ORIGINAL ARTICLE

POTENTIAL OF MYCOBACTERIAL EXCRETORY SECRETORY PROTEIN ANTIGENS (SEVATB ES-31, ES-43, EST-6 AND ES-20) AS BIOMARKERS TO DETECT MYCOBACTERIUM TUBERCULOSIS BACILLI

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ABSTRACT

There is a need for a simple and reliable method to identify *Mycobacterium tuberculosis* from nontuberculous mycobacteria (NTM). The utility of mycobacterial ES-31, ES-43, EST-6 or ES-20 antigen as a biomarker for differentiation of *Mycobacterium tuberculosis* bacilli from nontuberculous mycobacteria was explored using Fluorescein isothiocyanate conjugated antibodies against these antigens. Detection of these antigens was done from *M.tb* H₃₇Ra and H₃₇Rv DSS antigen. The presence of antigen in bacilli using FITC labelled antibody was indicated by green fluorescence on the cell surface while, its absence by no fluorescence under microscope. In *M.tb* H₃₇Ra and H₃₇Rv bacilli, fluorescence was observed on addition of FITC labelled anti ES-31 and anti ES-43 antibody; whereas no fluorescence was observed in case of EST-6 and ES-20 antibody conjugates. However all the antigens were detected in detergent soluble sonicate antigen of tubercle bacilli on addition of FITC conjugates. Fluorescence was not observed for ES-31, ES-43, EST-6 and ES-20 antigen in any of the tested NTM as well as in *Escherichia coli*. SEVA TB ES-31 and ES-43 may be used as biomarkers to distinguish *M.tuberculosis* bacilli from NTM.

KEY WORDS

Biomarker, *Mycobacterium tuberculosis*, Non-tuberculous *Mycobacteria*, Immunofluorescence.

INTRODUCTION

Mere presence of acid fast bacilli in clinical specimen can't differentiate *Mycobacterium tuberculosis* (M.tb.) from nontuberculous mycobacteria (NTM) (1). Many people harbour NTM in their respiratory secretions without any symptoms. The immunosuppressed individuals infected by human immunodeficiency virus (HIV) infection have become most significant risk factor for disseminated NTM disease and of these, 95% are due to *Mycobacterium avium* complex (MAC)

(2, 3, 4). Clinical differentiation of M. tb infection and other mycobacteriosis is difficult due to overlapping domain of symptoms (5). Treatment of atypical mycobacterial infections depends upon the infecting organism (6). Hence, M.tb should be distinguished from NTM so that correct treatment may be given. Differentiation of M.tb. from NTM depends upon use of time consuming conventional morphological and biochemical tests or expensive genotypic methods (3, 7). Thus, there is a need for exploration of newer simple and rapid methods to detect and differentiate M. tb. from NTM in cultures.

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In earlier studies from our laboratory, we have reported isolation of SEVA TB ES-31 (ES-31) protein antigen, a serine protease, from culture filtrate of M. tb (8, 9). The detection of circulating ES-31 antigen (Ag) using affinity purified anti ES-31 antibody was found to be useful in detection of tuberculosis (TB) by Penicillinase ELISA (8, 10). Microtitre plate Peroxidase sandwich ELISA for detection and quantitation of circulating

ES-31 antigen has also shown to be useful in diagnosis of PTB cases (11). Utility of mycobacterial ES-31 antigen as a marker to distinguish *M. tb.* bacilli from *M. chelonae*, *Nocardia farcinicum* and *Escherichia coli* using Fluorescein isothiocyanate (FITC) conjugated anti-ES-31 antibody has been reported (12). A cocktail of affinity purified antibodies against ES-31, ES-43 and EST-6 antigens was shown to be useful for circulating antigen detection in TB by Penicillinase ELISA (13). Diagnostic potential of affinity purified antibodies against ES-20 antigen was also found useful in detection of tuberculosis cases (14, 15). In this study the utility of mycobacterial ES-31, ES-43, EST-6 or ES-20 antigen as a biomarker was explored to detect and distinguish *M. tb.* bacilli from NTM using Fluorescein isothiocyanate (FITC) conjugated antibodies against these antigens.

MATERIALS AND METHODS

Bacilli: Bacilli used in the present study include *M. tb.* H₃₇Ra bacilli and *M. tb.* H₃₇Rv bacilli; NTM such as *Mycobacterium chelonae* (*M. chelonae*), *Mycobacterium diernoferi*, *Mycobacterium flavescens*, *Mycobacterium asiaticum*, *Mycobacterium szulgai*, *Mycobacterium intracellulare*, *Mycobacterium gordonae*, *Mycobacterium fortuitum*, *Mycobacterium simiae*, *Nocardia farcinicum* and *Escherichia coli* (*E. coli*). The NTM strains were provided by Microbiology Department, MGIMS.

