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31 MAY 2012

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Via E_mail
CONFIRMATION VIA COURIER

Sub. New Patent Application # 1629/DEL/2012 dated 30.5.2012 on "A Diagnostic Kit based on Rapid Liposomal Agglutination (TB/M Card) Test for Detection of Antigens in Patients with Meningial, Pulmonary Tuberculosis" - Filing Intimation

Dear Dr Bisht

This is to inform you that a patent application, along with the complete specification covering the subject matter of above-mentioned invention, has been filed at Patent Office, New Delhi on 30.5.2012 and the said application has been accorded application # 1629/DEL/2012.

A copy of the complete specification, as filed, and Official Filing Receipt (OFR) is being mailed to you through courier today for your reference and records.

Please note that Request for Examination, along with requisite fee of Rs. 10,000/-. Is being submitted at IPO, New Delhi to get the application examined without ant delay upon its publication in the Officail Journal of Patent Office after the expiry of 18 months from the priority date i.e date of filing (30.5.2012)

Our next action will be initiated after receiving the First Examination Report (FER). We expect to receive this FER after expiry of 2 years from the date of filing i.e. around 30.5.2014. However, the request for expedited publication could be filed, if you so desire. The requisite fee for expedited publication is additional Rs. 10,000/-. In such a case, the application will be published after 1 month of payment of such fee and accordingly the examination will be expedited. Please let us know if you wish to adopt the expedited publication route.

In all your future correspondence with us, please quote our File Ref.# UD1629.

In case, you have any query in this connection, please do not hesitate to call us.

Thanking you

Sincerely yours

(C M Gaind)

Enclosed : As stated

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Serial Number	Reference Number/Application Type	Application Number	Title/Remarks	Amount Paid	Amount Computed	Fee Payment
1	F-18(iii)/1053/2012-DEL	1332/del/2004	--	800	800	Full
2	ORDINARY APPLICATION Pages:-44 , Claims:-12	1629/DEL/2012	A DIAGNOSTIC KIT BASED ON RAPID.	11200	11200	Full
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THE PATENTS ACT, 1970

COMPLETE

SPECIFICATION

SECTION 10

TITLE

"A Diagnostic Kit, based on Rapid Liposomal Agglutination (TB/M Card) Test, for Detection of Antigens in Patients with Meningial Pulmory Tuberclosis".

APPLICANT

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The following specification particularly describes the invention and the manner in which it is to be performed.

FIELD OF INVENTION

This invention relates to a diagnostic kit based on rapid liposomal agglutination (TB/M Card) test for detection of glycolipid antigens in patients with Meningial, pulmonary and other extra-pulmonary tuberculosis.

BACKGROUND OF INVENTION

Tuberculosis is the second most infectious disease and is the leading cause of death in the World. As per the estimates of World Health Organization, the Mycobacterium tuberculosis infects 1.7 billion people every year with an annual mortality rate of approximately 3 million. In India TB is known as the king of diseases having long survival in dormant state and marked ability to develop resistance. Although TB is a curable disease with the use of appropriate antibiotics, the major hurdle in the treatment lies in the late diagnosis of the disease due to unavailability of sensitive, simple and cost effective diagnostic test. Timely and accurate identification of persons infected with M. tuberculosis and rapid laboratory confirmation of tuberculosis are two effective public health measures that can be taken to combat the tuberculosis epidemic. Thus there is an urgent need to develop strategies to effectively tackle the problems associated with diagnosis and chemotherapy thereafter[1].

Tuberculosis has been declared a global emergency. The main requirement for its control is the rapid and accurate identification of infected individuals. However, the diagnosis of tuberculosis continues to pose serious problems, mainly because of difficulties in differentiating between patients with active tuberculosis and those with healed lesions, normal mycobacterium boris BCG (Bacillus Calmette Guerin) vaccinated individuals, and unvaccinated Manteux positives. Physicians still rely on conventional methods such as Ziehl-Neelsen (ZN) staining, fluorochrome staining, sputum

culture, gastric lavage, and other non-traditional methods [8]. Although the tuberculin test has aided in the diagnosis of tuberculosis for more than 85 years, its interpretation is difficult because sensitization with nontuberculous mycobacteria leads to false-positive tests. A number of proteinaceous and nonprotein antigens (such as acyltrehaloses and phenolglycolipids) have been explored from time to time for the development of such assays but they have not proved to be clinically useful. It has been difficult to develop an ELISA utilizing a suitable antigen because *M. tuberculosis* shares a large number of antigenic proteins with other microorganisms that may or may not be pathogenic. With the advent of molecular biology techniques, there have been significant advances in nucleic acid-based amplification. The detection of mycobacterial DNA in clinical samples by polymerase chain reaction (PCR) is a promising approach for the rapid diagnosis of tuberculous infection. However, the PCR results must be corrected for the presence of inhibitors as well as for DNA contamination.

Detection of mycobacterial antigens in CSF, serum, and sputum have been investigated with sensitivity between 45-88% and specificity 91-100%. Previously, an antibody based enzyme immunoassay for serodiagnosis of tuberculosis was devised by using a shotgun immuno-expression library in the λgt11 vector. The system offered a sound demarcation between BCG-vaccinated healthy subjects from patients with active tuberculosis and seemed an effective marker in detecting pulmonary and extra-pulmonary tuberculosis with 84.33% sensitivity and 93.62% specificity. An attempt has been made to develop "TB Screen test", which detects antiglycolipid antibodies present in the serum samples with 94.0% sensitivity and 98.3% specificity. However, both tests were found inadequate in detecting antibodies in CSF and tissue biopsy samples.

To overcome these problems, an effort has been undertaken to devise a simple, rapid, cost-effective, sensitive, and specific liposomal agglutination card test for detection of mycobacterium glycolipid antigens in various biological specimens during active tuberculosis infection. These glycolipid antigens have been found as components of the mycobacterial cell or as their metabolites present in the serum, CSF, and/or tissue biopsy samples. Moreover, surface glycolipid antigens of *M. tuberculosis* in the samples reacted with TB/M card test reagent, agglutinated within 4 minutes, and clearly differentiated between patients with active tuberculosis, those with previous vaccination (BCG), and healthy subjects with 97.3% sensitivity and 96.6% specificity.

A rapid liposomal agglutination test (TB/M Card test) for the detection of mycobacterial glycolipid antigens in patients with active tuberculous meningitis, pulmonary and other extra-pulmonary tuberculosis has been developed. Glycolipids of Mycobacterium tuberculosis H37Rv which were employed in antibody based TB Screen test developed earlier by our group (Indian Patent, US Patent, World Patent and European Patent)1- 4 were used to raise polyclonal antibodies in Rabbit. serum was obtained from the rabbit and anti-glycolipid antibodies (IgG) were purified by affinity column chromatography. The purified antibodies were coupled to activated liposome particles (size 0.2 0.4µm) in presence of phosphatidyl ethanolamine (PE), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and N-hydroxysuccinamide (Fig.3) and used as working reagent to detect glycolipid antigens of M. tuberculosis in serum / plasma /tissue biopsy extract and other biological fluids from patients with pulmonary or extra-pulmonary tuberculosis. Liposome conjugated antiglycolipid antibodies, when incubated on plastic slide/ card with the serum or plasma or biological fluid of subjects suffering from meningial,pulmonary and extra-pulmonary tuberculosis exhibit dark blue agglutination (clumping) which is visible to the naked eye within 4 minutes (Fig.1). The test facilitates early diagnosis of pulmonary as well as

extra-pulmonary tuberculosis. The test had been validated at laboratory level for sensitivity as well as specificity. The test was proved to be highly sensitive (97.4%) and specific (96.9%) for early diagnosis of tuberculosis. The test clearly differentiates between patients with active tuberculosis, those with previous vaccination (BCG), and healthy subjects. The analytical sensitivity of the TB/M card test was found to be 1ng/ml and no cross reactivity was reported when tested with culture filtrates of sonicated non-mycobacterial and mycobacterial strains 5. The test is an effective and rapid tool not only for diagnosis but also for monitoring the efficacy of anti-tuberculous chemotherapy. The test is found highly cost effective and does not require ice-cold temperature either during transit or during storage. No diagnostic technology with such a combination of unique features (rapidity associated with desired sensitivity, specificity, simplicity, cost effective ness and stability of reagents (at ambient temperature) is available in the world for diagnosis of pulmonary and extra pulmonary tuberculosis.

