Eur J Clin Microbiol Infect Dis (2002) 21:1–5
DOI 10.1007/s10096-001-0651-7

ARTICLE

V. Choudhry · R.K. Saxena

Detection of *Mycobacterium tuberculosis* Antigens in Urinary Proteins of Tuberculosis Patients

Published online: 20 December 2001

Abstract A sensitive sandwich enzyme-linked immunosorbent assay (ELISA) that can detect up to 0.5 ng of culture filtrate proteins (CFPs) of *Mycobacterium tuberculosis* strain H37Ra is described. This detection system features several special characteristics: (i) the use of CFPs from the H37Ra strain of *Mycobacterium tuberculosis* to generate capture and detection antibodies in rabbits and mice, respectively; (ii) affinity purification of CFP-reactive antibodies and selection of the antibody preparations for best performance in the sandwich ELISA system; and (iii) the use of urine-derived protein preparations for antigen detection. The sandwich ELISA could detect up to 0.5 ng of CFPs of the H37Ra strain of *Mycobacterium tuberculosis*. The assay did not detect antigens of *Escherichia coli*, *Candida albicans* or *Saccharomyces cerivisiae* but efficiently detected CFP preparations from nine different clinical isolates of *Mycobacterium tuberculosis*. Significant variations, however, were noted in the relative efficacy of the assay to detect CFPs from different clinical isolates of *Mycobacterium tuberculosis*. The procedure was utilized for detecting tubercular antigens in urine samples from 29 patients with confirmed (sputum-positive) tuberculosis and from 25 healthy controls. Significant levels of antigen could be detected in 22 of the 29 samples tested.

Introduction

Previous reports from our laboratory [1] as well as from many other laboratories [2] have described immunodiagnostic tests for tuberculosis based upon detection of *Mycobacterium tuberculosis* antigens. Some of these tests, especially those using CSF and sputum samples, have claimed high sensitivity as well as specificity for antigen detection [3, 4, 5]. There have, however, been no reports on detection of *Mycobacterium tuberculosis* antigens in urine samples. One study in which urine samples were used reported a failure to detect mycobacterial antigens in these samples [6]. The mechanism of kidney filtration is unlikely to specifically retain *Mycobacterium tuberculosis*-derived components. Therefore, mycobacterial antigens must be present in urine samples of tuberculosis patients, albeit in extremely low quantities, which may explain the earlier failures to detect these antigens in urine. The present study was aimed at developing a sensitive sandwich enzyme-linked immunosorbent assay (ELISA) system to detect a broad spectrum of *Mycobacterium tuberculosis* antigens and to utilize this system to detect *Mycobacterium tuberculosis* antigens in urine samples. Our strategy included (i) the use of *Mycobacterium tuberculosis* culture filtrate antigens for generating capture as well as detection antibodies; (ii) the use of affinity columns to purify culture filtrate protein (CFP)-reactive antibody preparations and to select those preparations that performed best in the sandwich ELISA system; and (iii) the use of proteins isolated from urine samples for estimation of *Mycobacterium tuberculosis* antigens. In a preliminary screening, our sandwich ELISA detected up to 0.5 ng of H37Ra CFPs; 75% of the urine samples from sputum-positive tuberculosis patients were found to be positive by this test.

V. Choudhry · R.K. Saxena (✉)
School of Life Sciences, Jawaharlal Nehru University,
New Delhi 110067, India
e-mail: rajivksaxena@hotmail.com
e-mail: rks0200@mail.jnu.ac.in
Fax: +91-11-6187338

Materials and Methods

Animals

Outbred Swiss albino mice (8–12 weeks old) and New Zealand white rabbits (6 months–2 years old) were used for raising antisera against CFPs. All the animals were bred and maintained in the animal house facility of the Jawaharlal Nehru University, New Delhi.

