Detection of antibodies to a cocktail of mycobacterial excretory–secretory antigens in tuberculosis by ELISA and immunoblotting

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The seroreactivity of a cocktail of purified mycobacterial excretory–secretory (ES) antigens ES-31, ES-41 and ES-43 was assessed by ELISA and immunoblotting in patients with pulmonary tuberculosis. The ES-31 antigen was isolated by affinity chromatography and ES-41 and ES-43 were isolated by fast protein liquid chromatography from *Mycobacterium tuberculosis* H_{37}Ra culture filtrate. Seven of 27 pulmonary tuberculosis sera were not reactive to ES-31 antigen by ELISA. However, 6 out of 7 turned positive, when a cocktail of ES-31, ES-41 and ES-43 antigens was used in ELISA. Seroreactivity pattern of cocktail antigen was studied in immunoblotting using tuberculous sera. Addition of ES-41 and ES-43 antigens helped in increasing the sensitivity compared to ES-31 alone. Further, ELISA was observed to be more sensitive than immunoblotting using a cocktail of antigens.

TUBERCULOSIS (TB) is re-emerging as a deadly disease affecting both developing and developed countries in recent years. The success of TB control programmes depends not only on successful completion of treatment, but also on sound support from sensitive diagnostic tests for early diagnosis and constant monitoring. Various mycobacterium antigens[1–3] have been identified, such as 38, 65, 30/31, 23, 19 and 14 kDa and are being widely used for serodiagnosis of TB. But no single antigen is consistently a target for an antibody response and is found to work satisfactorily to meet the requirement of ideal serodiagnostic test for TB. Therefore, multiantigen assay (or cocktail of purified antigens) has been explored for developing a test with higher sensitivity and specificity[4,5]. The appropriate combination of antigens may be important for developing a good assay for detection of antibody to *Mycobacterium tuberculosis*. In our earlier studies, the purified ES-31 antigen has been extensively evaluated and found to have diagnostic potential in tuberculosis[6–9]. Similarly, other antigens like ES-41 and ES-43 have been isolated[9–11]. Studies using these antigens[6–11] demonstrated good sensitivity and specificity, indicating the diagnostic value of using a mixture of these antigens. In the present study we have analysed the seroreactivity of cocktail of these purified antigens (ES-31, 41 and 43) in pulmonary tuberculosis (PTB), using latest separating and probing techniques like ELISA and immunoblotting.

Blood samples were obtained from patients with confirmed PTB and disease controls attending Civil Hospital, Wardha and Kasturba Hospital, Sevagram, Maharashtra, India. Twenty-seven PTB cases comprised of 20 sputum-positive (S+) and 7 sputum-negative (S–), but clinically suspected and ATT responded cases. Blood samples taken from 5 cases of nontuberculous pulmonary disease like COAD ($n = 1$), pneumonia ($n = 2$) and leprosy ($n = 2$) served as disease control and 10 healthy individuals of this locality served as healthy control. Sera were separated and stored at –20°C with 0.1% sodium azide.

ES-31 antigen was isolated from *M. tuberculosis* H_{37}Ra culture filtrate by affinity chromatography using anti ES-31 antibody coupled sepharose-4B column as described earlier[12]. ES-41 antigen was isolated from culture filtrate by FPLC using superdex 75 HR 10/30 gel filtration column (Pharmacia Biotech, Sweden) followed by Resource 'S' 1 ml cation exchange column, as described earlier[9]. ES-43 antigen was isolated by 50% ammonium sulphate solubilization followed by SDS–PAGE fractionation and further fractionation on Resource 'Q' 1 ml anion exchange column, as described earlier[11]. All the antigenic proteins ES-31, ES-41 and ES-43 were mixed together in equal proportion to prepare a cocktail antigen (ES-31, 41 and 43).

