

*Full Length Research Paper*

# Estimation of cord factor antigen of *Mycobacterium tuberculosis* in pleural fluid and its diagnostic significance

Cinta Rose<sup>1</sup>, Neelima R.<sup>1</sup>, Mathai A.<sup>1</sup>, Harikrishnan V. S.<sup>2</sup>, Radhakrishnan V. V.<sup>1\*</sup>

<sup>1</sup>Department of Pathology, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum-695 011, Kerala State, India.

<sup>2</sup>Division of Laboratory Animal Science Biomedical Technology, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum-695 011, Kerala State, India.

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**In this study, a sandwich enzyme linked immunosorbent assay (ELISA) was developed to measure the concentration of cord factor (CF) antigen of *M. tuberculosis* in the pleural fluids of 42 patients with tuberculous pleural effusion (TPE). The assay was also simultaneously performed in 43 pleural fluid specimens with malignant pleural effusion (MPE). The assay detected CF antigen in 83.3% in patients with TPE effusion in whom *M. tuberculosis* was not demonstrated in pleural fluid specimens by bacteriological methods. The data also indicated that cord factor antigen was not present in significant titres in any one of the 43 pleural fluids of patients with MPE. It is concluded that estimation of cord factor of *M. tuberculosis* in pleural fluid specimens by the sandwich ELISA as developed in this study will support the diagnosis of tuberculous aetiology in pleural fluids, particularly in those patients in whom *M. tuberculosis* was not demonstrated by bacteriological methods.**

**Key words:** Pulmonary tuberculosis, tuberculous pleural effusion (TPE), *Mycobacterium tuberculosis*, cord factor (CF) antigen, trehalose 6,6' dimycolate, enzyme-linked-immunosorbent assay (ELISA), malignant pleural effusion (MPE) specificity.

## INTRODUCTION

Pleural effusion is one of the common clinical manifestations in patients with pleural and pulmonary tuberculosis. Pleural effusion usually occurs as a result of release of antigens of *M. tuberculosis* bacilli into the pleural space from a sub-pleural tuberculous lesion (Epstein et al., 1987). During the past decade, sophisticated imaging techniques have been developed as an aid for the diagnosis of TPE. In spite of these, precise aetiological diagnosis of pleural effusion in majority of patients with pleural *tuberculosis* remains a challenge because the imaging features in the thorax of patients with TPE often resemble that of MPE. This poses considerable diagnostic difficulties at bedside diagnosis and management of patients with pleural effusion. Distinction effusion.

between TPE and MPE becomes extremely relevant because management modalities differ vastly in these two groups of patients. A confirmatory diagnosis of TPE depends upon the demonstration of the causative agent of the disease, *M. tuberculosis* bacilli in pleural fluid specimens by a bacteriological method. Owing to the paucibacillary status, *M. tuberculosis* bacilli are seldom demonstrated in pleural fluid specimen by the conventional bacteriological method (Anie et al., 2007). Hence, development of alternative laboratory parameters is not only essential but also becomes extremely relevant. In the earlier published studies, several non-bacteriological diagnostic parameters have been evaluated for the early laboratory diagnosis of TPE. These include (a) histopathology in pleural biopsy (Diacon et al., 2002), (b) nucleic acid amplification assay (Dinnes et al., 2007; Trajman et al., 2007), (c) estimation of biomarkers such as adenosine deaminase (Chen et al., 2004; Trajman et al., 2007), (d) antibody to mycobacterial glycolipids

\*Corresponding author. E-mail: [vvr@sctimst.ac.in](mailto:vvr@sctimst.ac.in). Tel: 91-471-2524594, 91-471-2524508.

(Morimoto et al., 2006), (e) lipoarabinomannan (Yokoyama et al., 2005) and (f) IFN- $\gamma$  (Greco et al., 2003; Jiang et al., 2007). These above diagnostic approaches do hold promise, but the sensitivities and specificities reported in these studies are highly variable and hence restricted the routine application as a diagnostic tool in patients with TPE.

