Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 5, Issue 3, 2013

Research Article

# SEROLOGICAL IMMUNODIAGNOSIS OF TUBERCULOSIS USING PCR AND IN-HOUSE DEVELOPED CLINICAL ISOLATE BASED ELISA

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*Received: 09 Apr 2013, Revised and Accepted: 20 May 2013*

**ABSTRACT**

Tuberculosis (TB) is one of the major public health problems worldwide, mainly due to the appearance of drug resistant *Mycobacterium tuberculosis* strains that render TB control programs more cumbersome.

Objective: Diagnostic tests which are rapid, sensitive and specific for the identification of causative agent are required for successful health programs focussing disease control. Many diagnostic tools have been developed to improvise the need for rapid diagnosis of tuberculosis. Techniques that are sensitive and specific to detect *M. tuberculosis* in clinical samples are important for the diagnosis of patients with tuberculosis.

Methods: Ten clinical isolates were collected from patients clinically confirmed with TB and were enrolled into the study and tested for the presence of *M. tuberculosis* by Ziehl–Neelsen smear and LJ culture. KUMT09 was selected based on the biochemical and morphological characteristics. It was further subjected to 16S rRNA gene sequencing. In this study, sera of 200 patients were evaluated for the presence of antibodies against soluble extract and culture filtrate antigens by ELISA assay and compared with commercially available ELISA kit.

Results: KUMT09 was selected based on the biochemical and morphological characteristics. 16S rRNA gene sequencing showed ~ 99 % similarity with *Mycobacterium tuberculosis* in NCBI database. The sequence was deposited at GenBank with accession no. JQ394980. Out of 200 sera samples, 149 sera samples appeared to be positive for tuberculosis infection by using in-house developed ELISA methodology where as 168 sera samples appeared to be positive for tuberculosis infection by using commercially available ELISA kit. The sensitivity of the in-house developed ELISA kit was found to be 88.7 %.

Conclusion: The assay was simple, rapid and economical for the detection of *M. tuberculosis* infection and suitable for large scale screening of samples in endemic areas without any sophisticated equipment.

**Keywords:** Tuberculosis, ELISA, PCR, Antigens.

## INTRODUCTION

*Mycobacterium tuberculosis* (*M. tuberculosis*) is the causative agent of tuberculosis (TB), a disease having history back as far as the Egyptians in 2400 BC [1]. In developing countries, TB is a major cause of morbidity and mortality.

Tests based on detection of antibodies against *M. tuberculosis* are preferred over traditional methods for diagnosis of active tuberculosis as they can detect immune response induced during infection. The type of immunoglobulin detected would indicate the status of infection i.e. whether the infectious process is progressing or static. Serologic methods have the advantages like easy to perform, cheap and non invasive, but they are limited due to lack of highly specific and sensitive capture antigens. Enzyme linked immunosorbent assay (ELISA) has been used with increased sensitivity for detecting the antibodies [2, 3, 4]. Most of the antibody response in tuberculosis is directed towards antigens common to all members of mycobacteria genera. In TB patients, the serological response to mycobacterial antigens has been primarily evaluated using standard ELISA with in- house methodologies and protocols which certainly differ from laboratory to laboratory. Clinical differentiation of tuberculosis and other mycobacteriosis is difficult due to overlapping domain of symptoms [5]. In the last decade, studies of new assays employing various antigens and antibodies have been reported. The conventional approaches which rely on ELISA based serological tests are simple and inexpensive. A delay in the diagnosis together with misdiagnosed TB cases contributes to M. tuberculosis transmission and mortality. The rapid identification using a sensitive and specific test would prompt early treatment and prevention of transmission.

The present situation demands more specific, sensitive, rapid, simple, safe (non-hazardous) and cost-effective diagnostic test(s). This goal can be achieved either by developing new test(s) or by improving the existing ones. In this respect, to overcome this, the present study explores the potential of detecting mycobacterial antibodies in serum. The present study was, therefore, undertaken to standardize a rapid, sensitive and inexpensive immunoassay for the diagnosis of tuberculosis.

## MATERIALS AND METHODS

### Isolation of mycobacterial strain

Ten bacterial species were isolated from various clinical samples from different diagnostic laboratories as well as hospitals. These strains were named as KUMT01, KUMT02, KUMT03, KUMT04, KUMT05, KUMT06, KUMT007, KUMT08, KUMT09 and KUMT10. All clinical isolates were grown on Lowenstein-Jensen (LJ) slants and incubated at 37 °C for 8 weeks. The isolated bacterial strains were screened on the basis of colony morphology and biochemical tests.

### ATCC Strain

Mycobacterium tuberculosis H37Ra purchased from American Type Culture Collection Centre (ATCC 25177), USA, was used as reference strain.

### Morphological and biochemical characterization

### Growth rate and pigment production

Mycobacteria can be separated into two groups based on growth rate, optimal growth temperature and production of pigment with or without exposure of light [6]. The growth rate, growth temperatures, and pigment production of mycobacteria can be determined by observing growth of subcultures on LJ slants. This medium is preferred as the contrast color of the medium makes it easy to observe the presence and pigment of mycobacterial colonies grown on it.

### Ziehl –Neelsen stain (ZN)

Colony taken from LJ slant was emulsified in sterile saline (0.85 %) to make smear on glass slide. The smear was flooded with strong carbol fuchsin (basic fuchsin 2.5 gm, 100 % alcohol 25 mL, melted phenol crystals 12.5 mL and distilled water 250 mL) and heated with

spirit lamp from bottom till steam arises from the stain while taking care not to boil the stain. After 3 to 5 min, the smear was washed in running tap water and decolorized with 1 % acid alcohol (hydrochloric acid 10 mL and 70 % alcohol 990 mL) mixture until pink color stopped oozing out of the smear. Then smear was washed in running tap water for 5 min and again washed with distilled water. The smear was counter stained with methylene blue solution (5 mL of methylene blue 1.4 %) dissolved in 45 mL of distilled water) for 30 sec. The smear was washed in running tap water, air dried and observed under oil immersion objective.