In order to differentiate between the present invention from currently available diagnostic methods, it is necessary to understand the merits and demerits of laboratory diagnostic and immunodiagnostic methods which are summarized as follows:

(A) Laboratory Diagnostic Methods:

The currently used methods[8] for laboratory diagnosis of tuberculosis are outlined below:

1. Ziehl-Neelsen (ZN) test: The simplest rapid diagnostic method is the detection of acid fast bacilli by microscopy. No specialized skills are needed for microscopy.

2. Culture method: Examination of sputum culture is the most reliable method for detecting pulmonary tuberculosis in a clinical setting. The current methods used in clinical laboratories are growth-dependent and may take 2-4 weeks to obtain the result.

3. Mantoux test: The most commonly used method in the history of tuberculosis diagnosis is the Mantoux test. However, this test can not be used for definitive diagnosis as it fails to differentiate BCG vaccinated healthy individuals and TB patients at times.

4. Radiometric culture: The BACTEC 460 radiometric system (Becton Dickinson Instrument Systems, Sparks, MD) is an auto-mated method for detecting 14CO2 liberated by bacteria during metabolism and decarboxylation of 14C-labeled substrates. For detection of mycobacteria, the system uses 14C-labeled palmitic acid as the substrate in modified Middlebrook 7H12 broth.

5. HPLC:High-performance liquid chromatography (HPLC) is a rapid and highly specific method for detecting the unique pattern of mycolic acids for identifying mycobacterial species. The Sherlock mycobacteria identification system (SMIS) uses computer software to identify mycobacterial species on the basis of mycolic acid pattern generated by HPLC.

6. Tuberculostearic acid detection: One easily detectable marker/component of M. tuberculosis is tuberculostearic acid, which can be detected in femtomole quantities by gas-liquid chromatography. The presence of tuberculostearic acid in cerebrospinal fluid is thought to be a diagnostic marker for tuberculous meningitis and has been suggested to be useful in diagnosing pulmonary tuberculosis as well.

7. Mycobacteriophage-based methods: A reporter mycobacteriophage that can infect only M. tuberculosis specifically has been designed to detect viable/living mycobacteria in a patient specimen. The phage-amplified biological (Pha B) assay uses mycobacteriophage D29 to detect viable M. tuberculosis. The Phage Tek MB assay (Organon Teknika, Durham, NC, USA) is an inexpensive, phenotypic bacteriophage-based assay that (within 24 hrs.) detects viable M. tuberculosis complex organisms in decontaminated sputum samples with a sensitivity of 31.1% and specificity of 86.1%. Its low sensitivity makes it a poor screening test for tuberculosis.

A genetically engineered mycobacteriophage carrying the gene for luciferase has been used for detecting viable M. tuberculosis directly in clinical specimens. Luciferase enzyme oxidizes luciferin in the presence of adenosine triphosphate (ATP) and generates light. Viable mycobacteria that are infected with this reporter mycobacteriophage emit visible light when luciferin (substrate for luciferase) is added. The emitted light may be measured by a luminometer. This test is commercially available and provides results within 48 hrs. Since the test can differentiate between live and the dead mycobacteria, it can be used for sensitivity testing of anti-tubercular drugs. The FAST Plaque TB assay is a rapid manual test that uses mycobacteriophage for directly detecting M. tuberculosis in sputum. Samples containing 500– 5,000 mycobacteria generate a clear positive signal with 70.3–75.2% sensitivity and 98.7–99.0% specificity.

B: Imunodiagnostic Methods

Various immunodiagnostic Methods which are being used are described below:

1. Antibody detection tests: Some of the commercially available antibody detection tests are listed in Table 1. In a comparative study on seven serodiagnostic tests, a combination of two tests (ICT and Pathozyme-Myco) yielded the best results, with a sensitivity of 66% and specificity of 86%

Table 1: Some antibody detection test12

Test	Antigen used
Myco Dot (Dot blot assay)	Lipoarabinomannan (LAM)
Detect TB (ELISA)	Recombinant peptide
Pathozyme-Myco (ELISA)	38kDA (recombinant) and LAM
Pathozyme -TB (ELISA)	38kDA (recombinant)
Antigen A-60 (ELISA)	Antigen 60
ICT diagnostics (membrane based)	38 kDA (recombinant)

2. ASSURE TB rapid test (Gene labs Diagnostics Pte Ltd., Singapore): It is an indirect solid-phase immune-chromatographic assay for detecting antibodies in clinical samples (plasma, serum, or whole blood). The test employs an antibody-binding protein conjugated to colloidal gold particle and a "cocktail" of novel antigens—Mtb 11 (CF10), Mtb 8, Mtb 48, Mtb 81 and 38 kDa protein—immobilized on the membrane, in lateral flow devices.

In the recently patented TB screen test (Madhav Institute of Technology and Science, Gwalior & National Research Development Corporation, Government of India), a "cocktail" of purified cell wall-associated antigens of M. tuberculosis (H37Rv) were incorporated on to the surface of liposome particles. This "cocktail" of antigens reacts with specific antibodies present in clinical samples to produce a blue agglutination. The test differentiates healthy controls and BCG-vaccinated individuals from those with active tuberculosis. The TB screen test has the potential for use on (four minutes), high sensitivity (94%) and high specificity (98.3%).

3. Antigen detection tests: Free mycobacterial antigens may be detected in the various types of body fluids at a minimum concentration of 3–20 mg/ml. The most commonly used antigens are PPD, glycolipids, sulpholipids, lipopolysaccharides, antigen5 (38 kDa), antigen A60, 45/47 kDa antigen, antigen KP90, 30 kDa antigen, P32 antigen, lipoarabinomannan (LAM), cord factor (trehalose-6, 60dimycolate), and phenolglycolipid-lipid antigen (PLG Tb 1).

The antigen detection methods include—sandwich ELISA, inhibition ELISA, latex agglutination, and reverse passive haemagglutination tests. Enzyme-linked immunosorbent assay (ELISA) is useful for early diagnosis of all forms of tuberculosis. Capture ELISA is a quantitative test that detects LAM in urine samples. The Dipstick method detects LAM in both pulmonary and extra-pulmonary specimens semi-quantitatively. In a field test, the Dipstick method had a sensitivity and specificity of 93% and 95%, respectively.

4. Gamma interferon assay: The 6 kDa early secretory antigen TB is recognized by T-cells of patients of tuberculosis, but not by T-cells of the BCG-vaccinated or the healthy unvaccinated individuals. The levels of IFN-gamma increase in the peripheral blood in the treated, as compared to the untreated patients. In the Gamma interferon assay, mononuclear cells from the peripheral blood are stimulated in vitro and ELISA measures production of IFN-gamma from the sensitized T-cells. The sensitivity of this assay was not significantly different in extra-pulmonary and pulmonary cases (83% vs. 74%), and smear-negative and -positive cases (80% vs. 71%). The assay is, therefore, interesting but not feasible in smaller laboratories and demands technical expertise.

5. Approaches based on molecular biology: In any molecular diagnostic technique, a specific sequence of nucleic acid in the clinical sample is

hybridized with a complementary sequence (probe). This is followed by detection of the hybrid. The target sequences present in the clinical specimen are amplified using suitably designed primers before hybridization. Any sequence of nucleic acid can be amplified by using DNA polymerase, if the information on that sequence is available.

6. Target sequences with diagnostic relevance: For diagnosing tuberculosis, the target sequences including genes encoding the 32, 38 or 65 kDa antigens, or the dnaJ, groEl, or mtb-4 genes are identified. The most frequently used target sequence is the IS 986 or IS 6110 repetitive element that is present in multiple copies (up to 20) in most strains of M. tuberculosis complex.

Several amplification systems have been described for use with M. tuberculosis, including strand displacement amplification (SDA), polymerase chain reaction (PCR) amplification, transcription-mediated amplification (TMA), oligonucleotide ligation amplification, and Q-beta replicase amplification. The first four of these amplification systems are the best developed systems for mycobacteria. Species-specific SDA assays have been developed for M. tuberculosis, M. avium, and M. Kansasii.