Isolation of antibodies to *M. tb.* ES-31, ES-43, EST-6 and ES-20 antigens: *M. tb.* H₃₇Ra detergent soluble sonicate (DSS) antigen, was prepared from *M. tb.* H₃₇Ra bacilli. Briefly, bacilli were 5% phenol inactivated in 0.5M phosphate buffer (PBS, pH7.2) and incubated with sodium dodecyl sulphate (SDS) extraction buffer. The supernatant was dialysed against 0.01M PBS, pH 7.2 and used as an antigen source (16). 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 (17). Anti ES-31, anti-ES-43, anti-EST-6 and anti-ES-20 antibodies were isolated from anti-DSS IgG by affinity chromatography using ES-31, ES-43, EST-6 or ES-20 antigen coupled Sepharose-4B column (9).

Antibody – FITC conjugate: FITC labelled antibodies were prepared to localize ES-31 antigen. In brief, 2.5 mg anti-ES-

31 antibody diluted to 1ml with 0.145M sodium chloride solution. 12.5 µg FITC / 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 0.01M 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-membrane filtration and stored at 4°C. Similarly FITC-labelled antibodies were prepared against ES-43, EST-6 and ES-20 antigen.

Immunofluorescence assay for detection of antigen: The detection of ES-31 antigen in bacilli in cultures was performed using FITC labelled specific antibodies. In brief, a loopful of bacilli was incubated with 100 µg FITC conjugated antibody in a conical vial for 1 hour at 37°C. Bacilli were washed twice with 0.05 M PBS and mounted using mounting medium (50% glycerol, 50% PBS and 0.1% Sodium azide). Bacilli were observed under Nikon Labophot Microscope with episcopic fluorescent attachment for fluorescence. In the present study, each sample had been analyzed in duplicate. Similarly, the detection of ES-43, EST-6 and ES-20 antigens in bacilli was performed using specific FITC labelled antibodies. The detection of antigens in bacilli on slide was also performed using FITC labelled antibodies with incubation for 1 hour at 37°C in humidified chamber followed by washing with 0.05 M PBS. Similarly detection of ES-31, ES-43, ES-20 and EST-6 antigens using their FITC labelled specific antibodies was done on slides containing *M. tb.* H₃₇Ra and H₃₇Rv DSS antigen instead of bacilli.

RESULTS

The presence of ES-31, ES-43, EST-6 and ES-20 antigen in bacilli using FITC labelled antibody was indicated by green fluorescence while its absence was indicated by no fluorescence when bacilli were observed under microscope. In *M. tb.* H₃₇Ra bacilli and *M. tb.* H₃₇Rv bacilli green fluorescence was observed showing presence of ES-31 and ES-43 antigens (Fig 1 and 2) on cell surface while no fluorescence was observed for EST-6 and ES-20 antigens (Fig 3). Fluorescence was not observed for any of the above mentioned antigen (ES-31, ES-43, EST-6 and ES-20) in any of the tested NTM i.e., *M. chelonae*, *M. diernoferi*, *M. flavescens*, *M. asiaticum*, *M. szulgai*, *M. intracellulare*, *M. gordonae*, *M. fortuitum*, *M. simiae*, *N. farcinicum* as well as in *E. Coli* (Fig 4). This study shows that ES-31 and ES-43 antigens are located on cell surface and are specific for *M. tb.* and not detected on NTM as well as in bacilli other than mycobacteria (*E. coli*). Fluorescence was observed in DSS

Fig 1: Detection of ES-31 antigen in *M. tb. H₃₇Rv* bacilli using FITC labelled anti- ES-31 antibody. A green fluorescence indicating the presence of SEVA TB ES-31 serine protease on cell surface

Fig 2: Detection of ES-43 antigen in *M. tb. H₃₇Rv* bacilli using FITC labelled anti- ES-43 antibodies. A green fluorescence with specific antibody conjugate indicating presence of SEVA TB ES-43 antigen on cell surface

Fig 3: Control: Staining of *M. tb. H₃₇Rv* bacilli using FITC. Absence of fluorescence indicating absence non-specific fluorescence. Similar observation was made with addition of FITC labelled anti EST-6 and with anti ES-20 antibodies

Fig 4: Detection of ES-31 antigen in *M. chelonae* bacilli (NTM) using FITC labelled anti- ES-31 antibodies. Absence of fluorescence with specific antibody conjugate indicating absence of SEVA TB ES-31 antigen on cell surface

Fig 5: Detection of EST-6 antigen in DSS antigen of *M. tb. H₃₇Ra* bacilli using FITC labelled anti- EST-6 antibodies. A green fluorescence with specific antibody conjugate indicating presence of SEVA TB EST-6 antigen. Similar observation was made with addition of anti ES-20, anti-ES-31, and anti-ES-43 antibodies.

antigen of *M. tuberculosis* H₃₇Ra and H₃₇Rv showing presence of ES-31, ES-43 EST-6 and ES-20 antigens in the detergent soluble sonicate antigen of tubercle bacilli (Fig 5, Table).