**Arylsulfatase test**

Arylsulfatase is an enzyme that hydrolyzes the bond between the sulfate and aromatic rings of tri potassium phenolphthalein. Free phenolphthalein can be recognized by the formation of red color when an alkali is added. The organism was inoculated on to Middlebrook 7H9 broth and incubated at 37 °C. After 3 days of incubation, 6 drops of sodium carbonate solution (2 N) was added [7] and observed for color formation.

**Catalase test**

The enzyme catalase splits hydrogen peroxide into water and oxygen which is released as bubbles. Catalase activities were measured at 68 °C and quantified at 37 °C. All mycobacteria except some isoniazid-resistant strains of *M. tuberculosis* and *M. bovis* display catalase activity [8].

(i) At 68 °C: Heavy suspensions of the organisms were made with 0.5 mL of 0.067 M phosphate buffer (pH 7) in a screw-cap tube. Then incubated at 68 °C in a water bath for 20 min and cooled to room temperature. The catalase reagent (0.5 mL) (equal volume of 10 % Tween 80 and 30 % $H_2O_2$) was added to each tube and tubes were recapped loosely. The tubes were observed after 5 min. Negative tubes were held for 20 min before discarding.

(ii) Semi quantitative: The surfaces of LJ deep (butt) tubes were inoculated with 0.1 mL of 7 day-old liquid culture. The slants were incubated at 37 °C in 10 % $CO_2$ incubator for 2 weeks (caps loosened). 1 mL of catalase reagent (0.5 mL of 30 % $H_2O_2$ and 0.5 mL of 10 % tween 80 in 0.067 M phosphate buffer) was added to each tube and observed for 5 min. The height of the column of bubbles were measured (in millimetres) above the surface of the medium. The tubes were observed for effervescence.

**Iron uptake test**

LJ slants were inoculated with barely turbid liquid suspension of the organism and incubated until there was visible growth. Eight drops of ferric ammonium citrate solution were added to each tube and incubated for up to three weeks and observed for development of rusty brown color in the colonies every weak and tan discoloration of the media [9].

**Sodium chloride (NaCl) tolerance test**

A barely turbid suspension of growth was prepared from LJ slant and inoculated on to a LJ slant with NaCl (5 %) and a control slant without NaCl. The slants were incubated at 37 °C with 10 % $CO_2$.The tubes were observed once a week for growth [6].

**Niacin accumulation test**

All members of mycobacteria genera produce niacin during growth. Sterile distilled water (1.5 mL) was added to a 4 weeks old LJ culture slant. The surface of the medium was cut and stabbed several times with a sterile needle. The tubes were tilted so that the liquid covers the surface of the medium and allowed 30 min to 2 h for niacin extraction. The extract (0.6 mL) was removed to a sterile test tube and 0.25 mL of 1.5 % O-tolidine and 0.25 mL of 10 % cyanogen bromide were added. The tubes were stoppered tightly and left at room temperature for 15 to 20 min, with occasional gentle agitation. Immediately the tubes were observed for development of pink color at the bottom of the tube against white background [10].

**Nitrate reduction test**

Mycobacteria differ in their ability to reduce nitrate. This is denoted by the development of color when nitrite reacts with the appropriate reagents. The nitrate (0.1 M sodium nitrate in 0.45 M phosphate buffer) broth was inoculated with a loopful of growth from an actively growing culture. The broth was shaken and incubated in an incubator at 37 °C for 2 h. One drop of 1:1 solution of concentrated HCl and sterile $H_2O$ was added. Two drops of sulphanilamide (0.2 %) and 2 drops of b-N-(1-naphthyl) ethylene diamine dihydrochloride (0.1 %) were added and shaken well. The tubes were observed for development of deep red color [11].

**Pyrazinamidase test**

In the absence of enzyme pyrazinamidase, pyrazinamide (PZA) is not converted to pyrazinoic acid and ammonia. Resistance to PZA is indicated by the inability to convert PZA to pyrazinoic acid. The surfaces of two tubes of LJ medium were inoculated from a fresh culture slant and incubated at 37 °C in an incubator. One tube was tested after four days and the other tube after seven days. To the tubes, 1.0 mL of freshly prepared ferrous ammonium sulfate (1 %) was added. The tubes were placed at room temperature for 30 min and then refrigerated at 4 °C to prevent growth of contaminants. After 4 h, tubes were examined for a pink band in the agar. The tubes were read by holding the tubes against a white background by using incident room light [12].

**Tellurite reduction test**

Most mycobacteria have the ability to reduce tellurite to metallic tellurium [6,12], visible in liquid cultures of mycobacteria as a fine black precipitate. Middlebrook 7H9 liquid medium was inoculated and incubated at 37 °C in 10 % $CO_2$ for 7 days. The broth should contain sediments on the bottom of the tube. Two drops of sterile potassium tellurite solution (0.2 %) was added to each test culture. Tubes were shaken to mix and re-incubated at 37 °C in 10 % $CO_2$ for 3 days, without shaking. On the third day, sedimented cells in each culture tube were examined for black precipitate of metallic tellurium, without mixing [13].

**Tween 80 hydrolysis test**

In this test, tween 80 acts as a lipid, binding the neutral red indicator and causing the solution to yellow. If the mycobacterial lipase hydrolyzes the tween 80, the neutral red indicator is no longer bound, and it reverts back to its normal red color at pH 7.2. 0.5 mL of tween 80 in 0.067 M phosphate buffer (0.5 %) and 2 mL of neutral red solution (1 %) were added to 1.0 mL of sterile deionised water. A loopful suspension of an actively growing culture was taken from a slant and inoculated to the tween 80 mixture. The mixture was incubated aerobically at 37 °C in an incubator and examined at 1, 5, and 10 days. The tubes were observed for the appearance of red color without shaking [14].

**Urease test**

The urease test is used to differentiate mycobacteria on the basis of their abilities to hydrolyze urea [6, 10] to form ammonia and $CO_2$. Loopful suspension of young, actively growing (21 days old) culture of the mycobacteria was inoculated to 5 mL of urea broth (0.01 M phosphate buffer system, pH 6.7, which was autoclaved; while still hot, 3 g of urea and 1 mL of phenol red (0.1 %) were added per 100 mL). The broth was incubated at 37 °C in an incubator for up to 72 h. The broth was observed for development of dark pink color.