7. Polymerase chain reaction (PCR): PCR based on (a) conventional DNA amplification, (b) nested PCR, or (c) real time are available in reference laboratories. PCR targets DNA, rRNA, insertion and repetitive elements, and various protein-encoding genes. Several Indian investigators have used separate gene targets like 38 kDa, TRC 4, and IS 1081 for this purpose. Most strains belonging to M. tuberculosis complex carry multiple copies of the insertion element IS 6110. However, an Indian strain lacks this DNA insertion sequence. RFLP patterns of 36-bp direct repeat (DR) sequence and polymorphic GC-rich repetitive sequence

(PGRS) are stable, which are used for identifying the isolates. In general, the PCR amplification process can be completed in 2–4 hrs after obtaining the processed clinical sample. The detection assay requires an additional 2–24 hrs. Since, the storage of the sputum samples on a filter paper for 5 days at room temperature reportedly has no apparent adverse effect on the performance of the nested PCR, clinical samples from the peripheral facilities may also be subjected to PCR in a reference laboratory.

8. Transcription mediated amplification (TMA) test: TMA, an isothermal target-based amplification system developed by Gen-Probe Inc. (San Diego, CA), has been combined with a homogeneous detection method to detect. M. tuberculosis in clinical specimens. This test (the Gen-Probe amplified M. tuberculosis direct test (MTD) test) uses the sediments prepared by the standard NALC/NaOH method and lyses the mycobacteria by sonication. Ribosomal RNA (rRNA) is amplified via TMA such that the rRNA target sequences are copied into a transcription complex by using reverse transcriptase, and then RNA polymerase is used to make numerous RNA transcripts of the target sequence from the transcription complex. The process is then repeated autocatalytically. The amplified sequences are detected by using an acridinium ester-labeled DNA probe specific for M. tuberculosis in a homogeneous solution hybridization assay format similar to that used in the Gen-probe Accuprobe species identification system. The lowest detection limit is 100 organisms. The method demands high technical skills and far from the reach of peripheral health centers.

9. Ligase chain reaction (LCR): LCR, a variant of PCR, is potentially useful for screening persons at high risk for developing tuberculosis and extra-pulmonary tuberculosis. A pair of oligonucleotides is made to bind to one of the DNA target strands so that they are adjacent to each other. A second pair of oligonucleotides is designed to hybridize to the same

regions on the complementary DNA. DNA polymerase fills the gaps between the primers with suitable nucleotides, while ligase binds the primers. The LCR DNA amplification method was recently developed as a commercial test for the detection of M. tuberculosis. The method has a high sensitivity for both smear-positive (100%) and smear-negative samples, and is suitable for screening of M. tuberculosis in high-risk patients. Because this method requires costly kits and expertise, it will take quite some time for it to find widespread acceptance in routine clinical laboratories.

10. Q-beta replicase amplification: Q-Beta replicase enzyme is used for producing RNA in the amplification reaction at a fixed temperature. When a suitable combination of capture and detector probes is used, M. tuberculosis is detected with a sensitivity of up to one colony-forming unit (cfu).

11. The LCX M. tuberculosis assay (Abbot): This test primarily makes use of the respiratory specimens. It has a high sensitivity and specificity for smear-positive and -negative specimens. Currently, the use of this technique is limited by its high cost and lack of skilled personnel.

12. Branched DNA signal amplification: This assay involves construction of a bifunctional oligonucleotide probe that contains a sequence specific for the target mycobacterial species. An enzyme-modulated signal amplification system increases the sensitivity of the assay. This assay can theoretically detect as few as 100–1000 organisms in a clinical specimen.

13. Peptide nucleic acid (PNA) assay : Peptide nucleic acid (PNA) is a DNA replica in which the sugar phosphate backbone of DNA is replaced by a polyamide backbone. PNA probes hybridize DNA or RNA with excellent affinity. The sensitivity of PNA probes targeting M. tuberculosis and a

typical mycobacteria is reported to be 98% and 57%, respectively.

Although Ziehl-Neelsen test is being carried out on day to day basis in peripheral health centres, the test suffers from poor sensitivity. About 40–60% of patients with pulmonary disease and 75% of patients with extra pulmonary disease go undiagnosed as this method inherits the following limitations: (i) a minimum of 104 organisms/mL of sputum is necessary, (ii) it cannot differentiate Mycobacterium tuberculosis from the other non-pathogenic and the saprophytic mycobacteria and (iii) it cannot diagnose paucibacillary pulmonary and extra-pulmonary tuberculosis.

The culture method is deemed the "gold standard" for diagnosing tuberculosis; however, it can take up to 2-6 weeks. The process is lengthy and cumbersome, and requires the use of asceptic laboratory conditions for mycobacterial culture.

Chest radiography provides some clues, but the radiographic analysis is often ambiguous and not-very-specific for tuberculosis. Concomitant HIV infection may further vitiate the classical radiographic analysis of the lesions in pulmonary tuberculosis. Interpretation of the radiographic findings is often prone to inter-observer variations.

The Mountoux test suffers from inherent set backs. A positive tuberculin test may suggest active tuberculosis, past infection, BCG vaccination, or sensitization by environmental mycobacteria. A negative result in this test may not necessarily exclude tuberculosis..

Detection of tuberculostearic acid yields although specific and sensitive diagnosis of tuberculous meningitis, a trained doctor is needed to draw the cerebrospinal fluid and expensive Gas-chromatograph.

The gamma-interferon assay, although used widely now a day, requires the use of laboratory facilities to stimulate viable lymphocytes, and an enzyme iummunoassay to quantify IFN-g and the test is not cost-effective too.

With the BACTEC system, the number of positive cultures may or may not be higher than that obtained by conventional culture on solid media. The BACTEC system is expensive, and the disposal of radioactive waste precludes its use in peripheral health centers.

The HPLC based diagnosis demands very expensive equipment and high degree of technical skills and is out of reach for routine diagnosis.

The immunodiagnostic tests based on antibody detection fails at times to diagnose current infections. Cross-reaction by environmental mycobacteria may produce false positive results. The currently available methods for purifying mycobacterial antigens are not reproducible and, therefore, the results of antibody detection assays are variable in different settings. It is also unlikely that the immune systems of all patients will recognize a single mycobacterial antigen alike. There is apparently no dominant specific antigen and most of the antibody response in the infected host is directed toward shared mycobacterial antigens.

The enzyme linked immunosorbent assays although sensitive suffer from the requisite specificity. More over, technical expertise and expensive equipment are needed for the enzyme assays to be carried out.

Effectiveness of the PCR technique in diagnosing tuberculosis is related to (a) DNA concentration in the clinical sample, (b) size of the target DNA sequence, (c) repetitiveness of the amplified sequence, (d) choice of primers, and (e) expertise of the personnel conducting the assay. The tests are

currently expensive and confined to reference and established laboratories. These tests also demand high degree of technical expertise and expensive equipment are needed. Though available at research institutions, they are not feasible in most of the clinical laboratories, particularly in the developing countries. Moreover, the PCR tests are unable to differentiate between viable and the dead AFB, which may lead to disagreement between the sputum smear and PCR results. Under the field conditions, the specificity of PCR may be low. A quality control study of seven laboratories worldwide found that false positives ranged from zero percent to 77%. In a second quality control study, 56% out of 30 participating laboratories reported false positives ranging from 5% to more than 50% of the spiked samples tested.

All DNA amplification based methods such as are cumbersome, expensive and out of the reach of a common public. Moreover these tests are impossible to be carried out at peripheral health laboratories in developing world. The extra-pulmonary and disseminated forms of tuberculosis are generally diagnosed by histopathology. The shortcomings of this method may include difficulty in obtaining representative clinical samples without the non-specific features.

None of the TB diagnostic tests reported so far are rapid and lack desired sensitivity and / or specificity but for 'TB screen test'. The 'TB screen test however, is based on antibody detection which may likely to fail in differentiating immediate past and current tuberculous infections. All the kits based on immunological (except for TB Screen test) or PCR based methods demand ice-cold chain during transport and storage and not at all cost effective. Moreover, almost all the existing tests but for TB screen test demand expensive equipment and high degree of technical expertise and out of reach to the common public.

The existing diagnostic tests are either poor in sensitivity and time taking

or very expensive and out of the reach of a common man. More over, the peripheral health centers are not equipped with the requisite infrastructure and technical expertise to carry out enzyme immunoassays or PCR and other DNA amplification based tests.

A diagnostic kit, involving the liposome based antigen detection test (TB/M Card test) developed under the present invention, is the simplest diagnostic kit with desired sensitivity and specificity available so far for tuberculosis. The test kit is suitable to screen various biological fluids as discussed in prior art and is qualified for diagnosis of active meningitis, pulmonary and other pulmonary tuberculosis subjects in peripheral laboratory setting and remote areas as well. The test is very simple and neither equipment nor special expertise is needed for conducting the test. The result is obtained within five minutes and can be carried out successfully at peripheral health centers by paramedical staff. The diagnostic reagents / kit can be transported and stored at ambient temperature and no refrigeration is required and is highly suitable for developing and under developed countries. The technology developed is highly cost effective (manufacturing cost less than Rs. 10/-). The test is proved highly specific and sensitive than the currently available commercial kits for tuberculosis.

