Indian J Med Res 135, May 2012, pp 745-755

Whole cell & culture filtrate proteins from prevalent genotypes of *Mycobacterium tuberculosis* provoke better antibody & T cell response than laboratory strain $H_{37}Rv$

Gavish Kumar[1], Hari Shankar[1], Mamta Chahar[2], Pragya Sharma[2], Virendra Singh Yadav[3], Devendra Singh Chauhan[2], Vishwa Mohan Katoch[4,+] & Beenu Joshi[1]

Departments of [1]Immunology, [2]Microbiology & Molecular Biology, [3]Bio-statistics & Epidemiology, National JALMA Institute for Leprosy & Other Mycobacterial Diseases (ICMR), Agra & [4]Indian Council of Medical Research, New Delhi, India

Received January 11, 2011

***Background & objectives*: The immune responses to different antigens of *Mycobacterium tuberculosis* $H_{37}Rv$ vary from patient to patient with tuberculosis (TB). Therefore, significant difference might be documented between the $H_{37}Rv$ with long histories of passages and recent clinical isolates of *M. tuberculosis*. In the present study, immune response of TB patients and healthy controls against 39 clinical *M. tuberculosis* isolates was correlated with laboratory strain $H_{37}Rv$.**

***Methods*: The antibody response was studied coating whole cell extracts and culture filtrate proteins of *M. tuberculosis* isolates and laboratory strain $H_{37}Rv$ by enzyme linked immunosorbent assay (ELISA). Lymphoproliferation was studied by incorporation of tritiated thymidine and cytokines (IFN-γ and IL-4) by using commercially available kits.**

***Results*: Sero-reactivity to whole cell extract (WCE) of 11 clinical isolates was higher with pooled serum and individual's serum from tuberculosis patients showed significant reactivity ($P<0.05$) to ten of these isolates using ELISA. Of the WCE of 39 clinical isolates, 10 were found to be potent inducer of lymphoproliferation as well as cytokine secretion ($P<0.05$) in peripheral blood mononuclear cells from PPD+ healthy controls. Six culture filtrate proteins (CFPs) from these selected clinical isolates were also better inducers of antibody and T-cell response.**

***Interpretation & conclusion*: Overall, our results revealed that the clinical isolates belonging to prevalent genotypes; CAS1_Del (ST-26), East African-Indian (ST-11) and Beijing family (ST-1) induced better antibody and T cell responses compared to $H_{37}Rv$ laboratory strain. Further studies need to be done to purify and identify the dominant protein (s) using whole cell extract and culture filtrates from these immunologically relevant clinical *M. tuberculosis* isolates, which will be worthwhile to find out pathogenic factors, potential diagnostic markers and protective molecules for tuberculosis.**

Key words Antibody and cell mediated response - culture filtrate protein - clinical *Mycobacterium tuberculosis* isolates - whole cell extract

[+]*Present address*: Secretary, Department of Health Research, Government of India & Director-General, Indian Council of Medical Research, New Delhi 110 029, India

Tuberculosis (TB) continues to remain as one of the major killer contagious diseases with nearly 1.7 million deaths and more than 9.4 million new cases each year[1]. Early and specific diagnosis is essentially required for control of disease. The development of specific immuno-diagnostic tests for tuberculosis has been hampered by cross-reactive antigenic epitopes of *M. tuberculosis* between different mycobacterial as well as with other non mycobacterial strains[2]. Cross-reactivity of most of the *M. tuberculosis* antigens with BCG further complicates the immune based diagnosis of tuberculosis[3,4] as most of the people are BCG vaccinated especially in the endemic country like India.

Immune-based tests are known as the valuable tool to diagnose a disease during early phases of the infection. Several investigations have been done to develop a sensitive and specific immuno-diagnostic assay by extracting the antigens from *M. tuberculosis* strain $H_{37}Rv$, but these assays suffer from variable immune responses[5]. $H_{37}Rv$ is maintained in the laboratories from a long time by *in vitro* passages, and therefore, appear to have lost its high virulent factors and may be immunodominant antigens. Recently, it has been shown that two closely related *M. tuberculosis* Beijing isolates collected in South Africa have vastly different pathogenic characteristics in terms of their ability to transmit and cause disease in humans and to cause pulmonary damage in mice[6]. Therefore, systematic studies in different regions are needed to map the antigen profile of *M. tuberculosis* isolates from patients with TB to understand the relationship between antigenic feature of *M. tuberculosis* with the prevailing epidemiological situation. In India, being a high incidence country, features of *M. tuberculosis* bacilli may be distinct from place to place, which suggest an urgent need to search for dominant antigens from prevalent strains of *M. tuberculosis* present in the community, these antigens could be used as a diagnostic or vaccine candidate (s).

Therefore, this study was designed to evaluate the immune-reactivity of clinical isolates of *M. tuberculosis* obtained from different regions of India in comparison to laboratory strain $H_{37}Rv$ using enzyme linked immunosorbent assay (ELISA), lymphoproliferation assay and cytokine estimation.

Material & Methods

M. tuberculosis clinical isolates: Thirty nine clinical *M. tuberculosis* isolates from ten different regions of India (Agra, Kanpur, Delhi, Ranchi, Karnataka, North East, Jaipur, Ahamedabad, Allahabad and Bhopal) and laboratory strain $H_{37}Rv$ were collected from Mycobacterial Repository Centre of National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Agra, India. The isolates were arbitrarily selected in terms of geographical source and growth profile. These isolates were inoculated (10^8 cfu/ml) in tween-80 free Sauton's medium on a shaking incubator at 37ºC.

Spoligotyping of clinical isolates: DNA from *M. tuberculosis* isolates was extracted by the method of van Embden *et al*[7]. Spoligotyping to detect 43 spacers was done using commercially available kit (Ocimum Biosolutions, Hyderabad) as described previously[8]. Binary format results were converted into octal code and compared with the international Database SpolDB4.0[9]. Spoligopatterns not found in SpolD4.0 were analyzed with "Spotclust"[10].

Preparation of whole cell extracts (WCE): *M. tuberculosis* growth was collected from Sauton's medium by centrifugation at 10,000xg and washed twice with 150 mM phosphate-buffered saline (PBS, *p*H 7.4). The growth was suspended (0.2 g growth/ml) in PBS and extraction buffer (50 mM Tris buffer, 10 mM $MgCl_2$, 1 mM ethylene glycol tetra-acetic acid, 1 mM phenylmethylsulphonyl fluoride, *p*H 7.4). Bacteria were sonicated with a vibra cell probe ultra-sonicator for a total of 20 min using 50 per cent output control (100%=475 w) and 50 per cent duty cycle (on/off) at 4^0C[11]. WCE were collected by centrifugation at 23000xg for 30 min. The protein concentration of each cell extract was estimated using the Bradford's method[12]. These extracts were stored at -20^0C till used.