**Susceptibility to p-nitrobenzoic acid (PNB)**

PNB (0.5 g) was dissolved in 15 mL of dimethyl formamide and added to 1 L of LJ fluid, aliquoted in tubes (6 mL) and inspissated. The media was cooled to room temperature, inoculated with the bacterial suspension and incubated at 37 °C. The slants were observed for growth after 28 days of incubation [12]. Out of ten clinical isolates, KUMT09 strain was selected on the basis of its morphological as well as biochemical properties and was identified using 16S rRNA gene sequencing technique.

**Microbial identification using 16S rRNA sequencing**

**Extraction of DNA**

The 16S rRNA sequencing was done by Chromous Biotech (Bengaluru) as contract research. The cellular pellet of the organism

was mixed with 75 µL of sucrose tris EDTA (STE) extraction buffer (320 mM Sucrose, 10 mM Tris HCl, 20 mM EDTA, 75 mM NaCl and 2.5 mL of 20 % SDS) along with 5 mg of polyvinyl pyrrolidone and 0.1 g of silica powder and incubated at 65 °C for 10 min. Centrifuged the content at 13,000 rpm for 10 min. To the supernatant, equal volume of chloroform: isoamyl alcohol (1:1) was added and repeated the centrifugation. To the aqueous layer, added 2/3 volume of isopropanol and centrifuged at 13,000 rpm for 10 min. The pellet was washed with 70 % ethanol by centrifugation. The pellet was dried and dissolved in 50 µL TE buffer.

**PCR amplification using consensus 16s rRNA primers**

**PCR amplification conditions**

DNA: 1 µL, 16S forward primer 400 ng, 16S reverse primer 400 ng, dNTPs (2.5 mM each) 4 µL, 10 X Taq DNA polymerase assay buffer 10 µL, Taq DNA polymerase enzyme (3U/ µL) 1 µL, water 84 µL, total reaction volume: 100 µL.

**16S rRNA specific primer**

Forward primer: 5'-AGAGTRTGATCMTYGCTWAC-3'

Reverse primer: 5'-CGYTAMCTTWTTACGRCT-3'

The conditions were, initial denaturation at 96 °C for 1 min followed by 25 cycles of denaturation at 96 °C for 10 sec, hybridization at 50 °C for 5 sec elongation at 60 °C for 4 min. The amplified products were visualized on ethidium bromide stained agarose gel using UV transilluminator. Approximately, 1363 bp of PCR products were sequenced using ABI 3500 XL genetic analyzer.

**Sequence similarities and phylogenetic analysis**

Nucleotide sequence of 16S rRNA gene of strain KUMT09 was determined and compared for similarity level with the reference species of mycobacterium contained in genomic database bank. The BLAST program (http://www.ncbi.nlm.nih.gov/blast) was employed in order to assess the degree of DNA similarity.

**Identification software details**

Phylogenetic Tree Builder uses sequences aligned with System Software aligner. A distance matrix is generated using the Jukes-Cantor corrected distance model. When generating the distance matrix, only alignment model positions were used, alignment inserts were ignored and the minimum comparable position is 200. The tree was created using Weighbor with alphabet size 4 and length size 1000 [15].

**Collection of samples**

Two hundred serum samples collected from patients suspected with TB, obtained from various diagnostic laboratories as well as hospitals were used in this study. The serum samples were confirmed for its positivity by inoculating on to modified Middlebrook 7H9 broth with PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin) cocktail of antibiotics and incubated for 1 week at 37 °C. They were also confirmed by ZN method. The samples showing positivity in the culture were considered as positive and the samples that did not show any growth were considered as negative. Blood sample was procured from clinical suspected patients with tuberculosis. After collection, blood was transferred to penicillin bottle and kept in incubator at 37 °C, to make blood clot. After 30 min of incubation, the blood sample was centrifuged at 5,000 rpm for 15 min to separate serum from blood clot. Serum samples were stored at -20 °C after adding sodium azide (0.1 %) for preservation.

**Polymerase chain reaction**

All serum samples were confirmed for the presence of IS*6110* gene which is present only in positive samples by polymerase chain reaction.

**DNA extraction**

mL of clinical samples was centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the pellet suspended in 567 µL of TE buffer (Tris EDTA, pH 7.4), 30 µL 10 % SDS and 3 µL proteinase K (20 mg/ mL), mixed and incubated at 37 °C for 1 h. After incubation, 100 µL of 5 M NaCl and 80 µL of high-salt CTAB buffer (containing 4 M NaCl, 1.8 % CTAB was added and mixed followed by incubation at 65 °C for 10 min. An approximate equal volume (0.7–0.8 µL) of chloroform-isoamyl alcohol (24:1) was added, mixed thoroughly and centrifuged for 5 min in a micro centrifuge at 12,000 rpm. The aqueous viscous supernatant was carefully decanted and transferred to a new tube. An equal volume of phenol: chloroform- isoamyl alcohol (1:1) was added followed by a 5 min spin at 12,000 rpm. The supernatant was separated and then mixed with 0.6 volume of isopropanol to get a precipitate. The precipitated nucleic acids were washed with 75 % ethanol, dried and re-suspended in 100 µL of TE buffer. DNA was extracted from clinical samples and from *M. tuberculosis* standard strain $H_{37}Ra$. Each step of the extraction protocol was performed inside biosafety cabinet, using protected tips and dedicated pipettes at room temperature [16].

**PCR primers**

Two set of primers; genus specific and species specific were used for the assay.

The sequences of the genus specific primers were:

Forward primer: 5’ GAGATCGAGCTGGAGGATCC 3’,

Reverse primer: 5’ AGCTGCAGCCCAAACCTGTT 3’

These primers were used to amplify a 383 base pair fragment of a gene that codes for a 65 KDa protein present in all species of mycobacteria.

The sequences of the species specific primers were

Forward primer: 5’ CCTGCGAGCGTAGGCGTCGG 3’,

Reverse primer: 5’ CTCGTCCAGCGCCGCTTCGG 3’

These primers were used to amplify a 123 base pair nucleotide sequence in IS*6110* present in strains of the *M. tuberculosis* complex (*M. tuberculosis, M. bovis, M. africanum, M. microti*). The primers were purchased from Sigma–Aldrich Genosys, India. The primers were reconstituted and stored in 100 µM and 5 µM stock solutions at -20 °C.