CF antigen (Trehalose 6,6 dimycolate) is one of the major lipid antigens of *M. tuberculosis*, and it plays an important role in the immunopathogenesis of *tuberculosis*. CF potentiates macrophage proliferation (Baba et al., 1997) and induces granuloma in tuberculous lesions (Yamagami et al., 2001). Besides this, several other studies estimated the antibody response to CF in sera of patients with pulmonary *tuberculosis*. These studies showed a sensitivity of 81% and specificity of 96%, and emphasised its diagnostic application in sputum culture negative patients with pulmonary *tuberculosis* (Maekura et al., 1993; Julian et al., 2002; Fujitha et al., 2005). In the present study, we developed a simple ELISA to measure the CF antigen in pleural fluid specimens of patients with TPE. The sensitivity of the ELISA has been evaluated in culture proven patients with TPE, and specificity of the assay was assessed in patients with MPE. Prior to the initiation of the study, a project proposal of this study was duly submitted to the Ethics Committee of this Institute and approval was granted (reference # B-1262009).

## MATERIALS AND METHODS

### Patients and selection criteria

In this study, 85 patients, clinically diagnosed as pleural effusion specimens, were studied over a period of two years (January 2010 to December 2011). These patients were admitted to (a) Hospital for Chest disease, Pulayanarkotta, Trivandrum, Kerala State, India (n = 71), and (b) Sree Chitra Tirunal Institute for Science and Technology Trivandrum, Kerala State, India (n = 14). There were 62 male and 23 female patients, and the age of the patients ranged from 20 to 81 years. Relevant clinical and radiological features in the thorax were recorded from the individual case files of these patients. The most common clinical symptoms were productive cough, breathlessness, chest pain, and pyrexia, for more than four weeks duration. Other constitutional symptom, such as loss of appetite, reduction in body weight and severe malaise were recorded in most of (72/85) these patients. At the time of admission, 76 out of 85 patients showed a positive intradermal tuberculin test (> 12 mm), and elevated erythrocyte sedimentation rates (45 to 120 mm/1st h). The plain skiagrams of the thorax were suggestive of pleural effusion, and the underlying lung lesions in the skiagrams were considerably masked because of the pleural effusion. In the 67 patients, the pleural effusion was unilateral, and 18 patients had bilateral effusion. Radiological features of the thorax alone could not define the precise aetiology of pleural effusion, necessitating further analysis of pleural fluid in all 85 patients. Pleural biopsy could not be undertaken in any one of the patients; (a) consent for the procedure was not given by the patients and secondly, (b) owing to the serious respiratory distress of the patients at the time of admission. However, thoracentesis in these patients was indicated as therapeutic, as well as diagnostic measures. 12 ml of pleural fluid from each patient was collected in 0.5 ml 3.8% sodium citrate, dispensed into four sterile screw-capped glass tubes (3 ml

per tube) and simultaneously subjected to routine cytological, biochemical, and microbiological investigations. For the immunological study, the pleural fluid specimens were coded and stored in sterile aliquots (1 to 2 ml) at -70°C until used in the ELISA.

Routine biochemical, bacteriological and cytological parameters showed; (a) elevated protein 700 to 2300 mg%, and (b) reduced glucose, < 50 mg%. These biochemical features were non-specific and did not distinguish tuberculous from non-tuberculous pleural effusion. The cytospin smears in the majority of pleural fluid specimens showed a predominant population of lymphocytes. 43 out of these 85 cytospin smears of pleural fluid specimens showed the presence of malignant cells. The pleural fluid specimens did not demonstrate acid-fast bacilli in Ziehl-Neelsen stained smears in any of the pleural fluid specimens. However, *M. tuberculosis* was isolated by conventional Lowenstein-Jensen medium in 4 out of 85 pleural fluid specimens.

### Classification criteria

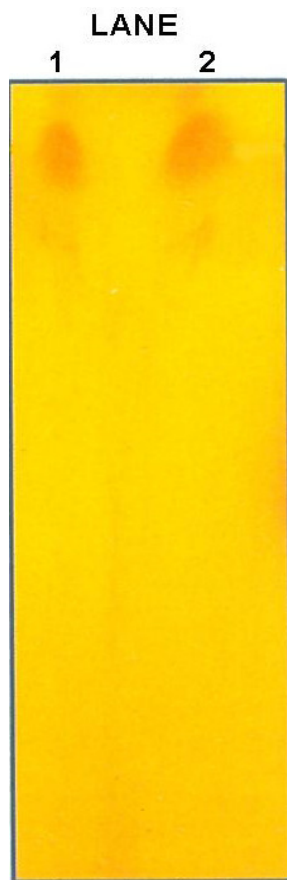
Based on the cytological and bacteriological investigations, the patients were classified into three groups: (A) MPE: Malignant cells were demonstrated in the cytospin smears of pleural fluids. *M. tuberculosis* or any other microbial agents were demonstrated by the bacteriological methods, (B) 'confirmed' cases of TPE (n=4) in whom *M. tuberculosis* bacilli were demonstrated by bacteriological culture, (C) the bacteriological investigations in 38/42 pleural fluid specimens did not yield *M. tuberculosis* or any other pyogenic bacteria or fungi. However, clinical features were suggestive of tuberculosis and hence, these patients were given the benefit of anti-*tuberculosis* therapy (ATT) for four weeks during their hospital stay. Most of the patients showed optimal clinical recovery, as well as resolution of pleural effusion following ATT, and hence they were classified as 'probable' case of TPE. The pleural fluid specimen from all the three group of patients were coded and stored in sterile aliquots (1 ml) at -70°C