Preparation of culture filtrate proteins (CFP): *M. tuberculosis* bacilli were removed and resultant culture filtrate was processed as described earlier[13]. Briefly, culture filtrate was sequentially filtered through 0.45 µm followed by 0.22 µm Millex GV PVDF membrane (Millipore, Bedford, MA, USA). SDS (10%) (Sigma, USA) was added to obtain 0.1 per cent final concentration (w/v) in the culture filtrate (CF) and kept in a boiling water bath for 5 min. CF was treated with trichloroacetic acid (TCA) (Sigma, USA) to obtain final concentration 10 per cent (w/v). Finally, this mixture was incubated at -20^0C for 5 h and the resulting precipitate was removed by centrifugation at 18,000xg for 30 min at 4^0C. Minimum volume of HPLC grade water (Qualigens fine chemicals, Mumbai, India) was added to disperse the pellet and then the whole suspension was washed with 1 ml of pre-chilled

acetone (Sigma, USA). Air dried pellets were dissolved in minimum volume of 2D rehydration buffer (Bio-Rad Laboratories, Hercules, CA, USA). CFP for cell proliferation assays were prepared by buffer exchange method using PBS. The protein concentration was determined by Bradford's method[12].

Study subjects: Serum samples from HIV negative tuberculosis patients (n=55, age range 18 to 60 yr) were collected from State Tuberculosis Demonstration Centre (STDC) and Department of Tuberculosis & Chest Diseases (TBCD), S. N. Medical College, Agra, India. All these patients were clinically diagnosed as pulmonary TB and confirmed bacteriologically by staining for acid fast bacilli/ isolation of tuberculous bacilli in cultures of Lowenstein-Jensen medium. These patients were categorized into three clinical groups (35 active untreated TB, 10 defaulter TB and 10 relapsed TB cases) as per the guidelines of Revised National TB Control Program, Central TB Division, Government of India[14]. Six samples from individuals who were taking standard treatment for TB for more than 5 months were also included in this study.

Serum samples from healthy controls (n=21) were collected from students (age range 18-25 yr) working in Immunology laboratory for short term projects of the Institute. Ten confirmed lepromatous leprosy (LL) controls were also included from out patient department (OPD) of National JALMA Institute for Leprosy and Other Mycobacterial Diseases (ICMR), Agra. All serum samples were collected and stored at -20^{0}C until used. The study was approved by Institutional ethical committee and informed consent was obtained from each patients.

Pooled serum preparation: Equal volume of 10 serum samples was pooled separately from different categories of individuals. *(i)* HPS (healthy subjects pooled serum); healthy BCG vaccinated subjects. *(ii)* FPS (fresh/new TB cases pooled serum); patients who had infection with *M. tuberculosis* for the first time and had no history of tuberculosis treatment. *(iii)* DPS (defaulter TB cases pooled serum); patients who had not taken the complete course of anti-tubercular treatment and the symptoms re-occurred. *(iv)* RPS (relapsed TB cases pooled serum); TB patients who were treated earlier for TB but symptoms re-occurred after completion of the treatment. *(v)* TPS (treated TB cases pooled serum); individuals who were taking standard treatment of TB for more than 5 months. *(vi)* LLPS (lepromatous leprosy cases pooled serum); confirmed lepromatous leprosy (LL) patients.

Enzyme linked immunosorbent assay (ELISA): ELISA was performed using standard protocols as described previously[15]. Briefly, maxisorp ELISA plates (Nunc Maxisorp, Roskilde, Denmark) were coated with 100 µl of 125 ng/ml whole cell extract/culture filtrate protein of clinical isolates and H_{37}Rv in carbonate bicarbonate (0.05 M, *p*H 9.6) buffer per well. The plates were read at 492 nm using an ELISA reader (Spectramax-M2 Reader, Molecular Devices, Sunnyvale, CA, USA).

T-cell proliferation assay: Peripheral blood mononuclear cells (PBMCs) were separated from heparinized blood of healthy individuals (10 individuals for WCE and 6 for CFP) by density gradient centrifugation over Ficoll hypaque[16]. The washed cells were counted and adjusted to 1x10^6 cells / ml in culture medium (RPMI 1640) and dispensed (100 µl/well) into 96 well flat bottom culture plates (Nunc, Roskilde, Denmark). Culture were set in triplicate with or without antigen in culture medium (100 µl/well) containing 5 per cent heat inactivated foetal calf serum supplemented with 2 mM L-glutamine and antibiotic & antimycotic and incubated for 5 days in a CO_2 incubator. Purified protein derivative (PPD) was taken as positive control in each experiment. Optimum doses (10 µg/ml for WCE and 1 µg/ml for CFP) for proliferation responses were determined by adding increasing amounts of WCE and CFP from clinical isolates at concentrations ranging from 1 to 25 µg of protein per ml to PBMCs (1x10^{6}cells/ml) suspension. The culture was pulsed with ^{3}H-thymidine (1 µci/well) for 18 h before harvesting, and the radioactivity of the harvested cells was measured in a β-liquid scintillation counter (LKB Rackbeta, Finland).

Cytokine ELISA: The culture supernatants from PBMC culture were harvested after 5 days of incubation in a CO_2 incubator and stored at -20ºC until use. Cytokine (IFN-γ & IL-4) levels were estimated by using e-Bioscience cytokine detection kit (San Diego, USA) according to the manufacturer's instructions.

Statistical analysis: Cut-off value, sensitivity and specificity for each antigen against individual serum were calculated by ROC (receiver operator characteristics) analysis using software STATA-7 (Strata Corporation, College Station, TX, USA). The cut-off was selected at the point which showed the best accuracy (correctly classifying individuals to their groups), sensitivity, and specificity by ROC. A positive predictive value (PPV) is defined as the probability to characterize a patient for the particular disease from the patient's population, and negative predictive value (NPV) is also needed

to exclude the disease. Scattergram was plotted using GRAPHPAD software prism version 5.0 (GraphPad Software, Inc., San Diego, CA, USA).

T-cell proliferation response of stimulated PBMCs was defined as stimulation index (SI) which was calculated by dividing the mean counts per minute (CPM) of experimental wells by mean CPM of control wells. T-cell proliferation and cytokine response was correlated and Spearman correlation coefficient (r) was calculated.