**Amplification of DNA**

DNA amplification by PCR was performed with a total reaction volume of 25 µL by using Eppendorf Thermo Cycler. Contents were well mixed and subjected to thermocycling as follows:

(a) For mycobacterium genus specific reaction, the conditions were:

The conditions were, initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 90 sec, annealing at 57 °C for 90 sec, extension at 72 °C for 90 sec and final extension at 72 °C for 5 min.

(b) For *M. tuberculosis* complex, the conditions were:

The conditions were, initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 2 min, annealing at 68 °C for 2 min, extension at 72 °C for 2 min and final extension at 72 °C for 5 min.

**Detection of amplified products and interpretation**

PCR products were detected on agarose gel (1.5 %) in 1X TAE buffer containing ethidium bromide at 10 µg/ mL concentration under ultra violet illumination. The molecular weight markers, positive control, negative control and samples were observed for the appropriate bands. When the single band of 383 bp and 123 bp were obtained, it was inferred that the sample was positive for Mycobacterium species and *M. tuberculosis* respectively.

**Quality control**

The quality of the amplification was monitored by the simultaneous testing of positive and negative controls in each run.

Positive amplification control: *M. tuberculosis* DNA standard strain $H_{37}Ra$

Negative amplification control: Reaction mixture without sample

Negative processing control: TE buffer in the lysis procedure

**Extraction of *M. tuberculosis* antigens**

**KUMT09 soluble extracts antigen preparation**

*M. tuberculosis* KUMT09 soluble extract antigen was prepared as described earlier [17]. *M. tuberculosis* KUMT09 was inoculated onto thyroxine supplemented LJ slants and incubated at 37 °C for 8 weeks. The bacilli were sub cultured on to Middlebrook 7H9 broth and Sauton's broth and incubated at 37 °C for 10 weeks. The bacilli were killed by autoclaving at 121 °C for 60 min. The medium was centrifuged at 5,000 rpm for 16 min. The bacilli were recovered as pellet, washed three times in 66 mM sodium phosphate buffer (PB) and extracted with sodium dodecyl sulphate (1 %) in PB at 80 °C. After incubation, bacilli were inactivated with 5 mL of phenol (5 %) for 1 h at 4 °C for 1 hr. After centrifugation at 10,000 rpm for 1 hr, the supernatant was filtered through a 0.22 µm pore size membrane. The supernatant was dialysed against phosphate buffered saline (PBS) for 48 h. The sediments were sheared by vortexing with 3 mm glass beads for 5 min. The suspension was dialysed against PBS for 48 h. Both concentrated supernatant and suspension were mixed in equal proportion (1:1). The protein content of the antigen was determined by Lowry [18] method. The antigen was stored at -20 °C till future use [19]. This antigen cocktail was marked as MTSE.

**KUMT09 culture filtrate antigen preparation**

*M. tuberculosis* KUMT09 culture filtrate antigen was prepared as described earlier [17]. *M. tuberculosis* $H_{37}Ra$ was cultured in synthetic Sauton's medium (Hi-Media, Mumbai) enriched with 0.5 % glucose, 0.5 % sodium pyruvate and 0.05 % tween 80 for 3 weeks (35 °C; 10 % $CO_2$). Then centrifuged at 10,000 rpm for 45 min at 4 °C, and the supernatant fluid was sterilized by filtration through a 0.22 µm-pore-size membrane (Sartorius Stedim Biotech, France). The proteins were precipitated with 80 % ammonium sulfate in cold overnight, dissolved in sterile phosphate-buffered saline (PBS) and dialyzed at 4 °C. The protein content was determined by Lowry's method using bovine serum albumin (BSA) standard [18] and adjusted to 5 mg/ mL [20]. The protease inhibitor phenylmethylsulfonyl fluoride was added to a concentration of 10 mM. The culture filtrate preparations were stored at -20 °C until required. This antigen was marked as MTCFA.

**Antibody detection ELISA assay using MTSE antigen**

**Coating of ELISA plates**

The ELISA plates were coated as described earlier [21]. Two 96 welled U bottomed polystyrene microtitre plates (Tarsons, India) were coated with the antigens (50 µg/ mL in carbonate bicarbonate buffer). This solution (50 µL) was transferred into the wells of ELISA microtitre plates. The plates were kept in the refrigerator overnight. The next day, plates were washed 3 times with PBS tween (PBST) solution. To 100 mL of PBST solution, skimmed milk powder (2 g) was added. The plates were decanted after overnight incubation and non specific binding sites were blocked by adding 150 µL of PBST milk (1 %) into antigen coated ELISA plates. Then the plates were incubated at 37 °C for 2 h. The plates were blot dried and stored in the refrigerator.

**ELISA assay**

ELISA was performed in 96 welled polystyrene microtitre plates as described earlier [21]. PBST milk (1 %) was prepared (0.5 g of skimmed milk powder was added to 50 mL of PBST solution, which served as the sample diluent). PBST milk (500 µL) was added into each dilution tube and 100 µL of serum sample was added to it. These 1:6 dilutions were mixed well. The microtitre plates were marked as MTSE IgG and MTSE IgM. The first well was left blank and to the second well positive control serum was added. From the dilution tubes, 50 µL of diluted serum sample was transferred to the ELISA plate wells in duplicates and plates were incubated at 37 °C for 2 h. After incubation, the plates were washed for four times with PBST solution and were blot dried. The conjugate IgG HRP and IgM HRP (Sigma, USA) of 5 µL were added to 15 mL (1:3000 dilution) and 5 mL (1:1000 dilution) of PBST milk (diluent). 50 µL of IgG conjugate was added to the plate MTSE IgG and 50 µL of IgM conjugate was added to plate MTSE IgM. The plates were then incubated at 37 °C for 2 h. After the incubation, the plates were washed with the wash buffer (PBST) for six times and were blot dried. 75 µL of the substrate (ortho phenlyenediamine dihydrochloride in 0.1 M phosphate citrate buffer and 6 µL of 30 % hydrogen peroxide) were added into each well of ELISA plates. The plates were then kept in a dark place for 30 min for colour development. The colour change was noted and the reaction was stopped using the stop solution 1N sulphuric acid (50 µL) into each well of ELISA microtitre plates. The plates were read using the ELISA reader at 492 nm.