Stick indirect ELISA was performed for detection of tuberculous IgG antibodies as described earlier[9], using purified ES-31 antigen and a cocktail of ES-31, ES-41 and ES-43 antigens (50 pg each). In brief, 5 μl of optimally diluted antigen (0.2 μg/ml) was applied to cellulose acetate membrane square fixed to a plastic strip, and used along with optimally diluted human sera (1 : 600) and antihuman IgG penicillinase conjugate (1 : 1000) in this assay. Sera showing complete decolorization of blue colour of starch iodine penicillin 'V' substrate at least 5 min earlier than the negative control, denote a positive reaction.

The cocktail antigen containing ES-31, ES-41 and ES-43 protein antigens was resolved by SDS–PAGE using 5–15% gradient gel. The proteins were transferred electrophoretically onto nitrocellulose membrane (NCP, BA 85 Scleicher and Schuell AG, Switzerland) at 0.6 ampere (90 volts) for 90 min at 4°C, as described by Towbin *et al.*[13].

Table 1. Detection of tuberculous antibody to a cocktail of antigens ES-31, ES-41 and ES-43 by ELISA in anti ES-31 IgG positive and negative PTB cases

Group	No. screened	No. (%) positive* to cocktail antigen
Pulmonary tuberculosis		
Anti ES-31 IgG positive	20	20 (100)
Anti ES-31 IgG negative	7	6 (86)
Healthy controls	10	–
Disease controls	5	–

*Sera showing positive reaction at 1 : 600 serum dilution.

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Table 2. Reactivity pattern to different combinations of antigen by immunoblotting in PTB

Reactivity to	S+, anti-ES-31 IgG +ve (*n* = 15)	S+, anti-ES-31 IgG –ve (*n* = 5)	Total (*n* = 20)	S–, anti-ES-31 IgG +ve (*n* = 5)	S–, anti-ES-31 IgG –ve (*n* = 2)	Total (*n* = 7)
31	12	–	12	2	–	2
41	6	1	7	1	–	1
43	11	3	14	2	1	3
31/41	12	1	13	2	–	2
31/43	13	3	16	3	1	4
41/43	11	3	14	2	1	3
31/41/43	13	3	16	3	1	4

S +ve, Smear-positive PTB; S –ve, Smear-negative PTB.

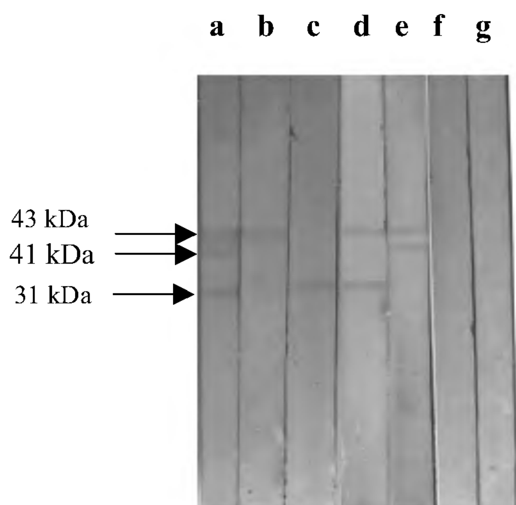

Figure 1. Representative immunoblot showing reactivity pattern. Tuberculosis sera showing reactivity to (a) ES-43, 41 and 31 antigens, (b) ES-43 antigen, (c) ES-31 antigen, (d) ES- 43 and 31 antigens, (e) ES-43 and 41 antigens and (f, g) control sera showing no reactivity.

Table 3. Comparative analysis of seroreactivity of cocktail antigen by ELISA and immunoblotting in PTB

Group	No. screened	Positivity to cocktail antigen in	
		Blotting	ELISA
Pulmonary TB			
Sputum-positive	20	16 (80)	19 (95)
Sputum-negative	7	4 (57)	7 (100)
Control group			
Healthy control	10	–	–
Disease control	5	–	–

After transfer, the NCP was blocked with quench solution (5% skimmed milk powder (SMP) in 0.05 M PBS), overnight at 4°C to saturate additional binding sites and then washed four times with PBS/T containing 2% SMP. The strips were then incubated with serum samples (1 : 50 dilution) and peroxidase-labelled antihuman IgG conjugate. Antibody binding was revealed using freshly prepared peroxidase substrate consisting of 0.05% diamino benzidine and 0.03% of hydrogen peroxide in citrate buffer pH 5.0 for 15 min. The reaction was stopped by rinsing the strips in water.