### Isolation of cord factor antigen from *M. tuberculosis*

*M. tuberculosis* H<sub>37</sub>R<sub>v</sub> strain was grown in Sauton's medium for 6 to 8 weeks. At optimum growth, the cultures autoclaved, and bacillary sediments in pellet form were recovered by centrifugation. The autoclaved pellets were weighed and sonicated in chloroform/methanol (4:1, vol/vol) for 15 min at +4°C. Deionized distilled water was added (1:20, total volume), and the aqueous phase was sequentially extracted with chloroform/methanol (3:1 and 2:1, vol/vol), centrifuged at 5000 g for 30 min, and the deposit was allowed to dry at room temperature, and was treated with acetone. The insoluble phase containing CF was separated by repeated centrifugation. The CF was precipitated by 'drop-wise' addition of methanol at +4°C and then dissolved in tetrahydrofuran and reprecipitated by 'drop-wise' addition of methanol at 4°C to a final ratio of 1: 2 tetrahydrofuran/methanol (vol/vol). The precipitated CF was then dissolved in chloroform/acetone (8:2, vol/vol) and then loaded onto a column of silica gel, and CF was eluted with chloroform/methanol (9:1, vol/vol). The elute containing CF was weighed, and the purity was assessed by thin layer chromatography (TLC) using 10 × 10 cm silica plates, in comparison with reference cord factor obtained from Colorado State University, USA (Figure 1). TDM at concentrations of 1000 pg/ml were dispensed in *n*-hexane and stored in sterile aliquots at +4°C.

### Antibody to cord factor

Two adult male albino rabbits were immunised with 300 µg of CF incubated at 37°C for 2 h. Following that, the plates were washed antigen mixed thoroughly in 1 ml of 0.1 M phosphate buffered



**Figure 1.** Thin layer chromatogram showing cord factor antigen. Lane 1: Cord factor isolated from *M. tuberculosis* H<sub>37</sub>R<sub>v</sub> strain in the laboratory, lane 2: reference cord factor antigen supplied by the Colorado State University, USA.

saline (PBS) containing 0.2% Tween-80 and 1 ml of incomplete Freund's adjuvant (Sigma Chemicals, St. Louis, USA). The immunisation was repeated on 14th, 21st, 27th days, following primary immunisation. The rabbits were bled on 35th day, and an indirect ELISA measured the antibody titre in immune sera. An end-point antibody titre at 1:2400 dilution was obtained following the 4th immunisation. 10 ml of venous blood collected from each rabbit and the sera were separated, dispensed in 1 ml sterile aliquots and stored at -70°C until use in ELISA.

#### ELISA for the estimation CF antigen in pleural fluid

Prior to assay in pleural fluid specimen, an ELISA was standardized with decreasing concentration of CF antigen (1000 to 1 pg/100 µl *n*-hexane). Briefly, each well in the microtitre ELISA plates (Nunc, Roskilde, Denmark) were coated with 100 µl of CF antigen and thoroughly with 0.1 M phosphate buffered saline in 0.05% Tween 20 (PBS-T) and quenched with 1% bovine serum albumin in 0.15 M PBS for 1 h. 100 µl of rabbit antibody to CF (1:20000 dilution) was added to each well and incubated for 2 h. The wells were washed, and 100 µl of (1:50000 dilution) anti-rabbit IgG biotin conjugate (Sigma) was added and incubated for 2 h. Subsequently, the plates were washed and 100 µl (1:1000 dilution) of avidin peroxidase (Sigma) was added and incubated for 1 h. The plates were then