OBJECTS OF INVENTION

The main objective of the present invention is to develop a diagnostic kit, involving liposomal agglutination card test for detection of glycolipid antigen in patients with pulmonary and extra-pulmonary tuberculosis,

Another objective of the present invention to provide a disgnostic kit highly suitable for use at peripheral health centers in developing and under developed world.

Yet another objective of the present invention is to devise a most rapid (< 5 minutes duration) and the simplest test kit

Still another objective of the present invention that the developed kit, technique, and technology should be such that the biological specimens of diverse nature viz., cerebrospinal fluid, Synovial fluid, serum, tissue biopsy extract could be screened.

Yet another objective of the present invention is to develop a diagnostic process wherein neither equipment nor technical expertise is needed either for conducting the test or for reading the result.

A further objection of the present invention is to develop a diagnostic method wherein no refrigeration of the diagnostic reagents / kit is needed either during transit or during storage.

An additional object of the present invention to devise a diagnostic method with high specificity and sensitivity than the currently available rapid commercial kits for tuberculosis.

Yet another additional object of the invention is develop a highly cost effective technology having a manufacturing cost less than ten Rupees.

The foregoing has outlined some of the pertinent objectives of the invention. These objectives should be construed to be merely illustrative of some of the more prominent features and applications of the intended invention. Many other beneficial results can be obtained by applying the disclosed invention in a different manner or modifying the invention within the scope of disclosure. Accordingly, other objectives and a full understanding of the invention and the detailed description of the preferred

embodiment in addition to the scope of invention are to be defined by the claims.

STATEMENT OF INVENTION

Accordingly the present invention provides a diagnostic test kit, based on Rapid Liposomal Agglutination (TB/M Card) Test, for detecting meningial, pulmonary and other extra pulmonary tuberculosis comprising liposomal antigen suspension, N-hydroxysuccinamide as working reagent, plastic slides, mixing stricks, negative and positive control .

BRIEF DESCRIPTION OF THE DRAWINGS

Further objectives and advantages of this invention will be more apparent from the ensuing description when read in conjunction with the accompanying drawings wherein:

Figure1: Bands 1 to 5 scratched from the TLC plate used for further studies

Figure 2: Immunoreactivity of antigenic glycolipids cahraterised on a TLC plate with a serum sample from a patient confirmed to have an active case of TB with the BACTEC 460 system. Eastern block of extracted and purified glycolipid antigen of M tuberculosis and their reaction with positive serum: A-1;Immunoactivity of antiglycolipid antibodies with serologically positive sera of patients with active pulmonary TB on a TLC plate: A-2; Immunoactivity of antiglycolipid antibodies with serologically positive sera of patients with Extra-pulmonary TB on a TLC plate: B-1;Immunoactivity of antiglycolipid antibodies with serologically significant antigen with sera from BCG vaccinated Human for cross recativity studies on a TLC plate: B-2;Immunoactivity of antiglycolipid antibodies with serologically significant antigen with sera from BCG unvaccinated human for cross recativity studies on a TLC plate. **Arrows indicate reactive bands**

Figure 3: Schematic representation of principles of TB antigen detection (TB/M card test) Kit.; Annexure – I : Designing of liposomes and their conjugation with specific antibodies. Annexure – II : Priciple involved in liposomal agglutination test TB/M Card Test.

While the invention is described in conjunction with the illustrated embodiment, it is not intended to limit the invention to such embodiment. On the contrary, it is intended to cover all alternatives, modifications and equivalents as may be included within the spirit and scope of the inventions disclosure as defined by the claims.

DETAILED DESCRIPTION OF THE INVENTION

For the purpose of promoting an understanding of the principles of the invention, reference is now to be made to the embodiment illustres in the drawings and specific language is used to describe the same. It is nevertheless to be understood that no limitations of the scope of invention is hereby intended, such alterations and further modifications in the illustrated bag and such further applications of the principals of the invention as illustrated therein being contemplated as would normally occur to one skilled in the art to which the invention relates.

In the current invention, an antigen based diagnostic test employing polyclonal antibodies raised against specific glycolipid antigens of Mycobacterium tuberculosis has been developed in the laboratory by the inventors. Affinity purified rabbit anti-glycolipid antibodies (IgG) were coupled to liposome particles (0.2-0.4 μm) in presence of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and N-hydroxysuccinamide. Following incubation (for 1-2 minutes) with tuberculous diagnostic specimen (any biological fluid) on a glass slide, the antibody-conjugated liposomes form a visible dark blue agglutination (clumping) within 2-4 minutes if M. tuberculosis antigens are present in the diagnostic specimen.

The description of the invention is detailed under the following two sub-heads viz:

A) Methodology adopted and

B) Results obtained

A) Methodology adopted:

A.1) Culturing of Mycobacterium tuberculosis

The culturing of M. tuberculosis H37Rv (ATCC 27294) obtained from the Central JALMA Institute for Leprosy and other Mycobacterial diseases, Agra, India was done on a Lowenstein-Jansen agar slant (2 x 109 CFU/ml). The bacterial culture was harvested by centrifugation (10,000 x g for 20 min) at 4°C, and the pellet was washed by resuspension in 100 ml of phosphate-buffered saline (PBS; pH 7.2). Finally, the bacterial pellet was resuspended in 10 ml of TEN buffer (pH 8.0; 10 mM Tris HCl, 1 mM EDTA, 100 mM NaCl), heat inactivated at 80°C (water bath) for 45 min, and then sonicated (15% pulse, 150 W) and lyophilized.

A.2) Extraction and isolation of lipid antigen(s) from Mycobacterium tuberculosis.

The lyophilized mycobacterial powder (5 g) was placed into a glass reagent bottle, and 100 ml of a chloroform-methanol mixture (2:1) was added to it. This mixture was stirred at room temperature for 60 min and filtered through What man no. 1 filter paper. A 1/5 volume of 0.7% KCl (20.0 ml) was added to the filtrate, and the mixture was shaken five to six times. The suspension was transferred to a separation funnel and kept at 2 to 8°C for overnight until two distinct layers were separated. The lower organic phase was washed with 1/5 volume of washing solvent (chloroform-methanol-water; 3:48:47) as described above by keeping it at 2 to 8°C for overnight. The upper aqueous phase was removed, and the lower organic phase was retained after filtration. The organic phase was dried by evaporating the

solvent in a rotary solvent evaporator at 40°C. The moisture was removed by flushing the dried mixture with nitrogen gas. Neutral lipids were removed from the dried mixture by adding 50 ml of chilled acetone while the mixture was vortexed for 10 min and then filtered through What man no. 1 filter paper. This step was repeated until the lipids in the flask became whitish or colorless. The contents of the flask were filtered through What man no. 1 filter paper, and the filtrate was discarded. The lipids present on the filter paper were dissolved with chloroform-methanol (2:1) and transferred to a round-bottom flask. The solvent was evaporated on a rotary evaporator under reduced pressure at 40°C. The crude preparation was reconstituted in 10 ml of chloroform-methanol (2:1) and stored at -20°C for further use.

A.3) Purification of lipid antigen(s) of M. tuberculosis.

Silica gel H activated at 110°C for 1 h (in a hot-air oven) was packed into a glass column (2.5 by 30 cm) with manual tapping, and a known quantity of crude material (1.0 g/5 ml of stock) was loaded on either side of the column. The column was run in an ascending direction in a chromatographic jar (4.5 by 25 cm) with purification solvent (150 ml; mobile phase) at a ratio of 65:25:4 (chloroformmethanol-water) and room temperature until the solvent reached the other end of the column. The column was removed from the chromatographic jar and placed in a fume hood to evaporate the solvent from the column. A 1-cm length of each fraction was carefully scrapped with a clean rod to separate the individual molecules that were adsorbed with the silica gel, depending on the mobility and retardation factor (Rf) value (percent mobilities of the five fractions, 46.6, 53.4, 58.3, 67.2, and 72.4%) of the individual molecule. The individual fractions were collected and placed into clean dry glass test tubes, which were labeled with the respective fraction number. Ten milliliters of extraction solvent (mixture of chloroform-methanol [2:1]) was added to each test tube, and the test tubes were kept at room temperature for 30 min. The purity of

the eluted material was analyzed by TLC, and the selected fractions were further filtered through What-man no. 1 filter paper to remove the silica gel from the samples. The pure fractions were pooled and run on preparative TLC plates to reconfirm the Rf value. After extensive study, the individual bands were scratched from the TLC plate, the silica gel was removed and the samples were analyzed by liquid chromatography with a mass spectrometer. The pure fractions were pooled and used (Fig. 1).