Sera from 27 confirmed PTB patients, consisting of 20 S+ and 7 S– cases were evaluated by ELISA and immunoblotting. It is well known that TB patients do not show antibodies against all antigenic substances of *M. tuberculosis* at all stages of the disease[14]. Hence in spite of good diagnostic potential, 7 out of 27 (5 S+ and 2 S–) sera were not reactive to ES-31 antigen by ELISA. However, when a cocktail of ES-31, ES-41 and ES-43 was used in ELISA, 6 of 7 cases showed positivity (Table 1). Further reactivity of cocktail antigen was analysed by immunoblotting, which allows the visible reactivity pattern to specific antigens. Reactivity pattern to different combinations of antigen are shown in Table 2 and Figure 1. Out of 20 S+ PTB sera, 12, 7 and 14 showed presence of antibody to antigens ES-31, ES-41 and ES-43 respectively. Similarly, out of 7 S– PTB sera 2, 1 and 3 showed presence of antibody to ES-31, ES-41 and ES-43 antigens respectively. With positivity to all three antigens combined (ES-31/41/43), 16 out of 20 S+ and 4 out of 7 S–PTB showed presence of antibodies (Table 2). It was observed that positivity increased on combining the reactivity to all three antigens (ES-31/41/43) compared to reactivity to ES-31, ES-41 and ES-43 alone. Thus it was observed that sputum-positive and sputum-negative cases which were not reactive to ES-31 antigen, were found reactive to either ES-41 or/and ES-43 antigens (Table 2). However, by ELISA, S+ and S– PTB sera showed overall positivity of 96% (26/27) compared to 74% (20/27) by blotting using cocktail antigen. Further, none of the control sera showed presence of antibody by ELISA and immunoblotting (Table 3). Thus addition of ES-41 and ES-43 antigens to ES-31 in a cocktail helped in increasing the sensitivity. Lu *et al.*[15] also observed in Western blotting that mycobacterial protein antigen of the 30,000–43,000 region was found to be more reactive in sera. Earlier studies had also shown improved diagnosis by use of a combination of different antigens. In a study by Bothamley *et al.*[16] a combination of 19 kDa antigen, lipoarabinomannan (ML 34 epitope) and hsp 65 (TB 78 epitope) was used and improved sensitivity and specificity in smear-negative PTB achieved. Okuda *et al.*[4] studied antibody response against glycolipid antigen, lipoarabinomannan and antigen 60 (A60) and found that com-

bined use of three separate antigens maximizes the effectiveness of serodiagnosis. Similarly, Julian *et al.*[5] found that sensitivity is improved when antibodies are detected together in a test based on different antigens (proteins and glycolipids) and suggested the use of a cocktail of specific antigens from *M. tuberculosis* to develop a serodiagnostic test. In our earlier studies[17], a cocktail of ES-31 and ES-41 was explored by ELISA and was found to be useful for screening of broad spectrum of TB sera in PTB and extrapulmonary TB compared to ES-31 or ES-41 alone. However, the present study showed that a combination of ES-31, ES-41 and ES-43 is still better and more sensitive in ELISA compared to blotting. Thus ELISA using a cocktail antigen is simple, sensitive and easy to perform and more sensitive compared to blotting and can be useful in analysis of a large number of samples for serodiagnosis of TB.

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Using satellite telemetry to mitigate elephant–human conflict: An experiment in northern West Bengal, India

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Satellite tracking of animals has advantages in the study of species that migrate across international borders, have large home ranges and occupy remote and inaccessible areas. The efficacy of this technology in dense tropical forests may, however, be limited. At the same time, its use in mitigating wildlife–human conflict has not been examined so far. Here we report the movement patterns and habitat utilization of an adult male Asian elephant, and a preliminary assessment of the potential use of satellite technology as an 'early warning system' for conflict mitigation. Data on the location of the animal were obtained from a Platform Transmitter

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