DISCUSSION

Rapid and accurate diagnosis of TB is essential, but often delay in confirmation of diagnosis is hampering TB control measures. In the present study, immunofluorescence method using anti- ES-31, anti-ES-43, anti-EST-6 and anti ES-20 antibody conjugate for detecting specific antigen as biomarker for *M.tb.* was explored. This assay is simple, rapid and gives results only within two hours. ES-31 and ES-43 were observed

on cell surface of mycobacterial tubercle bacilli.

Though EST-6 and ES-20 antigens were isolated from M.tb.H₃₇Ra, detection of these antigens did not show fluorescence in M.tb. H₃₇Ra or in any other mycobacteria. However both of these antigens were detected in M.tb.H₃₇Ra DSS antigen on slide indicating that EST-6 and ES-20 are not present on surface of M.tb.H₃₇Ra. These antigens may be completely covered by mycobacterial cell wall or may be present in association with other structural components from which they get separated during DSS preparation. In earlier studies from our lab it was shown that ES-20 and EST-6 antigens to be present in sera of TB cases (14, 15). This might be due to release of these antigens from TB bacilli during its interaction with host immune cells or bacilli may secrete these antigens during its life cycle.

In the present day practice, differentiation of M.tb. from NTM depends upon use of conventional, phenotypic and newer genotypic methods. Discrimination of M. tb. from NTM is very important for clinical management of case as treatment of NTM infections differs in several facets. Treatment depends upon the infecting organism and the severity of the infection (18). Misdiagnosis leading to wasting treatment on people who are not infected and they have to face many side effects of anti tubercular drugs (6). Individuals with respiratory disease due to NTM therefore, do not need to be isolated from others. Hence M.tb. should be differentiated from NTM so that proper treatment may be given. By conventional morphological and biochemical tests such as rate and temperature of growth, pigmentation, niacin and Catalase production, tellurite and nitrate reduction, tween-80 hydrolysis, arylsulphatase and urea hydrolysis, TCH sensitivity, growth on Mac Conkey agar, sodium chloride tolerance, etc., identification of mycobacterial strain requires 2 to 4 weeks, in addition to 4-6 weeks for primary isolation (5). Newer methods which include analysis of fatty acids by chromatography, hybridization of gene probe, gene amplification followed by restriction analysis, gene amplification analysis by sequencing are rapid but very expensive (5). Niacin test can distinguish M.tb. from NTM (19, 20). Kilburn and Kubica further modified the niacin test using strip impregnation (21). Another available method, paraffin slide culture technique for identification of NTM requires 7 days (2, 22, 23). The lag phase between clinical presentation and confirmed diagnosis is crucial period, as delay in TB treatment results in rapid spread of the disease. Utilizing tuberculosis secretory MPB64 protein antigen, Tomiyana et al developed a simple immunochromatographic assay using anti-MPB64 monoclonal antibody for detection of M. tb. in cultures (24). Further a multicentric study revealed the clinical efficacy of the

immunochromatography test for the differentiation of *M. tb.* from other mycobacteria in fresh clinical isolates and stock cultures of different mycobacterial species grown in several liquid and solid media (25). However the cost of rapid test is prohibitive and not used in hospital laboratories.

This is the first study to report the detection of SEVA TB ES-31 Serine protease or SEVA TB ES-43 protein as a biomarker to confirm *Mycobacterium tuberculosis* and differentiate from NTM in cultures.

Further studies are in progress to find the usefulness of SEVA TB ES-31 and SEVA TB ES-43 protein antigens using more sensitive FITC conjugated antibodies against these antigens in FNAC and biopsy clinical samples which were analysed histopathologically for presence of tubercular pathology.

Table: Reactivity of anti-ES-antigen antibodies with *Mycobacterium tuberculosis* H₃₇Rv bacilli and its DSS antigen

Antibody	Reactivity with mycobacterial cell	Reactivity with DSS antigen
Anti ES-31	Present	Present
Anti ES-43	Present	Present
Anti EST-6	Absent	Present
Anti ES-20	Absent	Present

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