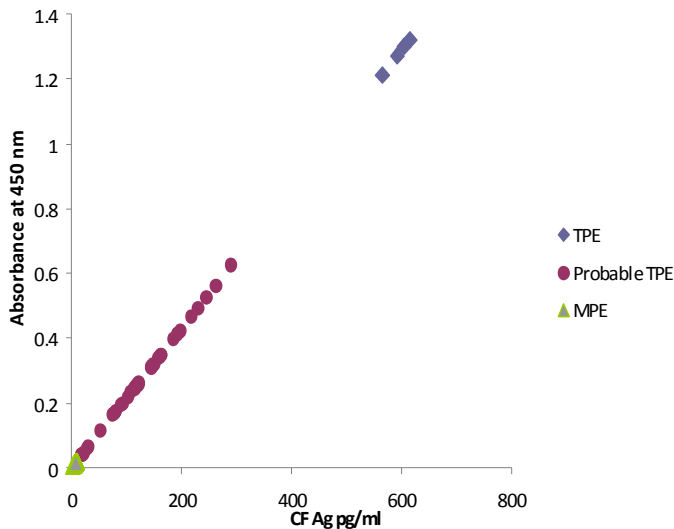
washed five times with PBS-T, and 100 µl of substrate containing o-phenylenediamine dihydrochloride added and incubated for 30 min. A color reaction was developed, and it was stopped at the end of 30 min by adding 100 µl of 2 M HCl. The blank well in the ELISA plate contained all the above reagents minus CF antigen. The absorbances in all the wells in the microtiter plates were measured at 450 nm with an ELISA reader (Bio-Rad, model 550, Bio-Rad Laboratories, Inc, Hercules, California) Absorbances at each CF antigen concentration (1000 to 1 pg/ml) and the blank wells in the ELISA were measured by the ELISA reader. The subtracted value in the absorbance in antigen and blank wells were plotted in a semi-log paper and a linear graph was obtained.

The data indicated that the standardization assay could detect the presence of CF antigen as low as 1 pg/ml. Prior to the estimation of CF antigen in pleural fluid of TPE and MPE, each pleural fluid specimen was individually treated with methanol. 200 µl of pleural fluid specimen was mixed with 500 µl of methanol and centrifuged at 3000 × g for 20 min. The deposit was discarded, and supernatant was allowed to dry at room temperature, to which 200 µl of *n*-hexane was added. From this, 100 µl was added to each well in the microtiter ELISA plates. The ELISA was performed using the identical technical protocol as described in the standardization assay. The assay was performed in batches of 5 pleural fluid specimens at a time. Positive control included in the assay was CF antigen standard 100 pg/ml, and the negative control was a blank solution containing 100 µl anti- CF antibody, enzyme conjugates in PBS-T. The CF antigen concentration in pleural fluids of patients with TPE and MPE was directly measured from the standard graph. In order to evaluate the reproducibility of the assay, as well as to assess the inter-observer variation, the assay on each pleural fluid specimen was repeated on two different occasions by two different investigators (CR and MA).

## RESULTS

Figure 2 and Table 1 represents the CF antigen concentrations in the pleural fluid specimens of patients with TPE and MPE. 43 pleural fluids from patients with MPE had CF antigen concentrations which ranged between 2 to 4.3 pg/ml (mean  $4.3 \pm 0.03$ ). In 38 patients with 'probable' TPE, CF antigen concentration in pleural fluids ranged between 4 to 325 pg/ml (mean  $120.5 \pm 16.5$  pg/ml). A test was chosen as positive in ELISA, when the CF antigen concentration was more 4.4 pg/ml (mean value in MPE with 3 SD). According to this criterion, pleural fluids in 32 out of 38 patients in 'probable TPE' gave positive results. In 6 out of 38 patients with 'probable' TPE, the CF antigen concentration was less than 4.4 pg/ml and hence was not suggestive of tuberculous aetiology. The pleural fluid specimen in these 6 patients was subjected to Polymerase chain reaction (PCR) for tuberculous aetiology and was also reported to be negative. In these six, pleural fluids cultures were reported to be negative for pyogenic bacteria and fungi. Despite extensive investigations, precise aetiology of pleural effusion in these six patients could not be established.

