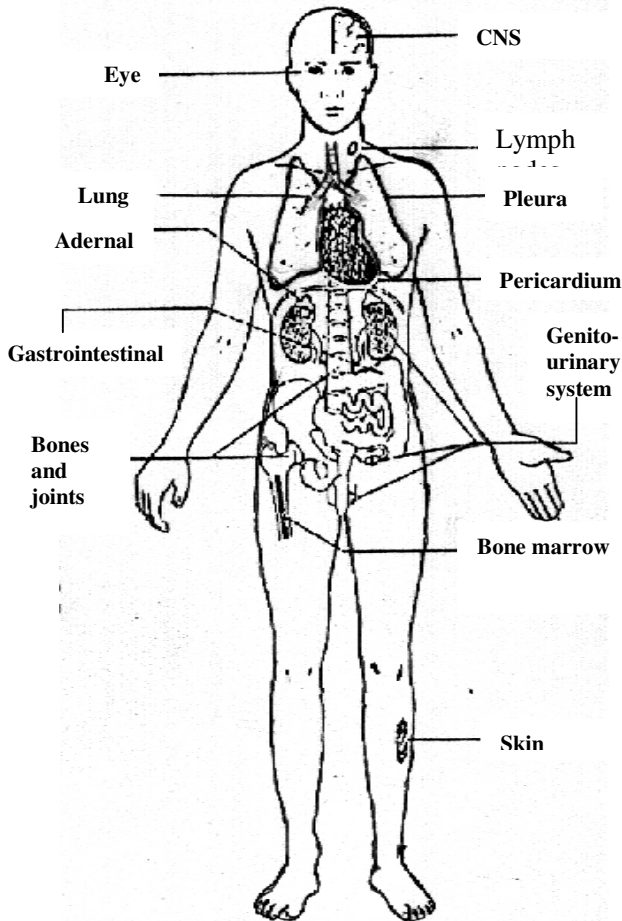


SEVA TB ELISA (IgG, Ag & IC-Ag) For Immunoscreening of Pulmonary & Extrapulmonary Tuberculosis and TB with HIV Coinfection



**Pulmonary & Extrapulmonary Sites
affected By Tuberculosis**

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TUBERCULOSIS

Historical Background:

Tuberculosis (TB) is a disease of great antiquity affecting mankind from very early times. Evidence of existence of tuberculosis has been found in the bones of Prehistoric man, found in Germany. Those remains date back to about 8000 B.C. Typical tuberculosis changes have been found in the spines of skeleton of ancient Egyptians dating from about 2500 to 1000 B.C. In the year of 1882, Robert Koch discovered the tubercle bacilli, the causative organism.

Present Scenario:

Tuberculosis (TB) is re-emerging as a deadly disease affecting both developing and developed countries. By the end of 2007, 202 of 212 countries and territories in the whole world had reported case notifications for 2006 and/or treatment outcomes for patients registered in 2005. These countries include 99.6% of the world's population.

Based on surveillance and survey data, WHO (2008) estimated that 9.2 million new cases of TB occurred in 2006, including 4.1 million new smear-positive cases. India, China, Indonesia, South Africa and Nigeria rank first to fifth in terms of incident cases. Among these new cases around 7.7% were HIV-positive.

HIV-TB Coinfection:

The HIV pandemic presents a massive challenge to global TB control. Globally, there were an estimated 709,000 new HIV-positive TB cases in 2006 (WHO-2008). TB is by far the most serious and prevalent opportunistic pulmonary infection among those infected with HIV. Among the 9.2 million new cases of TB in 2006, around 709 000 (7.7%) were HIV-positive. The African Region accounts for most HIV-positive cases: 85% in 2006. Out of the remaining cases, 6% are in the South-East Asia Region, mainly in India.

Global Emergency:

Although tuberculosis is a curable and to some extent preventable disease, its diagnosis sometimes, especially in Extrapulmonary tuberculosis, HIV-TB, Childhood TB, Smear Negative Pulmonary TB becomes difficult. The WHO declared tuberculosis as a Global Emergency in 1993. The diagnosis of TB is more difficult in patients with HIV due to less sensitivity of smear examination, less specific radiological findings, negativity of skin test, time consuming culture test. Patients with HIV more frequently have pulmonary infections, and such cases may be clinically suspected of TB, leading to over-diagnosis of TB. Hence prompt and accurate diagnosis of HIV-TB coinfection using serological test is required.