Results

Serological response of whole cell extracted and culture filtrate proteins: High optical density (>2 times) was observed using pooled serum from TB patients and low optical density taking pooled serum from lepromatous leprosy against total 22 clinical *M. tuberculosis* isolates (CI1,5,6,8,9,10,11,12,13,15,16, 19,20,22,23,24,25,27,31,32,33 and 39) in comparison to healthy subjects (data not shown). Finally, total eleven clinical isolates (CI1,5,6,8,11,20,23,27,31,32 and 39) representing eight different regions were selected for further serological analysis using individual serum from different types of tuberculosis cases (Fig. 1A, Table I). Overall antibody reactivity of TB patients' serum samples against 10 clinical isolates was significantly greater than healthy controls, however, reactivity was not significant against CI6 and *M. tuberculosis* $H_{37}Rv$ (Table I). Sensitivity was highest (88.57%) with WCE of CI20 and CI39 followed by CI5, CI11, CI23, CI27,

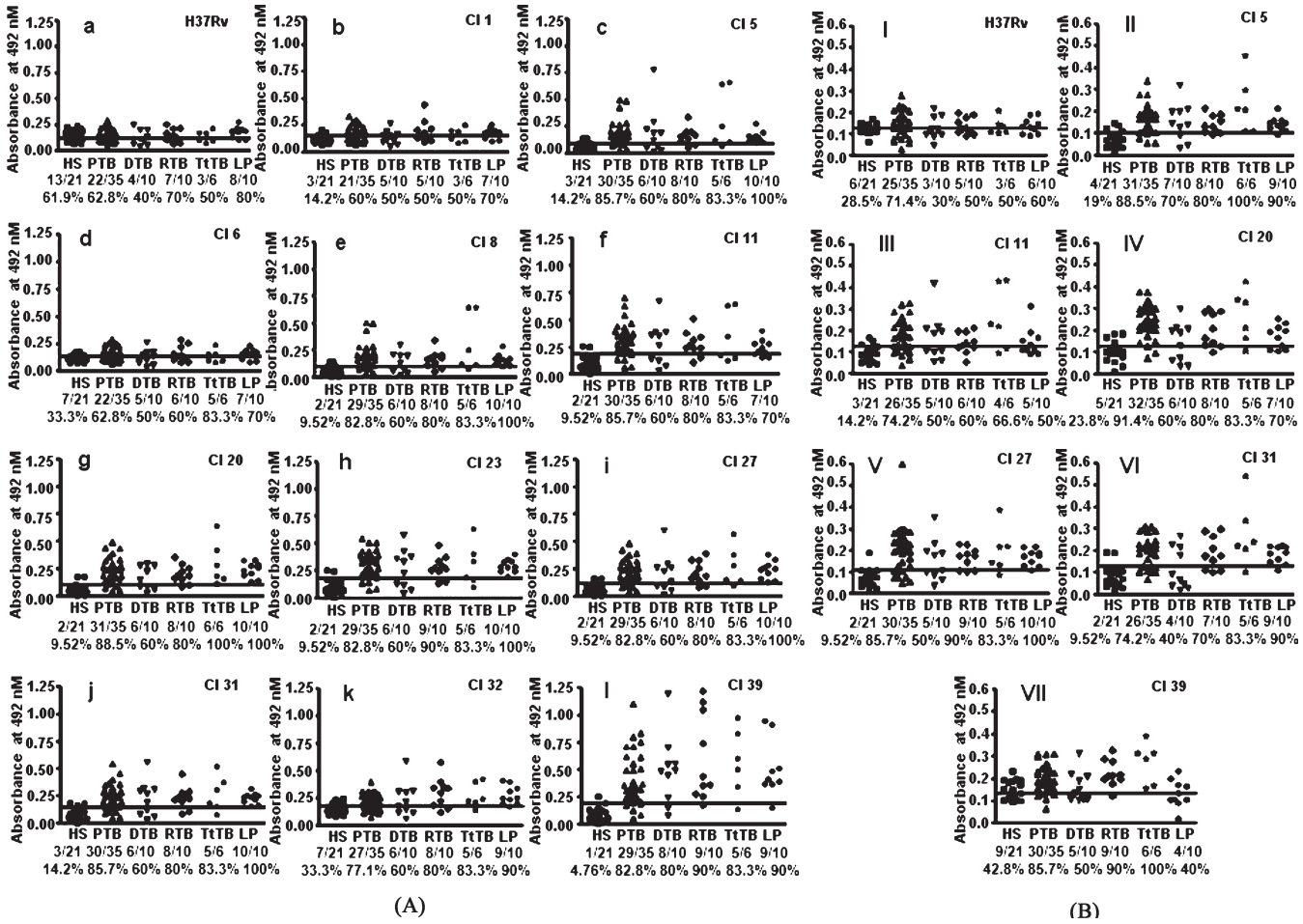

Fig. 1. (A) Scatter diagram of IgG antibody response to whole cell extract from various selected clinical isolates with individual serum from different groups of patients [patients with fresh pulmonary tuberculosis (PTB), defaulter TB (DTB), relapse TB (RTB), treated TB (TtTB), and lepromatous leprosy patients (LP)] and controls (HS, healthy subjects) (b-l) in comparison with *M. tuberculosis* $H_{37}Rv$ (a). **(B)** Quantitative antibody responses of healthy subjects (HS) and different groups of patients (PTB, DTB, RTB, TtTB, LP) to culture filtrate proteins of *M. tuberculosis* (I-VII). Solid lines represent cut-off value decided by ROC analysis. CI, clinical isolates.

Table I. Sensitivity and specificity of antibody response against whole cell extract from clinical *M. tuberculosis* isolates and $H_{37}Rv$ by ROC analysis

M. tuberculosis isolates	Cut-off	% Sensitivity	% Specificity	PPV	NPV	% Accuracy	ROC area	*P* value*
H37Rv	0. 119	65.71	38.10	67.9	42.9	55.36	0.499	
CI 1	0. 150	62.86	85.71	87.0	54.5	71.43	0.756	<0.05
CI 5	0.093	85.71	85.71	90.9	78.3	85.71	0.920	< 0.001
CI 6	0.135	65.71	66.67	76.7	53.8	66.07	0.713	>0.05
CI 8	0.101	82.86	90.48	93.5	76.0	85.71	0.923	< 0.001
CI 11	0.187	85.71	90.48	93.7	79.2	87.50	0.917	< 0.001
CI 20	0.109	88.57	90.48	93.9	82.6	89.29	0.940	0.001
CI 23	0.186	85.71	90.48	93.7	79.2	87.50	0.929	< 0.001
CI 27	0.125	85.71	90.48	81.1	73.7	87.50	0.933	< 0.001
CI 31	0.145	85.71	85.71	90.9	78.3	85.71	0.916	< 0.001
CI 32	0.170	74.29	66.67	78.8	60.9	71.43	0.751	<0.05
CI 39	0.194	88.57	95.24	96.9	83.3	91.07	0.954	< 0.001