Clinical Specimens

Urine samples from tuberculosis patients, diagnosed on the basis of radiological and smear examination but not treated for more than a month, were obtained from Sri Lala Ram Sarup Institute of

Table 1 Immunization schedule of rabbits

Antibody preparation	Rabbit no.	Primary immunization and booster doses (μg/rabbit)[a]	Antibody recovery (mg/ml antiserum)
1	I	(*50*), 12 weekly boosters (20), 9 weekly boosters (50)	0.18
2	I	[b] 22 (100), 23 (100), 24 (100), 25 (100), 26 (100)	0.21
3	II	(*100*), 1 (50), 2 (50), 3 (50), 4 (50)	0.26
4	II	[b] 5 (50), 6 (50), 7 (50), 8 (50)	0.20
5	II	[b] 9 (100), 10 (100), 11 (100), 12 (100), 13 (100)	0.17
6	III	(*100*), 1 (100), 2 (100), 3 (100), 4 (100)	0.12
7	IV	(*100*), 1 (100), 2 (100), 3 (100), 4 (100)	0.21
8	V	(*100*), 1 (100), 2 (100), 3 (100), 4 (100)	0.15

[a] Different rabbits were immunized intradermally and given primary immunization of culture filtrate proteins (CFPs) with incomplete Freund's adjuvant. Secondary immunizations (boosters) of different doses were given in PBS. Values in parentheses represent immunization doses in nanograms of CFPs, and values before parentheses denote the time of booster in weeks after the primary immunization. Figures in italics represent the primary immunization doses. Rabbits were bled by ear vein 1 week after the last booster dose, and specific anti-CFP antibodies were isolated from antisera by using affinity column chromatography, as described in Materials and Methods

[b] Continued immunization to the rabbit in row above

Tuberculosis and Allied Disease and the New Delhi Tuberculosis Center. Control urine samples were taken from healthy, young student volunteers. Urine samples (20–40 ml) were centrifuged at 300 × *g* for 20 min. Each sample was dialyzed overnight against tap water at room temperature using a 3.5 K dialysis membrane (Spectrapore, USA), dialyzed against 0.01 M phosphate buffer (pH 8.5) and loaded on a DEAE cellulose anion exchange column (Sigma, USA). The urinary proteins were eluted with 0.5 M NaCl in phosphate buffer (pH 8.5) and lyophilized. For antigen-detecting assays, lyophilized samples were dissolved in phosphate-buffered saline (PBS) and protein concentrations estimated by Bradford's method [7].

Culture Filtrate Proteins

Methods for culture of *Mycobacterium tuberculosis* strain H37Ra and some contemporary clinical isolates of *Mycobacterium tuberculosis* (JNU 4, JNU 7, JNU 18, JNU19, JNU 20, JNU 22, JNU 32, JNU 33 and JNU51), as well as preparation of CFPs from their culture supernatants, have been described previously [8, 9].

Generation and Affinity Purification of Antibody Reagents

Eight rabbit anti-CFP (H37Ra) antisera were obtained by immunizing and bleeding rabbits as per schedules given in Table 1. For primary as well as secondary immunizations, antigen was administered intradermally at four sites on the back of the rabbits. Swiss albino mice (8–12 weeks old) were primed intraperitoneally with 10–20 μg of CFPs with incomplete Freund's adjuvant per mouse, and four boosters of 5–15 μg of CFPs in PBS per mouse were given intraperitoneally at an interval of 7 days. On day 34, mice were bled through the eye. Blood samples were allowed to clot at 37°C for 1 h, after which clot retraction was allowed at 4°C for 16 h. Serum was collected by centrifugation at 2000 rpm for 20 min at 4°C.

Sepharose CL-4B beads (Pharmacia Fine Chemicals, Sweden) were activated by the cynogen bromide activation method and coupled to CFP preparations as described previously [10]. Protein immobilization ranged from 0.66 to 0.9 mg/ml bed volume. CFP-conjugated beads were packed in a column and stored in 0.01% sodium azide in PBS. For affinity purification of antibodies, antisera diluted twofold with PBS were loaded on the affinity column pre-equilibrated with PBS. After adsorption of antibodies, the column was washed with 10 bed volumes of PBS. Glycine-HCl buffer (0.1 M, pH 2.8) was used to elute the bound antibodies. Purified polyclonal antibody preparations were assayed for protein by Bradford's method and stored in aliquots at –20°C. Recovery of affinity purified-antibodies ranged from 0.12 to 0.26 mg/ml of antisera.