Clinical Presentation of Tuberculosis:

The incidence of pulmonary tuberculosis is more frequent than extrapulmonary form of tuberculosis (EPTB). EPTB is increasingly recognized since last decade because of the emergence of AIDS. Tuberculosis is an air borne disease, being transmitted via respiratory route on inhalation or ingestion of droplet nuclei containing varying quantities of *M tuberculosis* bacilli. The man with active disease when coughs, sneezes or spits, aerosolizes droplet nuclei. The risk of infection progressing to disease varies with age, the risk being greatest in children below 3 years, followed by young adult and the elderly people.

Pulmonary tuberculosis:

This is the most common form and affects the lung. The onset is usually insidious and illness remains unnoticed by the patients for some time. It reaches full extent within few weeks. The extent of the disease varies from minimal infiltrates that produce no clinical illness to massive involvement with extensive cavitation and debilitating constitutional and respiratory symptoms. In the absence of effective therapy, this pursues a

chronic and progressive course with growing bacterial colony. With the progression of pulmonary disease the normal pulmonary architecture is lost. The overall death rate of untreated pulmonary tuberculosis approaches 60%.

Extra pulmonary tuberculosis:

This occurs following haematogenous and lymphatic dissemination of tubercle bacilli from primary pulmonary infection or a reactivated focus elsewhere in the body i.e. infected lymph node etc. Number of tissues or different organs may be infected such as :

- **Tubercular lymphadenitis** involve the unilateral cervical nodes especially those high in the neck are more frequent in children. This form of tuberculosis is the most common form of extrapulmonary tuberculosis constituting towards 30% of the total disease. As the disease progresses, sinus tracts are resulted. These may slowly respond to medication, rarely may require excision.
- **Bone & Joint tuberculosis** is more common in elderly, although seen in all ages. The involvement is usually a late manifestation of haematogenous spread of this disease. The site most frequently involved is the vertebral body representing 36-50% of the total bone and joint tuberculosis cases. Lumbar and low dorsal spines are commonly involved in older; high dorsal in young. The weight bearing bones / joints (knee, 12-15%) are also involved. However any bone or joint can be involved. The course of disease starts from synovial membrane, then inflammation followed by demineralization and caseous necrosis.
- **Abdominal tuberculosis** includes i) abdominal organ (GIT, liver, spleen etc.) ii) peritoneum iii) lymphatics. The intestinal tuberculosis can be related to swallowing sputum tubercle bacilli or the disease reactivation in peri intestinal

lymphatic tissue. The ileocecal area of small intestine is the most common site (91%) involved. Peritoneal TB presents with seeding of tubercles through out the peritoneal surface and occurs in ascitic (exudative) or plastic (adhesive or dry) forms. This form of TB is easily confused with other diseases such as irritable bowel syndrome, alcoholic cirrhosis etc. Usually the signs and symptoms are vague and non specific, increases with age and patient presents with abdominal swelling, vague pain and alternate diarrhoea and constipation.

- **Meninges-CNS tuberculosis** is most common amongst infants, children with extrapulmonary tuberculosis. The patient presents in different stages: early fever, headache, and malaise; later confusion, seizures and coma. The prognosis is related to stage.

- **Genitourinary tuberculosis** is rare in young, more frequent among females. This may involve kidneys, ureters, bladder, testes, epididymis, uterus, fallopian tubes etc. This may complicate to early or late obstructive uropathy, infertility etc.

Pleurisy with effusion occurs when the pleural space is seeded with the *M tb.* bacilli. This may be acute or indolent; severe or asymptomatic.

- **Tuberculous pericarditis** represents as extension of pleurisy. This causes, dyspnoea and vague discomfort. Exudative effusion occurs and patients present with fever and pericardial pain, chronic obstructive pericarditis being its last sequel.

- **Miliary Tuberculosis** is a disseminated form of TB where infection spreads at multiple sites. X-ray shows miliary modular pattern and biopsy shows presence of cavitation. Its name comes from a distinctive pattern seen on a chest X-ray of many tiny spots distributed throughout the lungs with the appearance similar to millet seeds, may infect any number of organs like lungs, liver, spleen etc.