A.4) Quantization of glycolipids isolated from M. tuberculosis

The glycolipid moieties present in the samples were quantized by staining with the periodate-Schiff reagent for the detection of phosphatidates and glycolipids. The presence of glycolipids in these samples was further confirmed by alpha-naphthol staining for glycolipids. These samples were analyzed in detail by TLC and stained with vapor from iodine crystals in a dry, humidity-free chromatography staining chamber. The glycolipid sample (0.1 ml) was dried in a clean glass test tube, and 2 ml of orcinol solution (5-methylresorcinol; 2 mg/ml of 70% [vol/vol] sulfuric acid) was added to the sample. The reaction mixture was heated at 80°C for 20 min, and after it cooled, the absorbance at 505 nm of the color that developed was measured. The amounts of sugar moieties present in the glycolipids were calculated by using a glucose calibration curve.

A.5) Characterization of glycolipid antigen(s).

(A) Immunostaining on TLC plates:

The immunogenicity of glycolipid antigens was characterized by immunostaining on TLC plates. The TLC plates (silica gel 11-50 on an aluminum sheet; Merck, Darmstadt, Germany) were cut to 2.5 by 6.5 cm and activated at 110°C for 10 min. The plates were taken out and kept at

room temperature (with the avoidance of moisture), a pencil dot was made 1.0 cm from either end, and 20 µl samples were gradually loaded. The samples were run in the descending direction in a chromatographic jar containing 5 ml of purification solvent (for glycolipids), which consisted of a mixture of chloroform-methanol-water (65:25:4), and the solvent was run to the other end. The TLC plates were carefully removed from the jar with forceps and were kept over a blotting sheet at room temperature so that they could dry. Serum samples from patients with clinically confirmed TB were diluted 1:20 (0.2 ml of serum and 3.8 ml of PBS [pH 7.2]), and the diluted serum sample was poured into a clean petri plate. The TLC plate was dipped in the serum sample without shaking and was kept at 37°C for 1 h. The TLC plate was rapidly washed twice (without shaking) with wash buffer (PBS and 0.1% Tween 20), followed by incubation at 37°C for 30 min with rabbit anti-human immunoglobulin G (IgG) conjugated with peroxidase enzymes (Calbiochem, La Jolla, Calif.) diluted to a final concentration of 1:5,000. The TLC plate was again washed and developed with diaminobenzidine (DAB; Sigma, St. Louis, Mo.) solution (0.1% DAB in 100 mM Tris (hydroxymethyl) aminomethane hydrochloride [pH 8.0], 100 mM NiCl2, 0.006% H2O2). The reaction was stopped by washing the TLC plates with distilled water. The compound reacted with Rf values (46.6, 53.4, 58.3, 67.2, and 72.4) similar to those described above (Fig. 2)

(B) ELISA:

Polystyrene 96-well microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with 100 µl of antigen solution (1.0 µg/ml in n-hexane) per well were dried overnight at 37°C and blocked with 1.0% polyvinylpyrrolidone (PVP; Himedia, Mumbai, India) in PBS (pH 7.5) at 37°C for 3 h. The plates were washed with wash solution (PBS and 0.1% Tween 20) for 1 min at room temperature and dried under vacuum in a desiccator. Dilution buffer (PBS, 1.0% PVP, 0.1% Tween 20 [pH 7.5]) and 10 µl of a

serum sample from a patient with clinically confirmed TB were added to each well, and the plates were incubated at 37°C for 30 min. The plates were washed five times for 5 min each time with wash buffer (PBS, 0.5 M NaCl, 0.1% Tween 20). Goat anti-human IgG conjugated to peroxidase (Calbiochem) was diluted at ratio of 1:50,000 in dilution buffer (PBS, 0.1% Tween 20, 1.0% PVP), 100 µl of this mixture was added to each well, and the plates were incubated at 37°C for 30 min. After the plate was washed as described above, 100µl of tetramethylbenzidene (1.0% in dimethyl sulfoxide [Sigma]) was prepared in substrate buffer (citrate phosphate buffer, 0.006% H2O2 [pH 5.0]) and added to each well. The plates were incubated at room temperature for 15 min in the dark. The reaction was stopped with 50µl of stop solution (2.5N,H2SO4), and the absorbance at 450 nm was measured with a microtiter plate reader (Dynatech, USA).

A.6) Polyclonal antibodies against glycolipid antigens of M. tuberculosis

After characterization of the antigens, a cocktail of Glycolipid antigens from M. tuberculosis H37Rv (ATCC-27294) were injected into two young rabbits subcutaneously (200µg/ Rabbit; 250µl-IFA, 250µl-10mM, PBS, pH 7.2) and polyclonal antibodies were generated. The IgG from the antiserum was purified by protein A sepharose CL-4B affinity column chromatography (Amersham Pharmacia Biotech, Sweden), and used for the development of the present invention.

A.7) Preparation of activated liposomes

Phosphatidyl Choline 90mg; cholesterol 10mg; Phosphatidyl ethanolamine 100mg (Sigma) and dye (100 µl; 1% Sudan black B in chloroform) were mixed in a ratio of 9:1:10 in a pre-dried round-bottom flask, in which 10 ml of diethyl ether and 10 ml of Phosphate buffer (150mM; pH 7.2) was added while gentle vortexing followed by sonication (MSE,

England) for 60 seconds. The liposome vesicles were formed by slowly removing the diethyl ether under reduced pressure at 35° C followed by centrifugation at 10,000g for 10 minutes at 4°C, resuspended in 10 ml of phosphate buffer (10mM, pH 7.2) and stored at 4°C. To this, 15 ml (2.5mg/ml) of 1-Ethyl-3- (3-dimethylamin opropyl) carbodiimide hydrochloride (EDC, Sigma) in phosphate buffer (150mM, pH 5) was gently added while vortexing. The liposome solution was kept on an end-to-end rotary shaker at 4° C for 120 minutes for activation, followed by addition of 30 micromoles N-hydroxysuccinamide (NHS) and reaction was allowed to continue for another 120 min at 4° C to promote the coupling efficiency (Fig .3-Annex I).

A.8) Preparation of liposome – antibody conjugates

Affinity purified rabbit anti-glycolipid IgG (1mg) was tenderly added to 10 ml activated liposome solution and pH was adjusted to 8 by addition of NaOH (100mM) and incubated at 4° C for 16 hours. The entire method was optimized and standardized using variable concentrations of protein to be conjugated. The reproducibility of the preparation of liposomes and their coupling efficacy was analyzed. No major variation, however, (±5%) in the coupling of protein with liposomes was observed when the presence of protein in washing solution was estimated .The liposome suspension was dialyzed against distilled water at 4° C for 8 hours followed by centrifugation at 12,000g for 10 minutes at 4° C. Resulting pellet was washed thrice with Phosphate buffer (10mM; pH 7.2), re-suspended in 10ml of RP-2 buffer (NaH_2PO_4 10 mM, KH_2PO_4 10mM, EDTA 10mM, Choline Chloride 10% and Thiomersol 0.1%, pH 7) and eventually 10.0 ml of liposomal suspension (working reagent of test) was prepared and stored at 2-8° C ((Fig .3).

A.9) Screening of biological fluids by liposomal agglutination assay for detection of M. tuberculosis antigen

The culture filtrate of heat-inactivated sonicated M. tuberculosis strain in phosphate buffer saline (PBS; 150mM, 0.01%, NaN3, 0.1% BSA pH 7.2) served as positive control whereas 1% BSA in PBS or serum of normal healthy subjects were used as negative control.