Sandwich Enzyme-Linked Immunosorbent Assay

Affinity-purified capture antibody was coated in ELISA wells at a concentration of 500 ng/well in PBS for 16 h at 4°C. Plates were washed 5 times with 0.02% Tween 80 and blocked with 3% bovine serum albumin (BSA) at 37°C for 2 h. After washing the plates, different concentrations of CFPs (or urinary proteins) suspended in 2% BSA were added, and incubation continued at 37°C for 1 h. Plates were washed and detecting antibody (affinity-purified mouse anti-CFP antibody) added (100 ng/well) in 2% BSA; incubation was continued at 37°C for 1 h. After washing the plates, a secondary antibody rabbit anti-mouse Ig-HRPO (horse reddish peroxidase) preparation suitably diluted in 2% BSA was added. After incubation at 37°C for 1 h, plates were washed and 100 μl of freshly prepared substrate solution of OPD (6 mg of orthophenylene diamine and 10 μl of 30% H_2O_2 added to 12 ml 0.1 M citrate phosphate buffer, pH 5.2) was added. The enzyme reaction was stopped after 15 min by adding 5N H_2SO_4 (50 μl). Absorbance of the wells was monitored by an ELISA reader (7520 Microplate reader; Cambridge Technology, USA). In negative control ELISA assay wells, PBS was used instead of urinary protein samples; the rest of the protocol was identical.

Results

Preparation and Selection of Capture and Detecting Antibodies

Affinity-purified rabbit antibody preparations against CFPs of H37Ra strain of *Mycobacterium tuberculosis* were used as the capture antibody (CAB) to be coated on ELISA wells. An affinity-purified mouse anti-CFP antibody preparation was used as the detecting antibody, followed by a rabbit anti-mouse Ig-HRPO conjugate preparation for generation of color. Anti-CFP antibodies were purified on an affinity column (CFPs immobilized on Sepharose beads). A total of eight affinity-purified CAB preparations were tested in an ELISA system to compare their relative binding affinities for CFPs. Results presented in Fig. 1A indicate that individual CAB preparations varied significantly in their reactivities with H37Ra CFPs coated on wells. If the antibody concentration required for attaining half the maximal