**Antibody detection ELISA assay using MTCFA antigen**

**Coating of ELISA plates**

The ELISA plates were coated as described earlier [22]. Two 96 welled U bottomed polystyrene microtitre plates (Tarsons, India) were coated with the antigens (40 µg/ mL in carbonate bicarbonate buffer). This solution (50 µL) was transferred into the wells of ELISA microtitre plates. The plates were kept in the refrigerator for overnight. The next day plates were washed 3 times with PBS tween (PBST) solution. To 100 mL PBST solution, skimmed milk powder (2 g) was added. The plates were decanted after overnight incubation and non specific binding sites were blocked by adding 150 µL of PBST milk (1 %) into antigen coated ELISA plates. Then the plates were incubated at 37 °C for 2 h. The plates were blot dried and stored in the refrigerator.

**ELISA assay**

ELISA was performed in 96 welled polystyrene microtitre plates as described earlier [23]. Briefly, PBST milk (1 %) was prepared (0.5 g of skimmed milk powder was added to 50 mL of PBST solution. This is the sample diluent). PBST milk (300 µL) was added into each dilution tube and 100 µL of sample was added to it. These 1:4 dilutions were mixed well. The microtitre plates were marked as MTCFA IgG and MTCFA IgM. The first well was left blank and to the second well positive control was added. From the dilution tubes, 50 µL was transferred to the ELISA plate wells in duplicates and plates were incubated at 37 °C for 1 h. After incubation, the plates were washed for six times with PBST solution and it were blot dried. The conjugate IgG HRP and IgM HRP (Sigma, USA) of 5 µL were added to 15 mL and 5 mL of PBST milk (diluents). 50 µL of IgG conjugate was added to the plate (1:3000 dilution) MTCFA IgG and 50 µL of IgM conjugate was added to plate (1:1000 dilution) MTCFA IgM. The plates were then incubated at 37 °C for 1 h. After the incubation, the plates were washed with the wash buffer (PBST) for 8 times and were blot dried. 75 µL of the substrate (ortho phenlyenediamine dihydrochloride in 0.1 M phosphate citrate buffer and 6 µL of 30 % hydrogen peroxide) were added into each well of ELISA plates. The plates were then incubated in dark at room temperature for 30 min in order to develop colour. The colour change was noted and the reaction was stopped by adding stop solution 1 N sulphuric acid (50 µL) into each well of ELISA microtitre plates. The plates were read using the ELISA reader at 492 nm.

**Cut-off point determination**

The ELISA cut-off point was determined as per the kit manufacturer's guidance. Briefly, cut-off point was determined from 8 negative samples and it was calculated as 1. Results with lower optical density (OD) than 0.9 is considered as negative and OD greater than 1.1 considered as positive results. Those results between 0.9-1.1 are considered as suspected results and should be re-evaluated with fresh samples after a while.

**Validation of ELISA assay**

For validation of test, all serum samples were tested with commercially available ELISA kit for IgG and IgM antibody detection. The procedure was followed as per the kit

manufacturer's guidance. The antigen coated ELISA plates were kept in room temperature 30 min prior the commencement of test. 10 X concentrated sample dilution buffer was made to 1 X (1 mL of sample dilution buffer mixed with 9 mL of distilled water) concentration. The serum sample (200 µL) was mixed with 500 µL of 1 X sample dilution buffer. The diluted sample (50 µL) was added to IgG and IgM ELISA plates and kept in incubator for 1 h and 30 min at 37 °C. After incubation, the plates were washed with washing buffer for six times provided in the kit and blot dried. IgG and IgM HRP labelled conjugates were diluted with conjugate buffer and 50 µL of the conjugates were added to IgG and IgM ELISA plates respectively. The plates were incubated at 37 °C for 1 h and washed again with washing buffer (PBST) for four times and blot dried. Chromogen substrate (75 µL) was added to both plates and incubated in dark at room temperature for 30 min. After incubation, the reaction was stopped by adding 50 µL of stop solution (1 N sulphuric acid) into each well of ELISA microtitre plates. The plates were read using ELISA reader at 492 nm.

**Calculation of sensitivity of ELISA assay**

The sensitivity of the ELISA assay was determined by using the following formula.

$$\text{Sensitivity} = \frac{\text{No. of positive cases in ELISA}}{\text{No. of positive cases in commercially available ELISA kit}}$$

**RESULTS AND DISCUSSIONS**

The result of the present study encompasses screening of clinical isolates for *M. tuberculosis*, their identification both by biochemical tests as well as 16S rRNA gene sequencing and detection of the infection by PCR as well as ELISA techniques.

**Screening and identification**

**Morphological and biochemical characterization**

In the present study, ten clinical isolates were assigned numbers from KUMT01 to KUMT10. The isolates were screened and selected based on their morphological and biochemical reactions (Table 1).

**Table 1: Biochemical reactions of clinical isolates**

| Test | KUMT 01 | KUMT 02 | KUMT 03 | KUMT 04 | KUMT 05 | KUMT 06 | KUMT 07 | KUMT 08 | KUMT 09 | KUMT 10 | ATCC 25177 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Ziehl–Neelsen stain** | P | P | P | P | P | P | P | P | P | P | P |
| **Optimum growth temperature (°C)** | 37 | 37 | 37 | 37 | 37 | 37 | 37 | 37 | 37 | 37 | 37 |
| **Colony morphology** | R | R | SR | R | R | R | R | SR | R | R | R |
| **Pigmentation** | Pi | NP | NP | Pi | Pi | NP | NP | NP | NP | NP | NP |
| **Arylsulfatase** | P | P | P | N | P | P | N | N | N | P | N |
| **Catalase 68 °C** | P | P | P | P | P | P | P | P | N | P | N |
| **Catalase semi quantitative (mm) 37 °C** | 48 | 45 | 47 | 50 | 43 | 44 | 39 | 49 | 45 | 43 | 45 |
| **Iron uptake** | N | N | P | P | P | P | N | P | N | N | N |
| **NaCl tolerance** | N | N | P | N | N | N | P | P | N | P | N |
| **Niacin accumulation** | N | P | P | N | N | N | P | P | P | P | P |
| **Nitrate reduction** | N | P | P | N | N | N | N | N | P | P | P |
| **Pyrazinamidase** | N | P | N | N | P | P | P | P | P | P | P |
| **Tellurite reduction** | N | P | P | N | P | N | N | P | N | P | N |
| **Tween 80 hydrolysis** | P | P | P | N | P | P | N | P | P | N | P |
| **Urease** | N | P | P | P | P | P | N | N | P | P | P |
| **Susceptibility to *p*-nitrobenzoic acid** | P | P | P | N | N | N | N | N | N | P | N |