Several hundreds of biological samples (Table-2) were collected from TB Hospital (Group, I II &III), Hamidia Hospital (Group, IV), Cancer Research Center (Group, V-A), Chhatrapati Shivaji maharaja hospital Kalwa, Mumbai (V-B) and Gandhi Medical College (Group, I-VA), Bhopal, India. Written consents were obtained from the Ethics Committee regarding selection of patients and from patients/volunteers before enrolling them in the study. Clinical diagnosis of the patients was based on history, signs, symptoms, chest radiography, sputum microscopy, histological findings, and/or results of tuberculin skin test, followed by further confirmation with PCR and BACTEC-460 methods. All samples were collected and processed under proper guidance and refrigerated in coded form with detailed history of the patients. Tissue biopsy specimens were sliced, homogenized (w/v) in PBS (10mM, pH 7.2), centrifuged at 10,000g for 30 minutes and supernatant was stored at 2-8°C for further use in which 0.01% sodium azide was added. Cerebrospinal fluids, sera and tissue biopsy samples from active tuberculosis subjects were used for investigation of sensitivity, whereas, sera from the healthy individuals (BCG vaccinated and non-vaccinated) or from patients with diseases other than tuberculosis were used for investigation of specificity of the TB/M card test.

Table 2: Details of different groups of subjects included in the study from various sources and status of their medications

S.No.	Group	No. of patients
I- A	Active TUM group (CSF)	
	(i) Culture+ve/Smear +ve	184
	(ii) Culture+ve/Smear -ve	120
	(iii) Culture-ve/Smear +ve	28
	(iv) Culture-ve/Smear -ve	16
	Total	348
I B	Control subjects (CSF)	
	(i) Aseptic Meningitis	50
	(ii) Septic Meningitis	30
	Total	80
II-A	Active pulmonary tuberculosis (Serum)	
	(i) Children TB	50
	(ii) Adults TB	50
	(iii) HIV co infected TB	98
	(iv) MDR tuberculosis	86
	Total	284
II-B	Extra pulmonary cases	
A	(i) Lymph node (Tissue biopsy)	40
	(ii) Abdominal (Tissue biopsy)	32
B	(i) Bone joint TB (Serum)	8
	(ii) Pleurisy (Serum)	10
	Total	90
II-C	Other extra-pulmonary tuberculosis	
	(i) Adult (Pleural fluids)	26
	(ii) Adult (Synovial fluids)	18
	Total	44
II-D	Control subjects	15
	(i) Gastrointestinal tract infection (Tissue biopsy)	25
	(ii) Other joint disease (Synovial fluids)	
	Total	40
III- A	Normal healthy blood donors (Serum)	50
(i)	Drug treated but sm. Negative subjects	50
(ii)	BCG vaccinated normal healthy subjects	58
(iv)	BCG Non vaccinated	75
	Total	233
III-B	Other respiratory diseases (Serum)	
(i)	Lung cancer	16
(ii)	Pulmonary fibrosis	11
(iii)	Bacterial pneumonia	48
(iv)	Bronchitis	12
(v)	Bronchial asthma	12
(vi)	Pulmonary aspergillosis	04
(vii)	Common cold disease	03
	Total	120

III C (i) (ii) (iii) (iv) (v)	Non-respiratory diseases (Serum) Other Mycobacterial disease Hepatitis U/C Malaria PV/Pf HIV (Non-Mycobacterial disease) Rheumatoid arthritis	16 21 23 35 20
	Total	115
Sub Total	For Sensitivity (I A, II-A, B, and C) For Specificity (I-B, II-C, III and IV)	766 594
	Gross total	1360

The sensitivity and specificity of the test was determined in comparison with the results of the culture and smear analysis of the same subjects, including patient's history, clinical manifestations, histopathological examination, PCR, BACTEC-460, and status of drug administration. For the calculating sensitivity and specificity, "indeterminate" samples were not included and were considered as "negative" test results.

The test reagents (liposomal suspension, positive and negative controls) were stored at 4-8°C (real-time) and 37°C (accelerated) for stability study. Shelf life of the test reagents was found to be one year when extrapolated with real-time as well as accelerated stability study.

B) Results obtained

B.1) Visualization of liposomal agglutination on glass slide or test card

The presence of glycolipid antigen in the biological specimens is indicated by a dark blue agglutination (clumping) on the glass slide / test card which is visible to the naked eye. The presence of agglutination considered as "positive" test for tuberculosis infection. During the first minute the clumps appear small; but with the progress of time the clumps become larger. Maximum clumping is observed within 4-5 min. No clumping indicates "negative" result. To validate the strength of agglutination and

reproducibility, technical and non-technical personnel confirmed the readings of TB/M card test and no major variations (±5%) were observed.

B.2) <u>Diagnosis of extra-pulmonary tuberculous meningitis by detecting glycolipid antigens employing liposomal agglutination test</u>

The ability of TB/M card test to diagnose tuberculous meningitis was determined by screening of about 348 CSF samples (Table 3A) from active tuberculous meningitis patients. 100% sensitivity was achieved with (n=184) culture positive and smears positive, 95.2% with (n=120) culture positive-smear negative, 87.5% with (n=28) culture negative-smear positive whereas 84.2 % with (n=16) culture and smear negative subjects. Thus, eventually 96.3% (335/348) sensitivity was achieved in subjects with tuberculous meningitis. Specimens (n=80) from non-tuberculous meningitis (Table 2B) but other CNS disorders (septic and aseptic meningitis) were enrolled as "control" samples. Out of 80 control samples 74 were negative, 5 false positive, and one showed ambiguity (75/80) with 94.1% specificity.

Table 3: Efficacy of liposomal agglutination test or TB/ M card test in detecting extra-pulmonary tuberculosis

S.No	Clinical status of Samples	No. of Sample tested	Specimen used	Results of TB/M card test			Sensitivity (%)	Specificity %
				+ve	-ve	Ind		
A(i)	Active TBM subjects Culture & Smear +ve (PCR+ve/BACTEC-460+ve)	184	CSF	184	0	0	100	-
(ii)	Culture +ve/ Smear -ve (PCR+ve/BACTEC-460+ve)	120	CSF	114	4	2	95.2	-
(iii)	Culture -ve / Smear +ve (PCR+ve/BACTEC-460+ve)	28	CSF	24	2	2	87.5	-
(iv)	Culture & Smear -ve, (PCR+ve/BACTEC-460+ve)	16	CSF	13	1	2	84.2	-
	Total	348		335	7	6	96.3	-
[illegible]	Control subjects (Other CNS Dis.) Aseptic Meningitis	50	CSF	3	46	1		94.3
[illegible]	Septic meningitis	30	CSF	2	28	0		93.7
	Total	80		5	74	1		94.1

(+Ve) indicates Positive, (-Ve) Negative and Ind. indicates indeterminate results with TB/M card test

B.3) Diagnosis of pulmonary tuberculosis by detecting glycolipid antigens employing liposomal agglutination test

The data presented in the Table 4A comprises 284 frank pulmonary tuberculosis patients, divided into four subcategories, which are, (i) children (10-15 years, n=50) (ii) adults (24-45 years, n=50) (iii) adults co-infected with HIV and tuberculosis (n=98) and (iv) Multi-drug resistant cases (n=86). Samples from children exhibited 96.1% sensitivity, whereas adults exhibited 98% sensitivity. Interestingly, samples from the latter two sub-groups; HIV-TB co-infected (97/98 with one false negative) and MDR cases (85/86 with one indiscriminate) showed about 99% of sensitivity. Final sensitivity of the TB/M card test in pulmonary tuberculosis was 98.2%.

B.4) Tissue biopsy extracts and serum samples from patients with extra-pulmonary tuberculosis

Attempts were made to evaluate the compatibility of the TB/M card test on specimens (n=90) from Patients with extra-pulmonary tuberculosis (Table-4B). These patients were divided in to two sub-categories: Patients in the 'a' sub-category (n=72) were subjected to tissue biopsy specimen, which were taken either from lymph node (n=40) or from abdomen (n=32). The test with the lymph node specimen showed 100% sensitivity (40/40), whereas the abdomen sub-category showed 96.9% (31/32 with one "indeterminate") sensitivity. Patients in the 'b' sub-category were either suffering from bone-joint tuberculosis (n=8) or pleurisy (n=10). Though the number of patients was relatively small, the trend was largely the same with 100% (8/8) sensitivity in bone-joint and 90.9% sensitivity in pleurisy TB patients with final sensitivity 97.8% in extra-pulmonary tuberculosis subjects. Pleural and synovial fluid could be used as specimens for pleurisy and other joint diseases.

Table 4: Efficacy of liposomal agglutination test or TB/M card test in diagnosing pulmonary and other extra-pulmonary tuberculosis.