CI, clinical *M. tuberculosis* isolates, study subjects; tuberculosis patients (N=55) and control subjects (healthy subjects; N=21, lepromatous leprosy subjects; N=10), ROC, receiver operator characteristics; PPV, positive predictive value**;** NPV, negative predictive value; *P value when area under ROC of H37Rv was compared with whole cell extract of clinical *M. tuberculosis* isolate

CI31 (85.71%), CI8 (82.86%) and CI32 (74.29%) as compared with $H_{37}Rv$ (65.71%). Similar reactivity to CI6 was noted as $H_{37}Rv$, and CI1 (62.86%) was comparatively less reactive. Accuracy of ELISA was high to all clinical *M. tuberculosis* isolates and it was 55.36% for $H_{37}Rv$ (Table I). All the clinical isolates showed higher reactivity in fresh tuberculosis cases than $H_{37}Rv$ except CI1, and CI6 (Fig 1A). Highest reactivity (80%) to defaulter tuberculosis cases (DTB) was noted against CI39 and slightly higher antibody (60%) was reported against CI5, CI8, CI11, CI20, CI23, CI27, CI31 and CI32 as compared with $H_{37}Rv$ (40%). Reactivity to clinical isolate1 and 6 was only 50 per cent in DTB group of patients. The reactivity to treated cases was highest (100%) against CI20 and to all other clinical isolates was 83.3 per cent except CI1 (50%). Only 50 per cent treated TB cases were reactive when antibody was detected against $H_{37}Rv$. The reactivity to serum from lepromatous leprosy patients was highest (100%) against WCE of CI5, CI20, CI23, CI27, and CI31 followed by 90 per cent for CI32 and CI39. Lowest reactivity (70%) was observed for CI1, CI6, and CI11. The reactivity against $H_{37}Rv$ was found 80 per cent with leprosy patient.

Antibody response to CFP collected at different time points (7, 14, 21, 28 and 35 days) from 11 selected clinical isolates of *M. tuberculosis* was analyzed by ELISA taking a panel of pooled serum samples from patients with various categories of tuberculosis and controls. Highest antibody levels were observed against 21 and 35 days culture filtrate. A total six culture filtrates from clinical isolates (CI5, 11, 20, 27, 31 and 39) which showed higher antibody (> 2 times than controls) to pooled serum of different categories of tuberculosis patients were selected for further analysis using different individual serum (Fig. 1B). Antibody reactivity to four culture filtrate of clinical isolates (CI5, CI20, CI27 and CI31) was significantly (<0.05) higher as compared with $H_{37}Rv$ (Table II). Most of the CFP of clinical isolates (CI5, CI20, CI27 and CI39) as well as $H_{37}Rv$ showed strong reactivity to PTB serum than their WCE. However, low antibody response to PTB was noted to culture filtrate of CI11 and CI31 as compared to their WCE. Defaulter tuberculosis cases (DTB) were also less reactive to CFP in comparison to WCE to four clinical *M. tuberculosis* isolates (CI11, CI27, CI31 and CI39) and $H_{37}Rv$ (Fig. 1 A and B). Similar reactivity of CFP to relapsed TB cases (RTB) was noted (CI5, CI20 and CI39) and lower

Table II. Sensitivity and specificity of antibody response against culture filtrate proteins from clinical *M. tuberculosis* isolates and $H_{37}Rv$ by ROC analysis

M. tuberculosis isolates	Cut-off	% Sensitivity	% Specificity	PPV	NPV	% Accuracy	ROC area	*P* value*
H37Rv	0.126	74.29	61.90	76.5	59.1	69.64	0.698	
CI 5	0.101	91.43	80.95	88.9	85.0	87.50	0.913	<0.05
CI11	0.127	80.00	85.71	93.3	72.0	82.14	0.861	>0.05
CI20	0.128	94.29	76.19	86.8	88.9	87.50	0.937	<0.05
CI27	0.108	85.71	90.48	93.7	79.2	87.50	0.91	<0.05
CI31	0. 127	80.00	90.48	93.3	73.1	83.93	0.916	<0.05
CI39	0. 134	88.57	57.14	77.5	75.0	76.79	0.797	>0.05

CI, clinical *M. tuberculosis* isolates; study subjects; tuberculosis patients (N=55) and control subjects (healthy subjects; N=21, lepromatous leprosy subjects; N=10), ROC, receiver operator characteristics; PPV, positive predictive value; NPV, negative predictive value; *P value when area under ROC of $H_{37}Rv$ was compared with culture filtrate proteins of clinical *M. tuberculosis* isolate

(CI11 and CI31) than their WCE. Only CFP of CI27 was showing high reactivity to RTB than WCE. The reactivity of treated tuberculosis patients (TtTB) against CI5 and 39 was higher than WCE of similar isolates. Lepromatous leprosy patients' serum was less reactive to CFP of all clinical isolates (CI5, 11, 20, 31 and 39) as well as to $H_{37}Rv$ except CI27 as compared with their corresponding WCE. Accuracy of all CFP was comparatively higher than $H_{37}Rv$ using cut-off determined by ROC analysis (Table II). These CFP of clinical *M. tuberculosis* isolates belonged to prevalent genotypes; CI5, CAS1_Del (ST-26); CI11, CAS1_Del

Table III. Lymphoproliferation and IFN-γ response against whole cell extract (WCE) and culture filtrate proteins (CFP) of selected clinical isolates of *M. tuberculosis* and $H_{37}Rv$

