1% sodium azide).

One hundred fields per slide were observed and presence of ≥1 bacillus/100 fields was considered as positive smear. Slides stained for ZN microscopy were examined under Olympus-GB (OIC-787783) microscope at 1000× magnification (100× objectives with 10× magnification for the eyepiece). Slides stained for fluorescent microscopy were examined under Nikon Labophot Microscope with episcopic fluorescent attachment at 400× magnification (100× objectives with 10X magnification for the eyepiece). Slides negative for ZN method were washed in xylene for removal of cedar wood oil; air dried; washed thrice with 0.05 M PBS and re-stained by immunofluorescence method. Slides negative for auramine-O staining method were washed thrice with 0.05M PBS and restained by immunofluorescence method. These restained slides were observed for presence of bacilli.

Three sets of slides stained by ZN, auramine-O and immunofluorescence methods were examined and the positivity of smear was considered on presence of ≥1 bacillus/100 fields. ZN staining method showed detection efficacy for *M. tb.* upto 1×10⁴ bacilli/ml while auramine-O method showed upto 1×10⁵ bacilli/ml (Table 1). The presence of bacilli was indicated by green fluorescence against dark black background on immunostaining using anti-ES-31 antibody conjugate. This method showed sensitivity upto 1×10⁴ bacilli/ml (Table 1). The slides which were negative for ZN and auramine-O methods showed positivity on restaining with immunofluorescent staining method upto 1×10⁴ bacilli/ml concentration, but with decreased fluorescence. This showed that immunofluorescent staining method using anti-ES-31 antibody had better efficacy for detection of *M. tb.* than ZN and auramine-O methods using laboratory strain. Each experiment was performed in triplicate and the slides were screened by the laboratory personnel and authors in double blinded manner.

The data of the present study demonstrated that utilization of immunofluorescent staining with anti-ES-31 antibody conjugate, Ziehl-Neelsen and Auramine-O staining methods of *M. tuberculosis* bacilli

<table>
<thead>
<tr>
<th>Concentration of <em>M. tb</em>. H37Ra (no. of bacilli /ml)</th>
<th>ZN</th>
<th>Auramine –O</th>
<th>Immunofluorescent staining⁸</th>
</tr>
</thead>
<tbody>
<tr>
<td>1×10⁷</td>
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<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
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<tr>
<td>5</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

*300 fields were examined for negative result and not less than 100 fields in case of positive results.

⁸Immunofluorescent staining using anti-ES-31 antibody conjugate.

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**Table 1**—Comparative analysis of anti-ES-31 antibody FITC conjugate, Ziehl-Neelsen and Auramine-O staining methods for detection of *M. tuberculosis* bacilli
using laboratory strain. The slides demonstrating negative results by ZN and auramine-O methods showed positive results by immunofluorescent staining upto 1×10 bacilli/ml concentration. The fluorescence was decreased in restaining procedure. It is possible that some of immunofluorescent restained smears lost bacilli during the washing process or, those bacilli failed to stain properly for immunofluorescent staining. The directly stained immunofluorescent staining smears provide a sensible point of comparison.

Earlier studies in our laboratory have shown that FITC staining of ES-31 antigen technique is useful in differentiating M.tb from nontuberculous mycobacteria (NTM) compared to ZN or Auramine staining. Auramine-O stains the mycolic acid of mycobacteria while ZN indicates the acid-fastness of the bacilli. Both of these methods cannot differentiate M.tb from (NTM). But, in earlier study, we have shown the utility of immunofluorescent staining method in differentiating M.tb from NTM directly on the basis of smear results . Thus, there may be no need of chemical or molecular method for differentiation of M. tb. from NTM. This may prevent the diagnostic delay and provide confirmed tubercular diagnosis. Immunofluorescent staining may also be useful for stoppage of starting of anti-tuberculosis therapy on mere AFB positivity which may be false due to presence of nonspecific NTM in clinical samples. The reason behind the more sensitivity of immunofluorescent staining method may be the incubation time (1 hr at 37°C) of antibody conjugate with bacilli which can be considered as the productive disadvantage of this method. Another advantage of the present method is that M. tb. fluoresce bright green on dark black field, making it very easy to detect positive slides.

More detailed study can be performed in the clinical sputum, culture and FNAC samples. But even this preliminary data indicate the superior sensitivity of immunofluorescent staining in comparison with ZN and auramine-O methods using laboratory strain. According to WHO publication—Fluorescent microscopy is about as sensitive as culture, but much faster. It can be used as a substitute for culture to diagnose tuberculosis. It is significantly more sensitive for detecting acid-fast bacilli than are sputum specimens centrifuged or directly applied to slides . Thus, this immunofluorescent staining method using anti-ES-31 antibody conjugate may be useful for control measures of tuberculosis by improving the diagnostic predicament particularly in low bacillary load material such as saliva. If further confirmed, this may permit immunofluorescence to be used effectively in areas where it is difficult to control the quality of sputum production and collection such as in childhood tuberculosis cases.

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