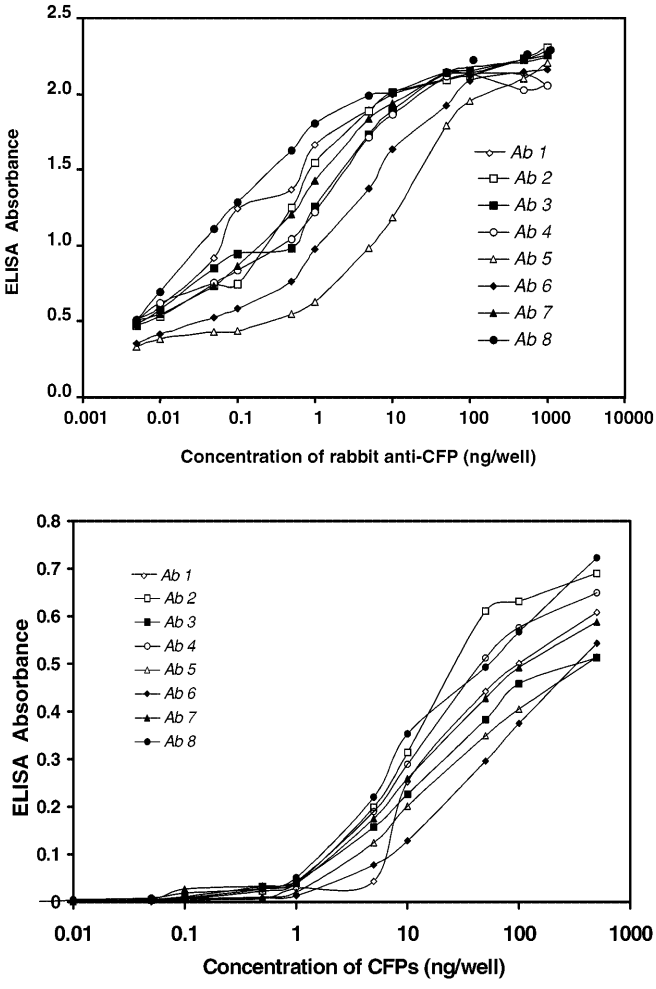

Fig. 1 Upper panel: ELISA reactivity of different preparations of affinity-purified rabbit anti-CFP and mouse anti-CFP antibodies. CFPs of H37Ra were coated in ELISA plates (1 μg/well, 4°C for 16 h), and ELISA reactivities of eight different preparations of rabbit anti-CFP antibodies were examined. Lower panel: Detection of antigen using different preparations of rabbit anti-CFP capture antibody in the sandwich ELISA system. Using standard assay conditions, the performances of different CAB preparations were compared in the sandwich ELISA

ELISA absorbance is compared for different CAB preparations, this value ranged from 0.1 ng (CAB 8) to about 10 ng (CAB 5).

Three batches of 40 Swiss mice each were immunized with CFPs, and pooled sera and anti-CFP antibodies were affinity purified to obtain the detecting antibody preparations. The mean concentration of affinity-purified antibody from pooled mouse antisera was 0.144±0.0058 mg/ml antiserum. No significant differences were observed in the reactivities of these preparations with CFPs (results not shown). Optimal concentrations of different antibody reagents, determined in initial standardization experiments, were found to be 0.5 μg/well for CAB, 0.1 μg/well detecting antibody and 1:80,000 dilution of the peroxidase-conjugated second antibody preparation.

The performances of eight CAB preparations to detect CFPs were compared in the sandwich ELISA. The results, shown in Fig. 1B, indicate that all CAB preparations gave good dose-response curves for detection of CFPs, yet there were significant variations. In sandwich ELISAs for the detection of CFPs, the order of CAB efficacy was CAB 8>CAB 2>CAB 4>CAB 7>CAB 3>CAB 5>CAB 6>CAB 1 (Fig. 1B), whereas in relative binding affinities, the order was CAB 8>CAB 1>CAB 2>CAB 7>CAB 3>CAB 4>CAB 6>CAB 5 (Fig. 1A). CAB 8 performed best by both parameters and was used in all subsequent experiments of antigen detection.

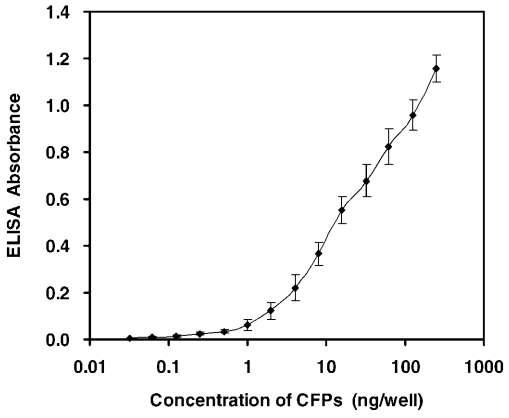

Fig. 2 Reproducibility of the sandwich ELISA. Standardized concentrations of rabbit anti-CFP antibody (500 ng/well) and mouse anti-CFP antibody (100 ng/well) and CFPs from different preparations were used in six independent sandwich ELISA assays. The mean ELISA absorbance curve of all the assays and the SD of ELISA absorbance at each point are shown

Detection Limit of the Sandwich Enzyme-Linked Immunosorbent Assay System

The dose-response curves obtained with sandwich ELISAs performed on different days showed good consistency. Fig. 2 shows an average curve with standard errors for six independent dose-response curves prepared on different days. Using the mean ELISA absorbance in absence of CFPs (0.192±0.0362) and based on the assumption that a positive absorbance for an antigen-containing well should be at least two standard deviations removed from the control values, we estimated the detection limit of the sandwich ELISA to be 0.5 ng/well.