M. tuberculosis isolates	T-cell proliferation response (Mean ± SE of SI)		IFN-γ detection in pg/ml (Mean ± SE)	
	WCE (n=10)	CFP (n=6)	WCE (n=10)	CFP (n=6)
$H_{37}Rv$	9.1 ± 2.1	13.8 ± 4.7	346.0 ± 71.8	610.0 ± 206.9*
CI1	21.1 ± 4.4*	12.7 ± 4.8	729.0 ± 109.4***	545.0 ± 298.8
CI5	38.7 ± 10.1*	22.7 ± 4.6*	779.5 ± 107.5***	925.0 ± 237.6*
CI6	18.0 ± 4.5*	14.5 ± 3.6	604.0 ± 104.4**	645.5 ± 122.9*
CI11	35.5 ± 10.4*	15.7 ± 4.7*	757.5 ± 121.3***	758.3 ± 293.7*
CI20	39.3 ± 11.9*	21.2 ± 5.8*	440.0 ± 91.0*	660.0 ± 182.8*
CI23	19.8 ± 5.3*	12.7 ± 3.1	387.5 ± 82.5*	393.3 ± 73.3
CI27	22.3 ± 6.7*	23.7 ± 7.0*	440.0 ± 113.9*	708.3 ± 248.1*
CI31	16.1 ± 3.0*	26.2 ± 9.0*	591.2 ± 96.9***	690.0 ± 155.9*
CI32	40.4 ± 13.8*	15.9 ± 3.4	603.3 ± 116.0***	730.0 ± 150.7*
CI39	40.7 ± 12.7*	23.1 ± 6.6*	603.6 ± 105.0***	1047 ± 136.1*

*, **, *** show statistically significant response $P<0.05$, $P<0.01$ and $P<0.001$ respectively, significance was calculated by comparing SI of WCE or CFP of $H_{37}Rv$ with clinical *M. tuberculosis* isolates for T-cell proliferation response; significance of IFN-γ secretion was calculated by comparing unstimulated PBMCs with WCE or CFP stimulated PBMCs; SI, stimulation index

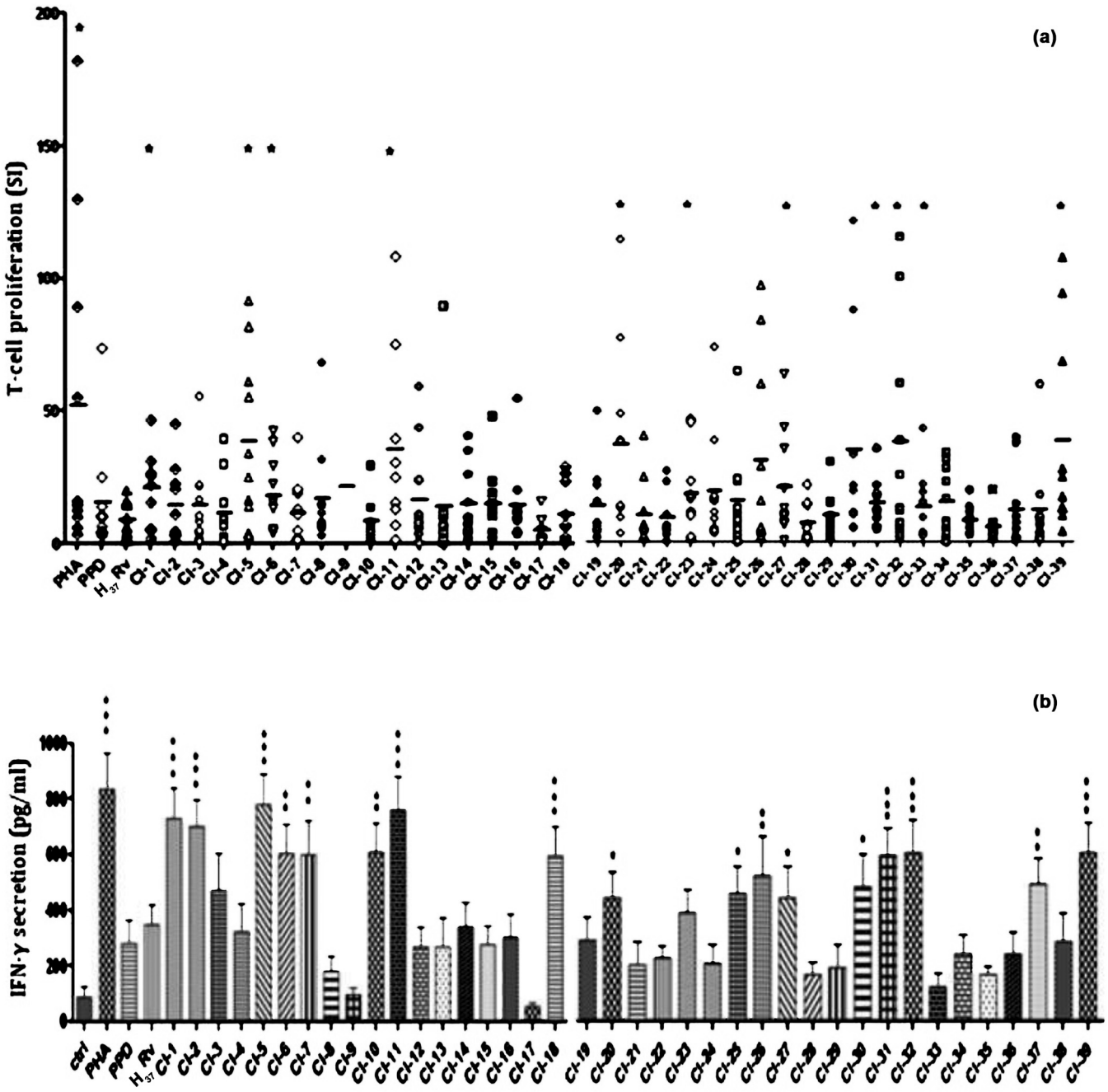

Fig. 2. (a) T-cell proliferation response and **(b)** IFN-γ levels secreted by PBMCs of healthy individuals induced by whole cell extracts from 39 clinical *M. tuberculosis* isolates (CI), H_{37}Rv, PPD standard, and Mitogen phytohemagglutinin (PHA); Ctrl, unstimulated PBMC, Bar represents the mean value of stimulation index (SI) for each isolate. Statistical significance of T-cell proliferation response was calculated using paired t-test by comparing the stimulation index values of H_{37}Rv and clinical isolates, *P* value for IFN-γ secretion was calculated by comparing unstimulated PBMCs (Ctrl) with WCE or CFP stimulated PBMCs, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