"P"- Positive, "Pi"- Pigmented, "N"- Negative, "NP"- Non- pigmented, "R"- Rough, "SR"- Intermediate in roughness.

The clinical isolates were screened and selected on the basis of their morphological and biochemical reactions. The clinical isolates KUMT01, KUMT02, KUMT03, KUMT05, KUMT06 and KUMT10 were positive for arylsulfatase test. The isolates KUMT01, KUMT04, and KUMT06 were pigment producers on LJ medium. All the clinical isolates except KUMT09 were catalase positive at 68 °C. *M. tuberculosi*s KUMT09 was selected for future studies based on the similarities between the reference strain (ATCC 25177).

TB patients are the most potent sources of transmission in the community [24, 25, 26]. Therefore, early detection of TB in clinical samples is important in the control of TB [27]. Isolation of the organisms by culture is absolutely needed for the diagnostic of this disease. Microscopic examination of clinical isolates for ZN stain is important for a definitive early diagnosis of TB. Nevertheless, in about 40 % of these cases, bacilli are not usually seen by ZN stain and 25 % of these are culture negative for *M. tuberculosis*. Because the conventional methods are low in sensitivity, time consuming or both, presumptive diagnosis of mycobacterium diseases is usually based on clinical findings such as persistent cough, fever and weight loss. Although tuberculin skin tests and radiologic findings may help, the diagnosis must be confirmed by isolation and identification of the etiologic agent [28]. The frequency with which acid fast bacilli (AFB) are seen on direct smears varies widely between different series, and depends on the time devoted to searching for AFB, the number of specimens examined, and the experience of the observer [29]. Mycobacterial strain KUMT09 was found to be identical to *M. tuberculosis* ATCC 25177. Based on the above morphological and biochemical tests, it was confirmed as Mycobacterium sp. *M. tuberculosis* KUMT09 strain was identified using 16S rRNA gene sequencing and was used for further studies.

**Molecular identification**

**Analysis of 16S rRNA gene sequence**

The selected isolate was further confirmed till the species level using 16S rRNA gene sequence alignment of *M. tuberculosis*. PCR amplification of the 16S rRNA gene was performed using the primers relative to *M. tuberculosis*. The 16S rRNA gene sequence of the isolate was compared with the available 16S rRNA gene sequence in GenBank database and the strain was identified as *M. tuberculosis* KUMT09. The partial nucleotide sequence for the 16S rRNA gene (1.5 kbp) was deposited in NCBI GenBank nucleotide sequence database with accession number JQ394980 (Fig. 1; 2 and 3).

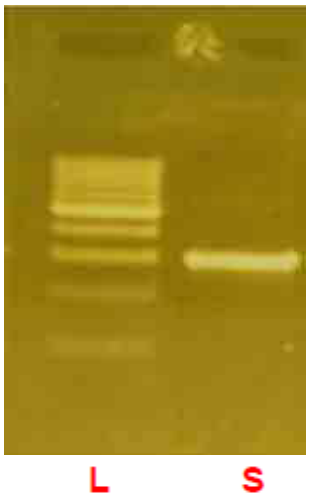
**Determination of ELISA cut off value**

During the past decade, several immunodiagnostic assays have been described as an alternative method of laboratory diagnosis of *M. tuberculosis*. With the development of the ELISA, serodiagnosis of TB has been studied by many investigators and each study claims some success. Most of the serological tests developed for TB diagnosis are based on the detection of anti-mycobacterial antibodies during different antigen preparation, ranging from crude mycobacterial extracts to purified antigens [30]. Antigen detection test has received little attention for tuberculosis. However, this type of assay could provide useful information for monitoring the efficacy of chemotherapy. The ELISA cut-off point was determined as per kit

manufacture's guidance. Briefly, cut-off point was determined from 8 negative samples and it was calculated as 1. Results with lower OD than 0.9 is considered as negative and OD greater than 1.1 considered as positive results. Those results between 0.9-1.1 are considered as suspected results and should be re-evaluated with fresh samples after a while.


Lane S: Sample; Lane L: 1kb DNA ladder

Fig. 1: Gel photo (PCR amplification of 16S rRNA gene *M. tuberculosis* KUMT09)




Lane L: 500bp DNA ladder; Lane S: ~1.5kb 16S rRNA gene fragment amplified

Fig. 2: PCR amplification of ~1.5kb 16S rRNA gene fragment from genomic DNA of the bacterial culture

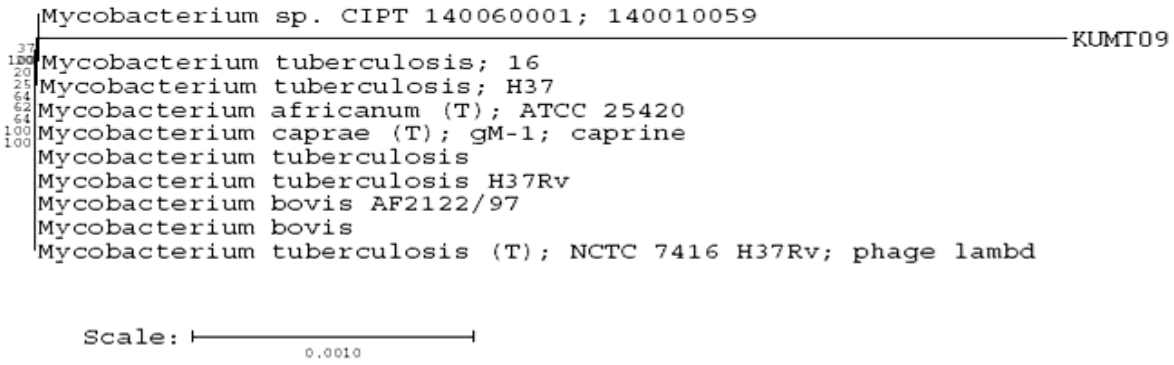



Fig. 3: Phylogenetic tree based on 16S rRNA gene sequence comparisons showing the relationship between members of genus Mycobacterium and isolate KUMT09