DIAGNOSTICS

Early diagnosis of tuberculosis is important both to individuals and to community, and is very crucial yet difficult to achieve. Presently the diagnosis of tuberculosis largely depends upon clinical, radiological, cytological & bacteriological examinations. Though the direct microscopy of sputum for acid fast bacilli (AFB) is reliable for pulmonary tuberculosis, it is not that sensitive, limitations being, it may give false negative results and require a high degree of bacillary load of 50000 bacilli / ml of sputum and is subject to inherent errors like contamination. Other demerit is that it is positive in open cases only. Thus it is not helpful in extrapulmonary form of tuberculosis and in childhood tuberculosis where sputum is not available. The culture method is cumbersome and time taking, requires 6-8 weeks for positive results. The radiological examination is non specific and not suitable for field studies in developing countries, like India. The tuberculin skin test is not reliable in discriminating active form from non active tuberculosis. Even the new techniques such as PCR, DNA Probes, RFLP, BACTEC system, etc. for early diagnosis of tuberculosis are no doubt are sensitive and help rapid diagnosis, but still do not find their way into routine diagnosis and more over not cost effective and practical in developing countries. Thus, there is a search for alternative test which will stand with its merits in vigorous clinical trails. Serodiagnostics have attracted considerable attention of the Investigators.

Immunodiagnosis:

SEVA TB ELISA (IgG & Ag) system based on the detection of tubercular IgG antibodies and antigen in tuberculosis has been developed and explored in several ways at this institute. The detection of IgG antibody (titre 1:600 and above) by indirect ELISA against SEVA TB Cocktail antigen (ES-31, 43 & EST-6 Ag) in pulmonary and extra pulmonary

tuberculosis suggests active tuberculosis infection. Free tubercular or immune complexed antigen (IC-AG) is detected using affinity-purified anti Cocktail antibody by sandwich ELISA. A serum with an antigen titre of 1:300 and above is considered as positive reaction. Combinatorial use of antibodies showed improved sensitivity and was thus observed to be better than single antibody. The specificity was observed to be 99% for immune-complexed antigen using cocktail antibody. Out of 68 smear-positive TB cases studied, using cocktail antibody, a sensitivity of 97% (66/68) for immune-complexed cocktail antigen and 91% (62/68) for free cocktail-antigen detection was observed, compared to 91% (62/68) for immune-compleied ES-31 and 79% (54/68) for free ES-31 antigen when anti-ES-31 antibody was used alone.

The test is quite helpful in childhood tuberculosis where it is difficult to obtain sputum sample. This test has been found useful in clinically suspected and anti tuberculosis therapy (ATT) responded cases, which were negative for bacterial examination. This test has also been useful in confirming tubercular etiology in extra-pulmonary tuberculosis (bone & joint, abdominal, CNS-meninges, lymph node, genito-urinary etc.). Antigen assay was found to be useful in detection of TB with HIV coinfection.

Antibody detection by indirect penicillinase ELISA and Antigen detection by Sandwich ELISA in Pulmonary and Extrapulmonary Tuberculosis:

The antibody detection test is based on the detection of specific IgG antibody to purified culture filtrate antigen of *Mycobacterium tuberculosis* H₃₇Ra (SEVA TB Cocktail Ag) by indirect penicillinase ELISA. The Cocktail antigen coated immunomodules (Nunc) are incubated with diluted human sera followed by addition of anti human IgG penicillinase conjugate (1:1000). After washing, the enzyme reaction is

detected using starch-iodine-penicillin 'v' substrate. The disappearance of blue color of substrate indicates the presence of antibody to cocktail antigen. 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. (Gupta et al. Curr. Sci. June 2005; 88(11):1825-1827).

The free and IC-Ag detection is done by sandwich ELISA. The immunomolecules sensitized with affinity purified goat antibody against *M.tb* cocktail antigen are incubated with appropriate test sera followed by addition of penicillinase labeled anti cocktail antibody. The positive reaction is detected by disappearance of blue color of the substrate. Free antigen and IC-Ag detection assays have given sensitivity of 91% & 97% respectively in sputum positive PTB cases. This test detected fresh cases (100%), chronic cases (Free ag-80%, IC-Ag-96%) and relapse cases (96%) (Harinath BC, Diag Microbio Inf Dis 2006; 55:65-68).

Bacteriologically confirmed EPTB patients (n=32) were analysed by antibody and antigen assay. Out of 32 bacteriologically confirmed EPTB sera screened 27 (84%) cases showed presence of antibody, 15 (47%) for presence of free antigen and 22 (69%) for presence of IC antigen. A sensitivity of 100% and specificity of 90% was observed for antibody / free antigen / IC-antigen assay, showing usefulness of the assay system. (Upadhye et al. Biomed Res 2007;18(3):161-166).