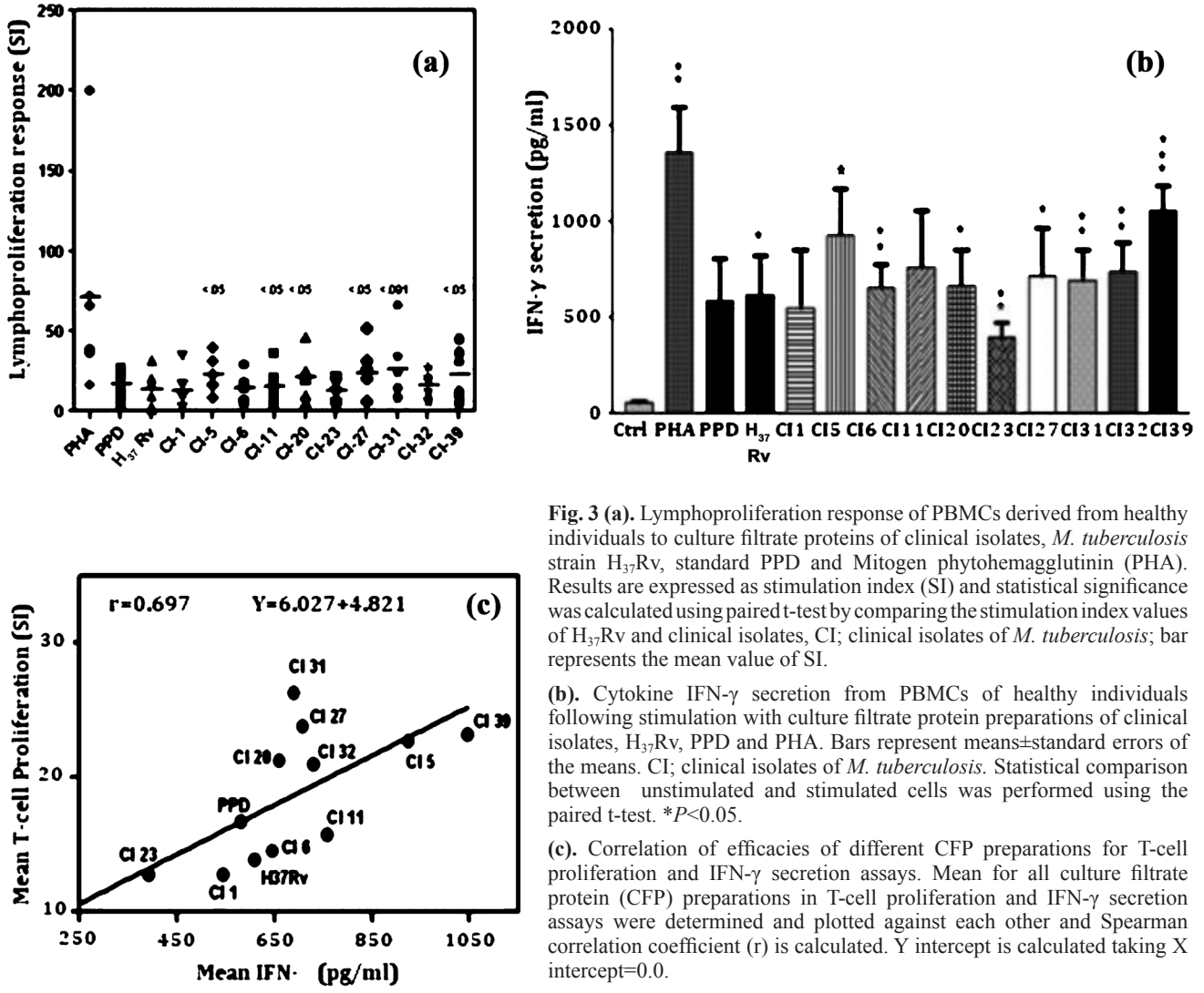

Fig. 3 (a). Lymphoproliferation response of PBMCs derived from healthy individuals to culture filtrate proteins of clinical isolates, *M. tuberculosis* strain $H_{37}Rv$, standard PPD and Mitogen phytohemagglutinin (PHA). Results are expressed as stimulation index (SI) and statistical significance was calculated using paired t-test by comparing the stimulation index values of $H_{37}Rv$ and clinical isolates, CI; clinical isolates of *M. tuberculosis*; bar represents the mean value of SI.

(b). Cytokine IFN-γ secretion from PBMCs of healthy individuals following stimulation with culture filtrate protein preparations of clinical isolates, $H_{37}Rv$, PPD and PHA. Bars represent means±standard errors of the means. CI; clinical isolates of *M. tuberculosis*. Statistical comparison between unstimulated and stimulated cells was performed using the paired t-test. **P*<0.05.

(c). Correlation of efficacies of different CFP preparations for T-cell proliferation and IFN-γ secretion assays. Mean for all culture filtrate protein (CFP) preparations in T-cell proliferation and IFN-γ secretion assays were determined and plotted against each other and Spearman correlation coefficient (r) is calculated. Y intercept is calculated taking X intercept=0.0.

(ST-26); CI20, CAS1_Del (ST-26); CI27, East African Indian (ST-11); CI31, Beijing family (ST-1) and CI39, CAS1_Del (ST-26) family.

Cell mediated immune response of whole cell extracted and culture filtrate proteins: The WCE of CI1, 5, 6, 11, 20, 23, 27, 31, 32 and 39 induced significantly higher levels of T-cell proliferation as compared to $H_{37}Rv$ and IFN-γ secretion as compared to unstimulated PBMCs (Fig. 2a, 2b and Table III) but IL-4 levels was not significantly higher for any WCE of clinical isolate as well as for $H_{37}Rv$ as compared to unstimulated PBMCs (data not shown). The relative efficacies of CFP preparations from 10 selected clinical isolates of *M. tuberculosis* were also analyzed by stimulating PBMCs from healthy donors. A comparison of the different CFP preparations revealed that the CFP of CI5, 11, 20, 27, 31, 32 and 39 clinical isolates were statistically more potent in inducing cell proliferation than those of $H_{37}Rv$ and PPD (P<0·05 for CI5, 11, 20, 27, 39 and <0·001 for CI31) (Fig. 3a). CFP preparations of clinical isolates which were significant inducers of T-cell proliferation were also the potent stimulator of IFN-γ secretion by PBMCs (Fig. 3b and 3c). Responses to different CFP preparations indicated that the CFPs derived from CI39 induced maximum IFN-γ, which was significantly higher (P<0.001) than the response to CFPs of $H_{37}Rv$ and PPD (Fig. 3b). IL-4 concentration was also determined in culture supernatants of PBMCs stimulated with or without CFPs (data not shown). A moderate increase in IL-4 secretion was induced only by some CFPs (CI5, CI32 and CI39). The relative

efficacy of different CFP preparations to induce T-cell proliferation and IFN-γ secretion were not similar for all PBMC preparations. Efficacy of different CFP preparations from clinical isolates in proliferation and IFN-γ assays were correlated and a positive correlation between the two parameters of T-cell activation was observed for clinical isolates (r, 0.697; Fig. 3c).