Detection of Antigens in Urinary Samples

Urinary proteins derived from 25 urine samples from healthy control donors and from 29 urine samples from sputum-positive tuberculosis patients were analyzed in the sandwich ELISA for levels of CFPs. In these experiments, urine proteins and standard CFP preparations were tested at different doses. Levels of tubercular antigens in urinary protein preparations were estimated by comparing the ELISA dose-response curves for the stan-

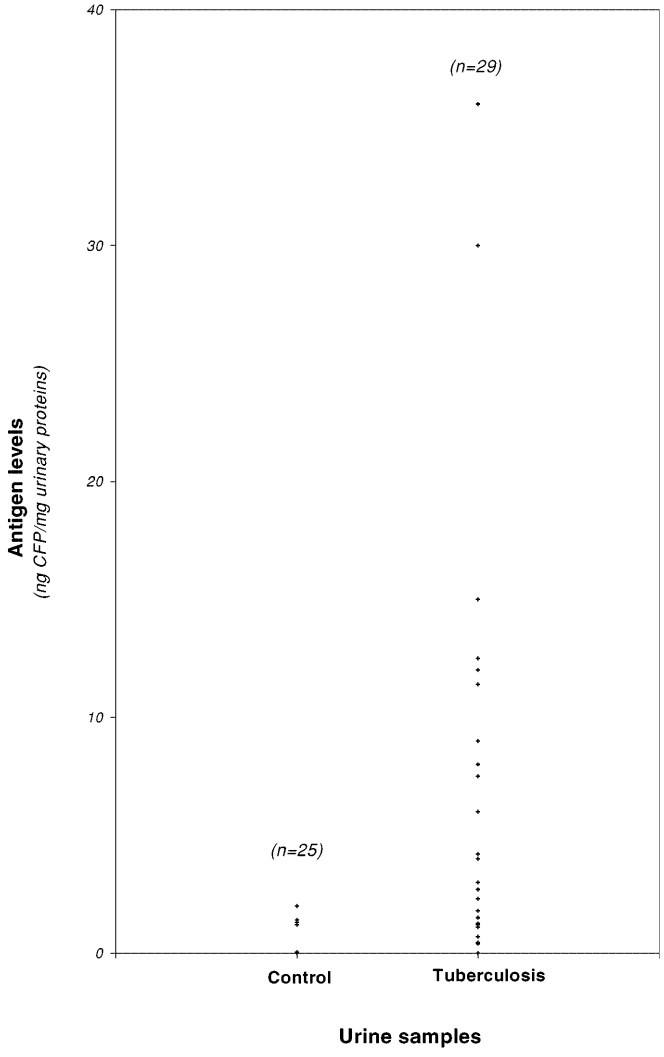

Fig. 3 Detection of CFPs in urine samples of tuberculosis patients. Urinary proteins were isolated from urine samples of tuberculosis patients using DEAE columns (as described in Materials and Methods) and used for antigen detection by sandwich ELISA. Antigen levels in urinary proteins were determined by comparing the ELISA dose-response curves for the urinary proteins and standard H37Ra CFP preparations. The number of points plotted may not be the same as the number of samples shown because of the overlap of points. For control samples, CFP amounts were nondetectable in 20 of the 25 samples and were 2.0, 1.4, 1.3, 1.2 and 0.04 ng/mg urinary proteins in the remaining five samples (group mean 0.24±0.55 ng/mg urinary proteins). For 29 samples from tuberculosis patients, the values were 36, 30, 15, 12.5, 12, 11.4, 9, 8, 7.5, 6, 4.2, 4, 3.8, 3.9, 2.7, 2.5, 2.3, 1.8, 1.5, 4.5, 2.3, 1.5, 1.2, 0.7, 0.4, 1.1, 1, 1.25, 0.0 (group mean 6.49±8.43 ng/mg urinary proteins)

dard CFP preparation and the urinary protein preparations. These results are summarized in Fig. 3. The mean ± 2 SD of CFP levels in control samples was 1.4 ng/mg urinary proteins. In 22 of the 29 (75%) urinary protein samples from tuberculosis patients, CFP levels were greater than 1.4 ng/mg urinary proteins.