HIV-TB Coinfection:

ES-31 antigen is one of the components of cocktail antigen showed usefulness in detection of TB with HIV coinfection. Studies on detection of antigen and antibody showed presence of antibody to ES-31 antigen in 46%, antigen in 62% and IC-antigen in 54% of cases of HIV-TB coinfecting cases. However on

combining the positivity of tuberculosis antibody, circulating free and circulating immune complexed antigen (Ab/Ag/CIC-Ag), a sensitivity of 97% was obtained in HIV –TB coinfection. (Shende et al Int J Tuberc Lung Dis, 2005; 9(8):915-919).

The microtitre plate peroxidase assay has been standardized. Peroxidase enzyme immuno assay showed a sensitivity of detection of 0.5 µg/ ml and levels of free and IC ES-31 antigens were 0.71 ± 0.64 and 0.82 ± 0.40 µg/ ml in AFB positive patients' sera.

The assay was evaluated in 27 clinical sera of sputum acid fast bacilli (AFB) positive and 10 AFB negative but anti-tuberculosis therapy responded pulmonary tuberculosis patients in a tertiary hospital and 20 normal sera as control. The assay for circulating free ES-31 antigen in this preliminary study showed a sensitivity of 74% for AFB positive cases and 70% for AFB negative cases with 90% specificity. The assay for IC-ES-31 antigen showed sensitivity of 77% for AFB positive and 70% for AFB negative cases with 90% specificity. (Majumdar et al. Biomed Res Sept-Dec. 2008; 19(3)).

These ELISA systems have been standardized using filter paper blood samples and results showed similar sensitivity & specificity at 1:50 dilution.

Immunomonitoring:

A follow up study of immune status during the course of ATT of tuberculosis patients showed an initial rise of tubercular antibodies in the first month of treatment followed by gradual decrease in the titers by the end of treatment. Circulating tubercular antigen levels showed gradual decrease during treatment. At the end of six months of ATT, about 75% showed the absence of circulating tubercular antigen. Thus presence of antigen may be used as a marker for elimination of tuberculosis infection as well as compliance of the patients with ATT. (Gupta et al J. MGIMS, 2004 Sept.; 9(ii): 16-21).

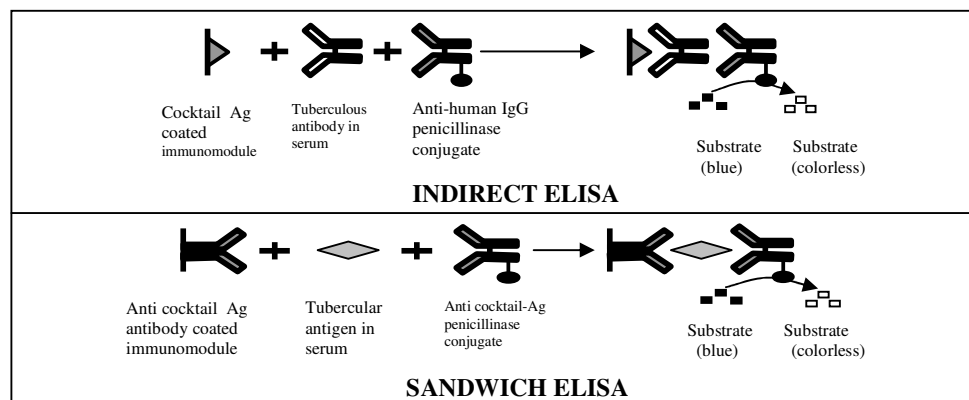


Table1: Detection of tuberculous antibody to a cocktail of antigens ES-31, ES-41 and ES-43 BY ELISA in anti ES-31 IgG positive and negative PTB cases. (Gupta S, Current Sci 2005;88(11)1825-1827.)

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 positivity at 1:600 dilution

Table 2: Analysis of seroreactivity of ES-6, ES-31 and ES-43 antigens by indirect ELISA in different stages of pulmonary tuberculosis and contacts. (Gupta S, Biomedical Research 2005;16(1):23-27)

Group	No. Screened	No (%) Positive for		
		6 kDa	43 kDa	31 kDa
Fresh cases	25	16 (64)	19 (76)	19(76)
Relapse cases	30	23 (77)	27 (90)	22 (73)
Chronic cases	25	20 (80)	19 (76)	23 (92)
Contact cases	10	4 (40)	1 (10)	1 (10)
Healthy control	20	2 (10)	2 (10)	1 (5)

* sera showing positivity at 1:600 dilution.