Discussion

Immune response of host could be a good indicator of exposure to *M. tuberculosis*. Failure of BCG in TB endemic countries is the major bottleneck for the control of this disease. Therefore, exhaustive research is going on to find new candidate antigens for the diagnosis and vaccine for TB. Most of the studies have been conducted on the purified antigens of laboratory strain H_{37}Rv which has been passaged over the years and might have lost some of the immunodominant antigens. It is possible that most recent and prevalent strains of *M. tuberculosis* could induce more specific and dominant immune response and characterization of these antigens could lead to identification of some new antigens. The present study was therefore undertaken to evaluate antibody response, lymphoproliferative, IFN-γ and IL-4 responses of PPD positive healthy individuals and TB patients to clinical isolates of *M. tuberculosis* belonging to different geographical regions of India. Significantly higher antibody reactivity was noted in TB patients with WCE of certain clinical isolates than H_{37}Rv. Most of the culture filtrates of clinical isolates were strongly reactive with fresh pulmonary TB cases in comparison to WCE of same isolate. In contrast, the reactivity to relapsed (RTB) and defaulter TB (DTB) cases was lower to culture filtrate than whole cell extracts of most of clinical isolates. This was a preliminary approach to find out immunologically relevant clinical isolates, as the cases of relapsed TB (RTB), defaulter TB (DTB) and lepromatous (LL) leprosy groups used in the present study were not sufficient to make a valid conclusion. Serum samples from leprosy patients showed strong antibody response to all clinical isolates. It appears that the immunodominant cross-reactive antigens of *M. leprae* are expressed in clinical *M. tuberculosis* isolates, some of which antigens are not expressed in H_{37}Rv. Further, reactivity of leprosy patients' serum samples against culture filtrate of most of the clinical isolates was less than that observed for whole cell extract. This finding suggests that *M. tuberculosis* secretes more specific molecules in culture medium.

Correlation of T-cell proliferation and IFN-γ secretion parameters with protective immunity has been shown[17,18]. In our study, T cell lymphoproliferative response and IFN-γ production of healthy individuals after stimulation with whole cell extracts of clinical isolates were significantly higher than the response with standard laboratory strain H_{37}Rv. Whole cell extracts of certain clinical isolates showed good correlation of antibody reactivity, lymphoproliferative response and IFN-γ production suggesting that these isolates could have promising molecules for immune based detection of tuberculosis and immune intervention. A study conducted in southern India also evaluated the potential of sonicated antigens from highly prevalent strains from south India in induction of protective immunity and humoral response[19].

M. tuberculosis isolates used in this study were cultured and harvested under the same standard culture conditions to minimize the differences in protein expression[20,21]. Various studies have demonstrated that antigens present in culture filtrate (CF) are inducers of protective immunity in mice and humans with TB[22,23]. Therefore, antibody, LTT and IFN-γ response of TB patients and healthy individuals to CF of clinical isolates was analyzed. High percentage of accuracy was noted for all six culture filtrates of clinical isolates than H_{37}Rv when antibody response was analyzed. Four out of five healthy individuals showed good proliferation response to culture filtrate of all clinical isolates whereas three healthy individuals showed good response to H_{37}Rv. CF of most of the clinical isolates induced higher IFN-γ than H_{37}Rv. IL-4 production was not significant, however in three isolates the culture filtrates induced moderately higher IL-4 response in most of the healthy individuals than that induced by H_{37}Rv suggesting both Th1/Th2 inducing nature of culture filtrate of these isolates. Siddiqui *et al* have also evaluated T cell proliferation, IFN-γ and IL-12 secretion against CF from six isolates of *M. tuberculosis* and found similar results as ours[24]. It has been shown that different *M. tuberculosis* isolates elicit different Th1 type immune response during the early phase of infection using mice model[25], as well as different levels of virulence[26]. It is interesting that immunologically relevant six culture filtrates identified in the present study belonged to prevalent genotypes [Central Asian Strain CAS1_Del (ST-26), East African-Indian (ST-11) and Beijing family (ST-1)] of *M. tuberculosis*. CAS1_ Del, East African Indian families of *M. tuberculosis* isolates are

of particular interest to provide insights into regional TB epidemiology because these are the most prevalent strains in Northern and other parts of India as defined by previous studies[27-31].

In conclusion, culture filtrate proteins are early recognized by host cell and, therefore, important for signaling pathways. Strong immune response to culture filtrate proteins extracted from prevalent clinical *M. tuberculosis* isolates highlighted the presence of immunodominant protein(s) in these clinical isolates that could be in addition to so far identified *M. tuberculosis* antigens. Therefore, systematic advanced proteomic and immunological profiling of these prevalent clinical *M. tuberculosis* isolates may lead to identify functional targets for pathogenic mechanisms, diagnosis and prevention of tuberculosis.

Acknowledgment

Authors thank Dr Bharat Bajaj, State TB Demonstration and Training Centre and Dr Rajesh Gupta, S.N. Medical College, Agra for providing clinically diagnosed samples of tuberculosis patients and Dr BK Girdhar, NJIL & OMD (ICMR) for providing well diagnosed serum samples of leprosy patients. This work was financially supported by Department of Biotechnology, New Delhi. The first Author (GK) is Senior Research Fellow of UGC-CSIR, New Delhi. Authors also thank Shriyut Malikhan Singh Tomar and MM Alam for their technical help.