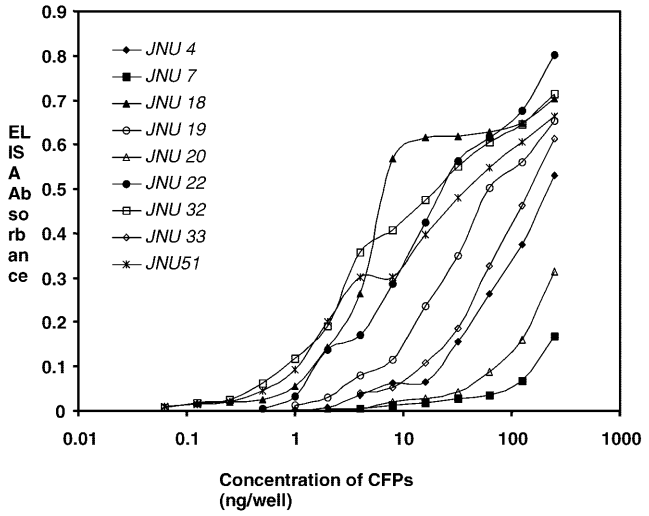

Fig. 4 ELISA dose-response curves for detection of CFP preparations derived from nine different clinical isolates of *Mycobacterium tuberculosis*

Cross-Reactivity of the Sandwich Enzyme-Linked Immunosorbent Assay System

Antigen preparations of *Escherichia coli*, *Candida albicans* and *Saccharomyces cerivisiae* showed no reactivity in the sandwich ELISA system (results not shown). The origin of CFPs in the urine samples of tuberculosis patients would be the *Mycobacterium tuberculosis* strain infecting the patient, and these strains could be different for different patients. It was therefore of interest to evaluate the ability of our sandwich ELISA, developed with antibody reagents prepared using CFPs derived from strain H37Ra, to detect CFPs of different contemporary clinical isolates of *Mycobacterium tuberculosis*. The ELISA dose-response curves for CFP preparations derived from nine contemporary clinical isolates of *Mycobacterium tuberculosis* are given in Fig. 4. Significant variations were found in the reactivities of CFPs derived from nine clinical isolates of *Mycobacterium tuberculosis*. Relative efficacies of detection were best for JNU-18, JNU-22, JNU-32 and JNU-51; intermediate for JNU-7, JNU-19 and JNU-23; and relatively poor for JNU-7 and JNU-20.

Discussion

In general, a sandwich ELISA for detection of antigens may be developed by using monoclonal antibodies that react only with a highly specific antigenic epitope of the infective agent. Such a detection system, while being specific, would nonetheless have poor sensitivity, since a positive test would require a sufficient concentration of that specific epitope in the body fluids of the patient. The use of a polyclonal antibody preparation, on the other hand, may render the test less specific by increasing the chances of cross-reactivity with other infective agents. The detection may be highly sensitive, however, since a large num-

ber of antigens are detected simultaneously. Our sandwich ELISA, developed by using affinity-purified polyclonal antibodies against CFPs of *Mycobacterium tuberculosis*, did not detect antigens of pathogens like *Escherichia coli*, *Saccharomyces cerivisiae* and *Candida albicans*. There was a good deal of cross-reactivity with secretory antigens of various clinical isolates of *Mycobacterium tuberculosis*, which permitted their detection in urine samples of tuberculosis patients. Interestingly, however, significant variations were noted in the detection limits for CFPs from different clinical isolates of *Mycobacterium tuberculosis* (Fig. 4). These results suggest that, if the infecting isolate of *Mycobacterium tuberculosis* secretes CFPs with low reactivity to the antibody reagents utilized for the sandwich ELISA, the system may fail to detect these antigens. Failure of the ELISA system to detect *Mycobacterium tuberculosis* antigens in seven urine samples (Fig. 3) could be related to poor cross-reactivity or the presence of a lower-than-detectable amount of antigen in the urine. If poor cross-reactivity indeed causes some urine samples from confirmed tuberculosis patients to test negative (as did 7 of the total 29 samples in this study), a solution may lie in developing polyclonal antibodies reagents by using a mixture of CFPs from several contemporary clinical isolates of *Mycobacterium tuberculosis*. This proposition, however, remains to be examined.