**Sample collection and identification by using PCR**

Two hundred serum samples were collected from patients suspected with tuberculosis were inoculated onto modified Middlebrook 7H9 broth and incubated at 37 °C. Out of 200 serum samples, 168 samples showed positive visible growth by forming magenta colored granules and were confirmed by AFB (ZN) staining. All serum samples were identified for tuberculosis infection by using polymerase chain reaction (PCR) technique by using genus specific (which amplified 383 base pair fragment of a gene that codes for a 65 kDa protein) and species specific (which amplified 123 base pair nucleotide sequence in IS*6110*) primers. Out of 200 serum samples, 169 samples appeared to be positive for tuberculosis infection.

**Detection of mycobacterial antibody by MTSE ELISA assay**

In the present study, two hundred serum samples were analysed for the detection of infection by using ELISA technique with MTSE (50 µg/ mL in carbonate bicarbonate buffer) antigen. Out of 200 samples, 149 samples gave positive results. By using MTSE ELISA assay, 70 samples showed high IgG positivity; 48 samples showed high IgM positivity and remaining 31 samples showed both IgG and IgM positivity (Fig. 4 and 5).

Studies reported that, 38 CSF specimens from patients with diagnosed tuberculous meningitis and 52 of non-mycobacterial etiology were examined for the presence of *M. tuberculosis* antigen by a sensitive and specific sandwich ELISA technique [31]. It is likely that demonstration of the bacillary components using specific probes may help in arriving at an accurate diagnosis of tuberculosis in such atypical situations. An alternative method to identify the presence of the causative microbe may be demonstrated the bacillary products, using their antigenicity.

The mycobacterium antigen detection assays are promising in this regard. Any test which is to replace direct microscopy or bacterial culture must offer advantages in terms of speed and ease of use and preferably have a higher sensitivity. Varying degrees of positivity were reported in cases of tuberculosis by different workers [32, 33]. Studies earlier [34] evaluated the diagnostic potential of a cocktail of 4 antigens encoded by regions of difference (RD) 1 and 2 of *M. tuberculosis*, that is, early secretory antigenic target-6, culture filtrate protein-10 (CFP-10), CFP-21, and mycobacterial protein from species tuberculosis-64 (MPT-64) on the basis of antigen and antibody detection by enzyme linked immunosorbent assay. Parallel detection of antigens and antibodies in the serum samples of pulmonary

tuberculosis (PTB) patients resulted in higher sensitivity as compared to either of the single tests in both smear positive (90 %) and smear-negative (60 %) PTB patients. In addition, combined detection of antigens and antibodies in the fluids of extrapulmonary tuberculosis (EPTB) patients could detect > 90 % of the patients with high specificity.