References

1. World Health Organization. *Global tuberculosis control; surveillance, planning, financing*. Geneva: WHO; 2009.
2. Lin YM, Reddy TBK, Arend SM, Friggen AH, Franken KLMC, van Meijgaarden KE, *et al*. Cross-reactive immunity to *Mycobacterium tuberculosis* DosR regulon encoded antigens in individuals infected with environmental, non-tuberculous mycobacteria. *Infect Immun* 2009; *77* : 5071-9.
3. Andersen P, Munk ME, Pollock JM, Doherty TM. Specific immune based diagnosis of tuberculosis. *Lancet* 2000; *356* : 1099-104.
4. Fine PE, Sterne JA, Ponnighaus JM, Rees RJ. Delayed type hypersensitivity, mycobacterial vaccines and protective immunity. *Lancet* 1994; *344* : 1245-9.
5. Lyashchenko K, Colangeli R, Houde M, Al Jahdali H, Menzies D, Gennaro ML. Heterogeneous antibody responses in tuberculosis. *Infect Immun* 1998; *66* : 3936-40.
6. Desouza GA, Fortuin S, Aguilar D, Pando RH, McEvoy CR, van Helden PD, *et al*. Using a label-free proteomic method to identify differentially abundant proteins in closely related hypo- and hyper-virulent clinical *Mycobacterium tuberculosis* Beijing isolates. *Mol Cell Proteomics* 2010; *9* : 2414-23.
7. van Embeden JDA, Cave MD, Crawford JT, Dale JW, Eisenach KD, Gicquel B, *et al*. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. *J Clin Microbiol* 1993; *31* : 406-9.
8. Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, *et al*. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *J Clin Microbiol* 1997; *35* : 907-14.
9. Brudey K, Driscoll JR, Rigouts L, Prodinger WM, Gori A, Al-Hajoj SA, *et al*. *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. *BMC Microbiol* 2006; *6* : 23.
10. Viotal I, Driscoll J, Kreiswirth B, Kurepina N, Bennet KP. Identification *Mycobacterium tuberculosis* complex strains families using spoligotypes. *Infect Genet Evol* 2006; *6* : 491-504.
11. Brodie AF, Kalra VK, Lee SH, Cohen AS. Properties of energy transducing system in different types of membrane preparations from *Mycobaterium phlei*; preparation, resolution and reconstitution. *Methods Enzymol* 1979; *55* : 175-99.
12. Bradford MM. A rapid and sensitive method for the quantitative of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* 1976; *72* : 248-54.
13. Kumar G, Shankar H, Bisht D, Sharma P, Singhal N, Katoch VM, *et al*. A simple and rapid method of sample preparation from culture filtrate of *Mycobacterium tuberculosis* for two-dimensional gel elecrophoresis. *Braz J Microbiol* 2010; *41* : 295-9.
14. Revised National Tuberculosis Control Programme (RNTCP) at a Glance. Page 5. Available from: *http://uttarkashi.nic.in/Dept/Health/RNTCP/RNTCP.pdf,* accessed on April 12, 2012.
15. Kumar G, Dagur PK, Singh M, Yadav VS, Dayal R, Singh HB, *et al*. Diagnostic potential of Ag85C in comparison to various secretory antigens for childhood tuberculosis. *Scand J Immunol* 2008; *68* : 177-83.
16. Boyum A. Separation of leukocytes from blood and bone marrow. *Scand J Clin Lab Invest* 1968; *97* (Suppl): 7.
17. Cooper AM, Dalton DK, Stewart TA, Griffin JP, Russel DJ, Orme IM. Disseminated tuberculosis in interferon-γ gene disrupted mice. *J Exp Med* 1993; *178* : 2243-7.
18. Almeida MGB, Chitale S, Boutsikakis I, Geng J, Doo H, He S, *et al*. Induction of *in vitro* human macrophage anti *Mycobacterium tuberculosis* activity: requirement for IFN-γ and primed lymphocytes. *J Immunol* 1998; *160* : 4490-9.
19. Rajavelu P, Das SD. Cell mediated immune response of healthy laboratory volunteers to sconicate antigens prepared from the prevalent strains of *Mycobacterium tuberculosis* from south India harboring a single copy of IS 6110. *Clin Diagn Lab Immunol* 2003; *10* : 1149-52.
20. Rosenkrands I, Slayden, RA, Crawford J, Aagaard C, Barry CE, Andersen P. Hypoxic response of *Mycobacterium tuberculosis* studied by metabolic labeling and proteome analysis of cellular and extracellular proteins. *J Bacteriol* 2002; *184* : 3485-91.
21. Stark J, Kallenius G, Marklund BI, Andersson DI, Akerlund T. Comparative proteome analysis of *Mycobacterium tuberculosis* grown under aerobic and anaerobic conditions. *Microbiology* 2004; *150* : 3821-9.
22. Andersen P, Askgaard D, Ljungqvist L, Bentzon MW, Heron J. T-cell proliferation responses to antigens secreted by

Mycobacterium tuberculosis. *Infect Immun* 1991; *59* : 1558-63.

23. Havlir DV, Wallis RS, Boom WH, Daniel TM, Chevenak K, Ellner JJ. Human immune response to *Mycobacterium tuberculosis* antigens. *Infect Immun* 1991; *59* : 665-70.
24. Siddiqui SM, Orme IM, Saxena RK. Effect of CFP preparation from Indian isolates of *Mycobaterium tuberculosis* to activate T-cells derived from healthy donors. *Int J Tuberc Lung Dis* 2000; *4* : 980-7.
25. Manca C, Tsenova L, Bergtold A, Freeman S, Tovey M, Musser JM, *et al*. Virulence of a *Mycobacterium tuberculosis* clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-α /β. *Proc Natl Acad Sci USA* 2001; *98* : 5752-7.
26. Dormans J, Burger M, Aguilar D, Hernandez-Pando R, Kremer K, Roholl P, *et al*. Correlation of virulence, lung pathology, bacterial load and delayed type hypersensitivity responses after infection with different *Mycobacterium tuberculosis* genotypes in a BALB/c mouse model. *Clin Exp Immunol* 2004; *137* : 460-8.
27. Sharma P, Chauhan DS, Upadhyay P, Faujdar J, Lawania M, Sachan S, *et al*. Molecular typing of *Mycobacterium tuberculosis* isolates from a rural area of Kanpur by Spoligotyping and Mycobacterial interspersed repetitive units (MIRUs) typing. *Infect Genet Evol* 2008; *8* : 621-6.
28. Bhanu NV, van Soolingen D, van Embden JD, Dar L, Pandey RM, Seth P. Predominace of a novel *Mycobacterium tuberculosis* genotype in the Delhi region of India. *Tuberculosis (Edinb)* 2002; *82* : 105-12.
29. Singh UB, Suresh N, Bhanu NV, Arora J, Pant H, Sinha S, *et al*. Predominant tuberculosis spoligotypes, Delhi, India. *Emerg Infect Dis* 2004; *10* : 1138-42.
30. Kulkarni S, Sola C, Filliol I, Rastogi N, Kadival G. Spoligotyping of *Mycobacterium tuberculosis* isolates from patients with pulmonary tuberculosis in Mumbai, India. *Res Microbiol* 2005; *156* : 588-96.
31. Suresh N, Singh UB, Arora J, Pant H, Seth P, Sola C, *et al*. *rpoB* gene sequencing and spoligotyping of multidrug-resistant *Mycobacterium tuberculosis* isolates from India. *Infect Genet Evol* 2006; *6* : 474-83.

Reprint requests: Dr Beenu Joshi, Scientist E, Department of Immunology, National JALMA Institute for Leprosy & Other Mycobacterial Diseases (ICMR), Taj Ganj, Agra 282 001, India
e-mail: bjjalma@gmail.com, beenuj2002@yahoo.co.in