S. No	Clinical status of subjects	No. of samples Tested	Clinical history	Results of TB/M card test			Sensitivity %	Specificity %
				+ve	-ve	Ind		
A (i) (ii) (iii) (iv)	Pulmonary tuberculosis (Serum) Children (age 10-15 yrs) Adults (age 24-45yrs) HIV +ve adults with active TB disease (18-40 years) MDR-Cases (Under therapy) (28-62 years)	 50 50 58 80	Chest X-ray+ve, PCR and Clinically +ve - - - -	 48 49 97 85	 1 - 1 -	 1 1 - 1	 96.1 98.0 98.9 98.8	 -
	Total	284	-	279	2	3	98.2	-
B a b	Extra-Pulmonary tuberculosis (i) Lymph node (tissue biopsy) (ii) Abdominal (tissue biopsy) (i) Bone joint TB (Serum) (ii) Pleurisy (Serum)	 40 32 8 10	Clinically +ve, PCR +ve - - -	 40 31 8 9	 - 1 - -	 - 1 - 1	 100 96.9 100 90.9	 -
	Total	90	-	88	-	2	97.8	-
C a b	Other extra-pulmonary tuberculosis Adult (Pleural Fluid) Adult (Synovial fluid)	 20 18	AFB, PCR, BACTEC 460 +ve - -	 20 18	 - -	 - -	 100 100	
	Total	38	-	38	-	-	100	-
	Sub total	412	-	411	2	5	98.3	-
D a b	Control subjects Gastrointestinal tract infection (Tissue biopsy) Other joint disease (Synovial fluid)	 15 25	AFB, PCR, BACTEC 460 -ve for TB -	 -	 15 24	 1	 -	 100 100
	Total	40	-	-	39	1	-	100
	Overall total	452	-	367	41	6	98.1	100

Healthy individuals and patients with diseases other than tuberculosis

It is evident from the data in the Table 5, out of 474 non-tuberculosis samples, 447 were found negative and 14 were false positive. In addition, 13 samples gave indiscriminate results with 96.6% accuracy. Specificity of TB/M card test against serum -from normal healthy subject with no information of BCG vaccination showed 100% (n=50), and -serum from drug treated cured M. tuberculosis subjects showed 96.1% (n=47, of these 2

found false positive and one indiscriminate). Moreover, serum from BCG vaccinated (n=58) and non-vaccinated (n=75) healthy individuals showed (57/58 and 74/75) >98% specificity.

Samples from other infectious group (non-tuberculous respiratory and non-respiratory disease) with TB/M card test exhibited promising results. Samples from patients suffering with lung cancer (n=16) and bronchial asthma (n=12) showed 100% specificity, whereas those suffering from - pulmonary fibrosis showed 93.3% (n=14), -bacterial pneumonia 94.1% (n=48), -bronchitis 92.3% (n=12), -pulmonary aspergillosis 90% (n=9), and - common cold 88.2% (n=15.The individual who suffered from other mycobacterial diseases (n=16), hepatitis B/C (n=21) and malaria (n=23) showed 100% specificity, which strengthens impetuosity of the TB/M card test. HIV-infected but not TB individuals (n=35) and those with rheumatoid arthritis (n=20) exhibited 97.2% and 95.2% specificity, respectively, each with one false positive result. The final specificity in patients with non-tuberculous respiratory and non-respiratory diseases was 94.0% and 98.2%, respectively (Table-5). The surface glycolipid antigens of M. tuberculosis in the samples reacted with TB/M card test reagent, agglutinated within 4 minutes, and clearly differentiated between patients with active tuberculosis, those with previous vaccination (BCG), and healthy subjects. Taken together, it may be concluded that the TB/M card test possesses a high degree of specificity.

Table 5: Evaluation of liposomal agglutination test or TB/M card test for it's specificity employing serum samples of normal healthy control subjects (Non-TB group), respiratory and non- respiratory disease (Non TB group).

S. No.	Clinical Status of subjects	No. of Sample Tested	Outcome	Results of TB/M card test			Specificity %
				+ve	-ve	Ind	

Normal healthy subjects-A	Normal healthy blood donors	50		Clinically healthy	-	50	-	100
	Drug treated but clin. Negative subjects	50		Cured TB patients (Clin. Healthy)	2	47	1	96.1
	BCG Vaccinated normal healthy subjects	58		Clinically negative	1	56	1	98.3
	BCG Non-Vaccinated Normal Health subjects	75		Clinically negative	1	72	2	98.6
	Total	233		-	4	225	4	98.3
Respiratory Diseases-B	Lung cancer	16		Symptoms	-	14	2	100
	Pulmonary fibrosis	14		Lab analysis	1	12	1	93.3
	Bacterial pneumonia	46		Gram staining	3	43	2	94.1
	Bronchitis.	12		Swab culture &	1	10	1	92.3
	Bronchial asthma	12		Clinical symptoms	-	11	1	100
	Pulmonary aspergillosis	9		Biochemical test	1	8	-	90.0
	Common cold	15		Symptoms	2	12	1	88.2
	Total	126		-	8	110	8	94.0
Non Respiratory Diseases-C	Other Mycobacterial disease	16		Clinically & PCR	-	16		100
	Hepatitis D/C	21		ELISA/Western Blot	-	21	-	100
	Malaria pv/pf	23		Std/rapid test	-	23	-	100
	HIV-1/2 (Not TB)	35		ELISA/Western Blot	1	33	1	97.2
	Rheumatoid arthritis	20		Symptoms	1	19	-	95.2
	Total	115		-	2	112	1	98.2
	Overal total	474		-	14	447	13	97.1

Analytical sensitivity and cross reactivity with other non-mycobacterium and mycobacterial species:

The analytical sensitivity of the TB/M card test was determined with purified glycolipid antigen, with whole cell lysate and the culture filtrate of a 3-week-old culture of M. tuberculosis after quantification. The test detected as low as 1ng of purified glycolipid/ml (Table-6).

Table 6: Determination of Analytical sensitivity with purified glycolipid antigens and culture filtrates of sonicated strains of M. tuberculosis

Sl. No	Dilutions of purified	Results of TB/M	Time taken	Dilutions of sonicated M.TB	Results of TB/M	Time taken

	Glycolipid antigen (Ref.53)	card test	(Min)	H37RV (ATCC-27294) strain	card test	
01	1000µg	4+ve	1Min.	1000µg	4+ve	2Min.
02	100µg	4+ve	2Min.	100µg	3+ve	3Min.
03	10µg	3+ve	2 Min.	10µg	2+ve	4Min.
04	5ug (1000ng)	2+ve	3Min.	1µg (1000ng)	2+ve	4Min.
05	100ng	2+ve	4 Min.	100ng	1+ve	4Min.
06	10ng	1+ve	4Min.	10ng	+ve	4Min.
07	5.0ng	>+ve	4Min.	5.0ng	+ve	4Min.
08	1.0ng	±ve	4Min.	1.0ng	±ve	4Min.
09	0.5ng	-ve	5 Min.	0.5ng	-ve	5Min.
10	0.25ng	-ve	5Min.	0.25ng	+ve	5Min.

The strength of agglutination is based on the concentration of antigen present in respective dilution and it doesn't increase or decrease after 4 minutes. The agglutination pattern ± indicated weak reaction

The cross reactivity of the liposomal agglutination test was examined with other lab strains and different clinical isolates. No cross reactivity was observed with other mycobacterial and non-mycobacterial species (Table-7).

Table 7: Cross reactivity study of TB/M card test with culture filtrates of non-Mycobacterial and Mycobacterial sonicated strains.

S.No	Name of bacterial strains	Type of strains used	Concentration of sonicated strains	Results of TB/M card test
A	Non Mycobacterium			
I	E.Coli *2	ATCC35695	100µg-1ng/ml	-ve
Ii	H.influenzae *1	Clinical isolates	100µg-1ng/ml	-ve
Iii	S.Pneumoniae *2	Clinical isolates	100µg-1ng/ml	-ve
Iv	S.aureus *2	Clinical isolates	100µg-1ng/ml	-ve
V	C.albicans *2	Clinical isolates	100µg-1ng/ml	-ve
B	Mycobacterium			
I	M.bovis *2	ATCC19210	100µg-1ng/ml	-ve
Ii	M.avium *2	ATCC-25291	100µg-1ng/ml	-ve
Iii	M.intracellulare *1	ATCC 13761	100µg-1ng/ml	-ve
Iv	M.Smegmatis *2	ATCC14468	100µg-1ng/ml	-ve
V	M.fortuitum *2	ATCC6841	100µg-1ng/ml	-ve

In a nutshell, antigen based liposomal agglutination test or TB/M card test is an effective and rapid tool for diagnosing patients with tuberculous meningitis, pulmonary, extra-pulmonary, MDR tuberculosis. The test can serve as a tool for monitoring the efficacy of chemotherapy. Thus the antigen based liposomal agglutination test is the first antigen based liposomal agglutination test with enormous sensitivity, specificity, simplicity. The diagnostic test is portable at ambient temperature and does not require sophisticated infrastructure or technical skill for diagnosing the tuberculosis infection.