The CF antigen concentration in the pleural fluid specimen from 4 culture positive patients with TPE ranged between 525 to 672 pg/ml (mean  $592.5 \pm 12.5$  pg/ml). These results also revealed that CF antigen concentration in pleural fluid specimen in culture proven patients with TPE



**Figure 2.** Showing the CF antigen concentrations in pleural fluid of TPE and MPE.

**Table 1.** Data showing the CF antigen (pg/ml) in pleural fluid specimens.

Parameter	TPE (n = 42)		MPE (n = 43)
	Confirmed (n = 4)	Probable (n = 38)	
Range	525-672	03-325	0 2-4.3
Mean	592±12.5	120.5±16.5	4.3±0.03
Sensitivity (%)	100	83.33	-
Specificity (%)	-	-	100

is statistically higher ( $P > 0.05$ ) than in patients with 'probable' TPE. This could be attributed to the higher bacillary load in pleural fluid in culture positive patients with TPE. The ELISA in all the pleural fluid specimens in all the three groups was repeated on two occasions, and there were no gross variation in CF concentration. The inter-observation variation (correlation coefficient  $r < 0.6$ ) was not statistically significant. Hence, it was established that the assay was reproducible.

## DISCUSSION

Tuberculous pleural effusion is a potentially curable infectious disease, provided an accurate laboratory diagnosis can be made, and an effective ATT can be instituted during the active stages of the disease. Untreated and undiagnosed patients with TPE may develop severe morbidity in the form of respiratory dysfunction. Hence, accurate laboratory diagnosis during active stages of pleural *tuberculosis* will form the "corner stone" in the management of TPE. Owing to the paucibacillary

status, *M. tuberculosis* bacilli are seldom demonstrated by bacteriological investigations. Hence, we developed a simple reproducible ELISA to measure the CF antigen concentration in the pleural fluid specimen of patients with TPE. Based on the diagnostic criteria used in the assay, the data indicated that CF antigen was not present in significant amounts in any one of the pleural fluid specimens of patients with MPE. Hence, ELISA developed in this study is highly specific for tuberculous etiology. One of the reasons for the high specificity is that CF antigen is present only in *M. tuberculosis* bacilli and not in any other bacteria or malignant cells. Secondly, the non-specific and cross-reacting protein antigens of *M. tuberculosis* and microbial agents in the pleural fluid are removed by treating the pleural fluid samples with methanol prior to assay.

Earlier published studies highlighted the application of several indirect assays as useful methods for the diagnosis of pulmonary *tuberculosis*. Among these, adenosine deaminase (ADA) and IFN- $\gamma$  estimations have been shown to possess the best specificity and sensitivity of 93% for ADA and 96% for IFN- $\gamma$  (Greco et al., 2003). However, it needs to be emphasized that estimation of ADA and IFN- $\gamma$  would only indicate the host inflammatory process in the pleural space and does not confirm the specific aetiological agent. Pleural biopsy is also reported to be a useful approach in the diagnosis of TPE (Diacon et al., 2002). However, positive diagnosis of TPE would depend upon the availability of representative of the biopsy specimen from the pleural lesion. At times, the histopathology of the pleural biopsy could be inconclusive or may yield false negative results. Moreover, pleural biopsy procedures may be difficult to be undertaken, particularly in those patients admitted with extensive respiratory distress. By contrast, estimation of CF antigen in pleural fluid specimens would support tuberculous aetiology, and hence holds promise for the laboratory diagnosis of pleural *tuberculosis*, particularly in those patients in whom the bacteriological methods were not helpful in making the diagnosis. In our earlier study (Anie et al., 2007), we estimated glycolipid antigen (TBGL) of *M. tuberculosis* in the pleural fluid specimens. Comparison of the data clearly revealed that CF antigen is more stable and specific than TBGL antigen. The assay yielded positive results in all the four culture positive patients with TPE, and hence this assay is sensitive. However, in order to strengthen the validity of the assay in terms of sensitivity and specificity, it will be essential to undertake the assay in larger number of pleural fluid specimens from culture positive patients with TPE, as well as in larger number pleural fluid specimens from patients with non-tuberculous infectious pulmonary diseases. The technical protocol used in the assay is user-friendly and therefore best suited to laboratories where there are in laboratory resources and expertise. Besides its high specificity, the assay is also reproducible. Storage of pleural fluid specimen at  $-70^{\circ}\text{C}$  for two years did not alter

the results. The results of this study also suggest that a patient with a positive ELISA has definite diagnostic value, and such patient may be given the benefit of ATT. However, prior to the assay standardization of the ELISA with CF antigen, anti-CF antibody is mandatory, as this will lend support to the validity of the ELISA. In conclusion, we believe that estimation of CF antigen in pleural fluid specimens as developed in this study has definite potential in the routine laboratory diagnosis, as well as bed-side management of patients with TPE.