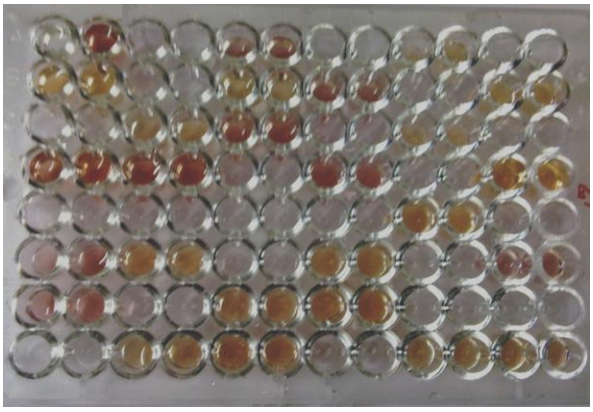


**Fig. 4: IgG ELISA assay using MTSE antigen**

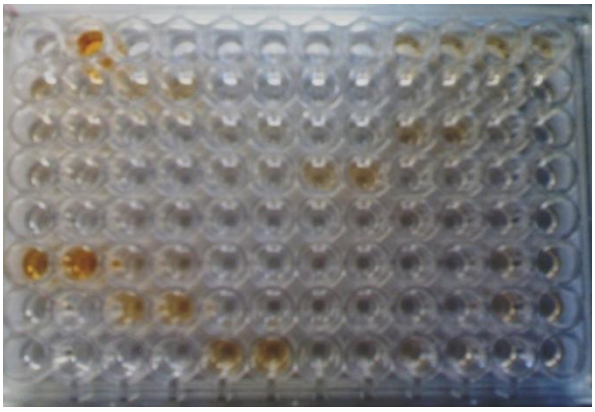


**Fig. 5: IgM ELISA assay using MTSE antigen**

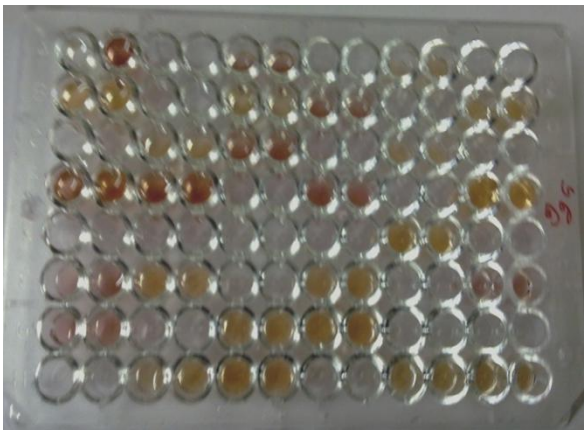


**Fig. 6: IgG ELISA assay using MTCFA antigen**

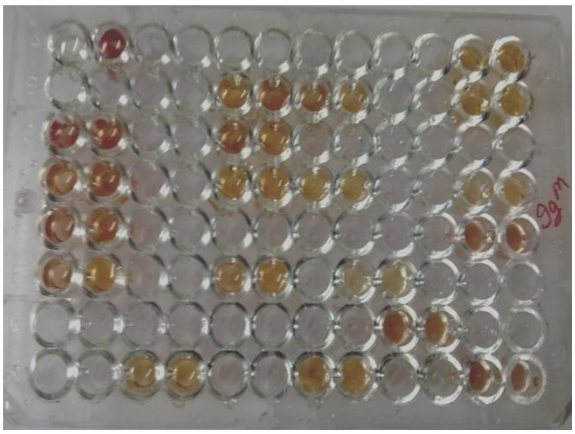


**Fig. 7: IgM ELISA assay using MTCFA antigen**

**Detection of mycobacterial antibody by MTCFA ELISA assay**

In the present study, two hundred serum samples were analysed for the detection of infection by using ELISA technique with MTCFA (40 µg/ mL in carbonate bicarbonate buffer) antigen. By using MTCFA ELISA assay, 65 samples showed high IgG positivity; 49 samples showed high IgM positivity and remaining 35 samples showed both IgG and IgM positivity (Fig. 6 and 7).

Earlier studies [33] demonstrated that *M. tuberculosis* culture filtrate antigen (CFA), responders were 17/21 (71 %) among PTB group. CFP-10 protein response of PTB was 48 % (13/21). Studies [4, 35, 36] have shown that antigen or antibody detection is useful in the diagnosis of pulmonary, meningeal, pleural and abdominal tuberculosis by radioimmunoassay. In a limited study of 10 CSF samples from patients with tuberculous meningitis showed the potential usefulness of ELISA in the detection of antigen [37]. 38 samples obtained from those meeting the diagnostic criteria for tuberculous meningitis, 23 were above the 0.25 µg/ mL level for positivity, whereas the remaining 15 were negative. Serodiagnostic tests like ELISA can show promise because of their ease of performance in field laboratories and cost effectiveness. Cocktail of different antigens has shown to be more useful than single antigen assay. The higher sensitivity in ELISA was possibly due to high turnover number of the enzyme and sensitive colour reaction in this assay.

**Validation of ELISA assay**

The use of *M. tuberculosis* specific antigen cocktail in ELISA provides an easy, cheap, and effective alternative to the molecular methods for the development of a specific test for TB in endemic countries. 200 serum samples were tested for diagnosis of infection by using commercially available IgG and IgM antibody detection kit for validating the results. Out of 200 samples, 168 samples showed positivity; 80 samples showed high IgG positivity; 54 samples showed high IgM positivity and remaining 34 samples showed both IgG and IgM positivity.

**Calculation of sensitivity of the test**

In the present study, the total number of cases in ELISA appeared to be 149. Total number of cases in commercially available kit appeared to be 168. Hence, the sensitivity of the test appeared to be 88.7 %. The ELISA for *M. tuberculosis* antigen detection is easy to perform, inexpensive, and allows simultaneous processing of multiple samples. The cost of diagnosis by means of examination of multiple serum samples is similar to that of the ELISA antigen detection assay. However, the ELISA assay developed in the current study will improve the diagnosis of infections because the sensitivity increases. In addition, it should improve *M. tuberculosis* diagnosis by eliminating false negative results caused by lack of training and experience of the laboratory personnel responsible for microscopic examination, untimely processing of samples.

The diagnostic value of a given test in clinical practice depends on its positive and negative predictive values. These values vary markedly with the prevalence of the disease in a given community. The high positive predictive values of tests would make a positive test result useful in strengthening the clinical suspicion, but a negative result would be less useful [38]. Moreover, the predictive values of positivity in high endemic areas may be achieved by increasing the specificity, i.e. by modifying the cut off point separating positive from negative test result. However, increased specificity can only be gained at the expense of the sensitivity [39].

**CONCLUSION**

Developing nations pose a major threat against tuberculosis. As its control relies on early detection of cases and effective treatment, timely diagnosis of TB is essential ensuring a favourable clinical outcome. Till date, the diagnosis of TB depends on clinical findings and various laboratory tests. Although several commercial diagnostic kits/ assays are available to identify and characterize the various species of mycobacteria, these cannot be put to use on large numbers of samples due to their high cost. Research for detection of a suitable antigen for serodiagnosis of tuberculosis has come a long way, and yet there is lot more to be explored. Therefore, immunodiagnosis seems to be ideally suited as a diagnostic method. Serodiagnostic tests like ELISA can show promise because of their ease of performance in field laboratories and cost effectiveness.

In this study, ten clinical isolates were screened and the selected bacterial isolate (KUMT09) was identified based on the morphological, biochemical and 16S rRNA gene sequence. The nucleotide sequence for the 16S rRNA gene (1363 bp) reported was highly related to *M. tuberculosis* exhibiting high similarity (~ 99 %). The sequence was initially analyzed at the NCBI server (http://www.ncbi.nlm.nih.gov) using the BLAST tool, corresponding sequences were downloaded, and the sequence homology analysis was performed. A phylogenetic tree was constructed by the neighbour joining method. The sequence was deposited at GenBank with accession no. JQ394980. In this study, sera from 200 TB patients were evaluated for the presence of antibodies against soluble extract (SE) and culture filtrate (CFA) antigens. By using MTSE ELISA assay, 70 samples showed high IgG positivity; 48 samples showed high IgM positivity and remaining 31 samples showed both IgG and IgM positivity. By using MTCFA ELISA assay, 65 samples showed high IgG positivity; 49 samples showed high IgM positivity and remaining 35 samples showed both IgG and IgM positivity. By using commercially available ELISA kit, 168 samples appeared to be positive out of 200 serum samples. 80 samples showed high IgG positivity; 54 samples showed high IgM positivity; and remaining 34 samples showed both IgG and IgM positivity. The sensitivity of the test appeared to be 88.7 %.

The detailed methodology provided here will enable different laboratories to standardize and carry out this test routinely. In addition, it does not require any sophisticated equipment or special skilfulness and offers the opportunity of investigating a large series of samples in a short period. The in-house assay for the surveillance of TB would be most suitable as the cost of commercial kits would be prohibitive for most TB control programs.

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