The use of ELISA or radioimmunoassay for detection of *Mycobacterium tuberculosis* antigens in sputum [2], plural fluid [11], CSF [3, 12] and serum [1] has been reported. Although high values of sensitivity and specificity have been reported for some of these tests [4], we are not aware of any successful attempt to detect antigens of *Mycobacterium tuberculosis* in urine samples. The present study demonstrates that *Mycobacterium tuberculosis* antigens are secreted in urine samples of tuberculosis patients and could be detected by using a sufficiently sensitive sandwich ELISA system. As suggested above, there is room for further improvement in this assay system. In addition, further analysis of a larger number of more diverse clinical samples (sputum, CSF, plural and ascites fluid, etc.) from various categories of tuberculosis patients would be needed to assess the value of the proposed sandwich ELISA for diagnosis of tuberculosis.

Acknowledgments This project was funded by research grants to RKS from the Potts Foundation and the Department of Biotechnology, Government of India.

References

1. Udaykumar, Sarin R, Saxena RK: Analysis of circulating immune complexes (CIC) in tuberculosis: levels of specific antibodies and antigens in CIC and relationship with serum antibody. FEMS Microbiology Immunology (1991) 76:135–142
2. Yanez MA: Determination of mycobacterial antigens in sputum by enzyme immunoassay. Journal of Clinical Microbiology (1986) 23:822–825
3. Araj GF, Fahwami BH, Chugh TD, Abu-Salim M: Improved detection of mycobacterial antigens in clinical specimens by combined enzyme-linked immunosorbent assays. Diagnostic Microbiology and Infectious Disease (1993) 17:119–123
4. Wadee AA, Boting L, Reddy SG: Antigen capture assay for detection of a 43-kilodalton *Mycobacterium tuberculosis* antigen. Journal of Clinical Microbiology (1990) 28:2786–2791
5. Pereir Arias-Bouda LM, Nguyen LN, Ho LM, Kuijper S, Jansen HM, Kolk AH: Development of antigen detection assay for the diagnosis of tuberculosis using sputum samples. Journal of Clinical Microbiology (2000) 38:2278–2283
6. Banchuin M, Wongwajana, Pumprueg U, Jearanaisilavong J: Value of an ELISA for mycobacterial antigen detection as a routine diagnostic test of pulmonary tuberculosis. Asian Pacific Journal of Allergy and Immunology (1990) 8:5–11
7. Bradford MW: A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the property of dye binding. Annals of Biochemistry (1976) 72:248–254
8. Chander A, Yadava R, Ghosh S, Rath PC, Yadava PK, Orm IM, Saxena RK: Strain differentiation among Indian clinical isolates of *Mycobacterium tuberculosis* by RFLP analysis. Molecular Biology Today (2000) 1:53–55
9. Siddiqui SM, Orme IM, Saxena RK: Efficacy of culture filtrate protein preparations from some Indian isolates of *Mycobacterium tuberculosis* to activate T cells derived from healthy donors. International Journal of Tuberculosis and Lung Diseases (2000) 4:1–8
10. Kohn J, Wilchek M: The use of cyanogen bromide and other novel cyanylating agents for the activation of polysaccharide resins. Applied Biochemistry and Biotechnology (1984) 9: 285–304
11. Samuel AM: Evaluation of tubercular antigen and anti-tubercular antibodies in pleural and ascitic effusions. Indian Journal of Medical Research (1984) 80:563–565
12. Radhakrishnan VV, Sehgal S, Mathai A: Correlation between culture of *Mycobacterium tuberculosis* and detection of mycobacterial antigens in CSF of patients with tuberculous meningitis. Journal of Medical Microbiology (1990) 33:223–226