Table 3: Analysis of free and immune-complexed serine protease (SEVA TB ES-31) antigen and its antibody in TB with HIV-coinfection (Shende N; Int J Tuberc Lung Dis 2005;9(8):915-919).

Group of patients	Serum Screened n	Positive reaction for			
		Ab* n (%)	Ag# n (%)	CIC-Ag# n (%)	Ag/ CIC-Ag/ Ab n (%)
PTB	30	26 (87%)	22(73%)	20(67%)	29(97%)
HIV+TB	24	11(46%)	15(62%)	13(54%)	21(87%)
HIV-infected PTB (S+)	14	6(43%)	10(71%)	9(64%)	13(93%)
HIV	30	2(7%)	-	1(3%)	2(7%)
Diseased control	30	2(7%)	-	-	2(7%)
Healthy control	30	1(3%)	-	-	1(3%)

* Serum showing positive reaction 1:600 dilution

Serum showing positive reaction 1:300 dilution

TB: tuberculosis; HIV: human immunodeficiency virus; Ab: antibody; Ag: Antigen; CIC-Ag: circulating immune-complexed antigen; PTB: pulmonary tuberculosis; S+: serum-positive; S-,C-: smear-negative, culture-negative; S-,C+: smear-negative, culture-positive.

Table 4: Reactivity of *M. tb* H₃₇Ra anti-cocktail antibody (anti ES-31, anti ES-43 and anti EST-6) for detecting circulating free and immune-complexed antigen in sputum positive pulmonary tuberculosis by sandwich ELISA (Harinath BC, Diag Microbio Inf Dis 2006; 55:65-68).

Group	No. screened	No.(%) showing positive reaction* for detection of	
		Cocktail Ag	
		Free Ag	IC [§] Ag
Sputum positive PTB	68	62(91)	66(97)
Healthy control	40	1(3)	NR
Disease control (COAD [#] - 12, Bronchial asthma - 9, Pneumonia - 5, PUO ^{##} - 10, Pleural effusion / empyema - 3, Leprosy -1)	40	3(8)	1(3)

* Sera showing positive reaction at 1:300 dilution; [§] IC Ag: Immune complexed antigen; [#] COAD = Chronic obstructive airway diseases; ^{##} PUO = Pyrexia of unknown origin

Table 5: Analysis of circulating free and immune-complexed (IC) cocktail antigen and its antibody in bacteriologically confirmed sera of extrapulmonary tuberculosis (Upadhye U, Biomed Res 2007;18(3):161-166).

Group	No. Screened	No.(%) showing positive reaction for detection of			
		Ab*	Ag**	IC-Ag**	Ab / Ag / IC-Ag
EPTB	32	27	15	22	32
TBLN	8	6	4	7	8
TBM	5	3	2	4	5
Bone and Joint TB	6	6	3	2	6
Abdominal TB	5	4	2	3	5
Pleural TB	4	4	2	2	4
Miliary TB	4	4	2	4	4
Healthy control	10	1	1	0	1

EPTB: Extrapulmonary tuberculosis; TBLN: tuberculous lymphadenopathy; TBM: tuberculous meningitis.

* Sera dilution 1:600

** Sera dilution 1:300

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ADVANTAGES OF SEVA TB ELISA

- The test can be done in simple hospital setting
- Helpful in childhood tuberculosis where sputum sample is difficult to obtain
- Helpful in clinically suspected tuberculosis cases negative by routine diagnostic methods like AFB smear & culture examination.
- Useful in confirming tubercular aetiology in extrapulmonary tuberculosis viz; tubercular lymphadenopathy, meninges-CNS tuberculosis, genito-urinary tuberculosis, bone & joint tuberculosis, abdominal tuberculosis etc.
- Useful in immunomonitoring for confirming elimination of infection and patient's compliance in tuberculosis cases during antituberculosis therapy
- Preferred over the insensitive, cumbersome or time consuming & widely unavailable, invasive & expensive tests such as Bacteriological, Radiological, FNAC, Laproscopic biopsy, USG, CT, Radionuclide bone scan, DNA probes, PCR etc.