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## REFERENCES

- Anie Y, Sumi S, Vargheese P, Madhavi LG, Sathish M, Radhakrishnan VV (2007). Diagnostic approaches in patients with tuberculous pleural effusion. *Diag. Microbiol. Infect. Dis.* 59:389-394.
- Baba T, Natsuhara Y, Kaneda K, Yano I (1997). Granuloma formation activity and mycolic acid composition of mycobacterial cord factor. *Cell Mol. Life Sci.* 53:227-232.
- Chen ML, Yu WC, Lam CW, Au KM, Kong FY, Chan AYW (2004). Diagnostic value of pleural fluid adenosine deaminase activity in tuberculous pleurisy. *Clin. Chimica Acta.* 341:101-107.
- Diacon AH, Van de Wal BW, Wyser C, Smedema JP, Bezuidenhout J, Bolliger CT, Walazf G (2002). Diagnostic tools in tuberculous pleurisy: a direct comparative study. *Eur. Respir.* 22:589-591.
- Dinnes J, Deeks J, Kunst H, Gibson A, Cummins E, Waugh N, Drobniewski F, Lalvani A (2007). A systematic review of rapid diagnostic tests for the detection of *tuberculous* infection. *Health Technol. Assess.* 11:1-196.
- Epstein DM, Kline LR, Albelda SM, Miller WT (1987). Tuberculous pleural effusions. *Chest* 91:106-109.
- Fujitha Y, Doi T, Sato K, Yano I (2005). Diverse humoral immune responses and changes in IgG antibody levels against mycobacterial lipid antigens in active *tuberculosis*. *Microbiol.* 151:2065-2074.
- Greco S, Girardi E, Masciangelo R, Capocchetta GB, Saltini C (2003). Adenosine deaminase and interferon gamma measurements for the diagnosis of tuberculous pleurisy: a meta-analysis. *Int. J. Tuberc. Lung Dis.* 7:777-786.
- Jiang J, Shi HZ, Liang QL, Qin SM, Qin XJ (2007). Diagnostic value of interferon-gamma in tuberculous pleurisy: a meta-analysis. *Chest* 131:1133-41.
- Julian E, Matas L, Perez A, Alcaide J, Laneelle MA, Luquin M (2002). Serodiagnosis of *tuberculosis*: comparison of immunoglobulin A (IgA) response to sulfolipid I with IgG and IgM responses to 2, 3 diacyltrehalose, 2, 3, 6-triacyltrehalose and cord factor antigens. *J. Clin. Microbiol.* 40(10):3782-3788.
- Maekura R, Nakagawa M, Nakamura Y, Hiraga T, Yamamura Y, Ito M (1993). Clinical evaluation of rapid serodiagnosis of pulmonary *tuberculosis* by ELISA with cord factor (trehalose-6, 6'-dimycolate) as antigen purified from *Mycobacterium tuberculosis*. *Am. Rev. Respir. Dis.* 148:997-1001.
- Morimoto T, Takanashi S, Hasegawa Y, Fujimoto K, Okudara K, Hayashi A (2006). Level of antibodies against mycobacterial glycolipid in the effusion for the diagnosis of tuberculous pleural effusion. *Respir. Med.* 100:17750-1780
- Trajman A, Kaisermann C, Luiz RR, Spherhacck RD, Rossetti ML, Feres Saad MH (2007). Pleural fluid ADA, IgA-ELISA and PCR sensitivities for the diagnosis of pleural *tuberculosis*. *Scand J, Clin, Lab, Invest.* 67:877-884.
- Yamagami H, Matsumoto T, Fujiwara N, Arakawa T, Kaneda K, Yano I, Kobayashi K (2001). Trehalose 6, 6'-Dimycolate (cord factor) of *Mycobacterium tuberculosis* induces Foreign - Body and Hypersensitivity -type granulomas in mice. *Infect. Immunol.* 69:810-815.
- Yokoyama T, Rikimaru T, Kinoshita T, Kamimura T, Oshita Y, Aizawa H (2005). Clinical utility of lipoarabinomannan antibody in pleural fluid for the diagnosis of tuberculous pleurisy. *Infect Cheomother.* 11:81-83.