The test has been developed, standardized, established and evaluated at internal and external laboratory settings for determination of sensitivity and specificity with several hundreds of various biological specimens. The

stability of diagnostic reagents at different temperature has (Storage temp;4-8°C,room temp 25-30°C,and accelerated temp 37/45°C) also been done for establishment of product self life.

All documents cited in the description are incorporated herein by reference. The present invention is not intended to be limited in scope by the specific embodiments and examples which are intended as illustration of a number of aspects of the scope of this invention. Those skilled in art will know or to be able to ascertain using no more than routine experimentations many equivalents to the specific embodiments of the invention described herein.

It is to be further noted that present invention is susceptible to modifications, adaptations and changes by those skilled in the art. Such variant embodiments employing the concepts and features of this invention are intended to be within the scope of the present invention which is further set forth under the following claims:

References

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We Claim

1. A diagnostic test kit, based on Rapid Liposomal Agglutination (TB/M Card) Test, for detecting meningial, pulmonary and other extra pulmonary tuberculosis comprising liposomal antigen suspension, N-hydroxysuccinamide as working reagent, plastic slides, mixing stricks, negative and positive control .

2. A kit as claimed in claim 1, wherein said liposomal antigen suspension is rabbit anti-glycolipid antibodies (IgG) coupled to liposome particles (0.2–0.4μm) in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride.

3. A kit as claimed in claim 1and claim 2, wherein said liposomal antigen suspension was prepared by:

   (a) mixing Phosphatidylcholine (75-125mg), Cholesterol (10-25mg), Phosphatidyl ethanolamine(100-150mg), and dye (50-150μl;2% Sudan blue in chloroform) in a pre-dried round-bottom flask;

   (b) adding 10-20 ml of diethyl ether and 10-20 ml of phosphate buffer (150-200 mM, pH 7.2-7.5) during gentle vortexing, followed by sonication for 60-120 seconds;

   (c) removing slowly the diethyl ether under reduced pressure at 35-40°C followed by centrifugation at 10,000-12,000 g for 10 -20min at 4°-8°C;

   (d) re-suspended in 10-20 ml of phosphate buffer (10-25 mM, pH 7.2-7.5);

(e)adding gently 15 ml (2.5 mg/ml) of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) in phosphate buffer (150-200 mM, pH 5-6) while vortexing;

(f) rotating the liposome solution at 4°-8°C for up to 120-180 min for activation, followed by the addition of 30-50 _mol N-hydroxysuccinamide (NHS) to promote the coupling efficacy;

(g) allowing reaction to continue for another 120-180 min.;

(h) adding gently affinity purified rabbit anti-glycolipid IgG (1-5 mg) to 10-20 ml activated liposome solution;

(i) adjusting the pH to 8-8.5 by the addition of NaOH (100 mM) and incubated at 4°C for 16-18 hours;

(j) dailysing the liposome suspension against distilled water at 4°-8°C for 8-10 hours, followed by centrifugation at 10,000-12 000 g for 10-20 min.;

(k) washing the resulting pellet three times with PBS (10 -25mM, pH 7.2-7.5), resuspended in 10-25 ml of RP-2 buffer (NaH2PO4 10-25 mM, KH2PO4, 10-25 mM, EDTA 10-25 mM, choline chloride 10-15% and thiomersol 0.1%, pH 7) to obtain the final 10-25 ml of liposomal suspension to be used as working reagent for the TB/M card test.

4. A kit as claimed in claim , wherein said negative control is prepared using 1% BSA in PBS(150-200 mM, pH 7.2-7.5) containing 0.01% NaN3.

5. A kit as claimed in claim 1, wherein said positive control is prepared from the culture filtrate of heat-inactivated sonicated M. tuberculosis strain H37Rv in PBS (150-200 mM, pH 7.2-7.5) containing 0.01% NaN3 and 0.1% bovine serum albumin (BSA).

6. A kit as claimed in claim 1, wherein the said test card is preferably a disposable plastic slides.

7. A kit as claimed in claim 1, wherein, Tissue biopsy extract, serum, cerebrospinal fluid (CSF), synovial fluid (SF), and pleural fluid (PF) could be used as specimen for testing, depending upon the availability and feasibility.

8. A method for testing of individuals using either specimen as claimed in proceeding claims, for detecting mycobacterial glycolipid antigen using the kit comprising positive control, negative control and samples to be tested by adding (25-50µl) in the circular zone of hydrophobic material coated plastic slides; evenly spreading over the circular zone having liposomal antigen suspension (25-50µl); manually rotating slides (25-50rpm/min.); clumping on the test card in case of positive control and test samples containing active tuberculosis infection observed within 4-5 minutes in a form of dark blue agglutination, rated as 1+ve - 4+ve depending on the rate of infection and presence of mycobacterial glycolipid antigen in the samples.

9. A kit as claimed in proceeding claims, wherein said anti-mycobacterial glycolipid antibodies were prepared after culturing of Mycobacterium tuberculosis H37Rv (ATCC-27294) strain on Sautons media and or Middle brook 7H9 or 7H12B broth supplemented with 10% ADC (albumin dextrose and catalase) at 37° C till late log phase following the heat inactivation at 75-80°C in water bath for 45-50 minutes, and a resulting cocktail of glycolipid antigens from M. tuberculosis H37Rv

(ATCC-27294) was extracted, purified and characterized, two young rabbits were immunized subcutaneously (200 µg/rabbit; 250 µl incomplete Freund's adjuvant [IFA], 250 µl -10-25 mM, phosphate buffer solution [PBS] pH 7.2-7.5) with the above antigen, and polyclonal antibodies (rabbit IgG) were purified by protein A sepharose CL-4B affinity column chromatography followed by characterization, and used for the development of the TB/M card test.

10. The process as claimed in claim 1, wherein the said buffer comprises NaH_2PO_4 $2H_2O$, KH_2PO_4, EDTA, choline chloride and thiomersol.

11. The process as claimed in proceeding claims, wherein the serum obtained from rabbits contain mycobacterial glycolipid antibodies (Rabbit IgG) were purified by protein A sepharose CL-4B affinity column chromatography which were coupled on the surface of liposome and used for development of Liposomal agglutination card test (TB/M Card test) for detection of mycobacterial antigens in patients with active tuberculosis or development of ELISA and or rapid chromatography test for tuberculosis.

12. The process based on TB/M card test as claimed in proceeding claims, for detection of mycobacteerial glycolipid antigens in patients with active tuberculous meningitis, pulmonary and extra-pulmonary tuberculosis suitable for the serodiagnosis in a human and or animals suspected with active tuberculosis infection.

Dated this 29th day of May, 2012

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Of L.S.Davar & Co.,
APPLICANT'S AGENT.

ABSTRACT

"A Diagnostic Kit, based on Rapid Liposomal Agglutination (TB/M Card Test, for Detection of Antigens in Patients with Meningial Pulmory Tuberclosis"

The invention relates to a diagnostic test kit, based on Rapid Liposomal Agglutination (TB/M Card) Test, for detecting meningial, pulmonary and other extra pulmonary tuberculosis comprising liposomal antigen suspension, N-hydroxysuccinamide as working reagent, plastic slides, mixing stricks, negative and positive control.

Fig. 1 Bands 1 to 5 were scratched from the TLC.

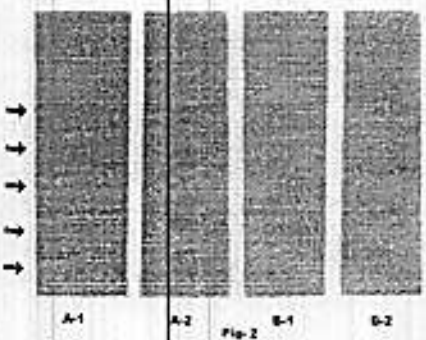

Fig. 2 Immunoreactivity of antigenic glycolipids characterized on a TLC plate.

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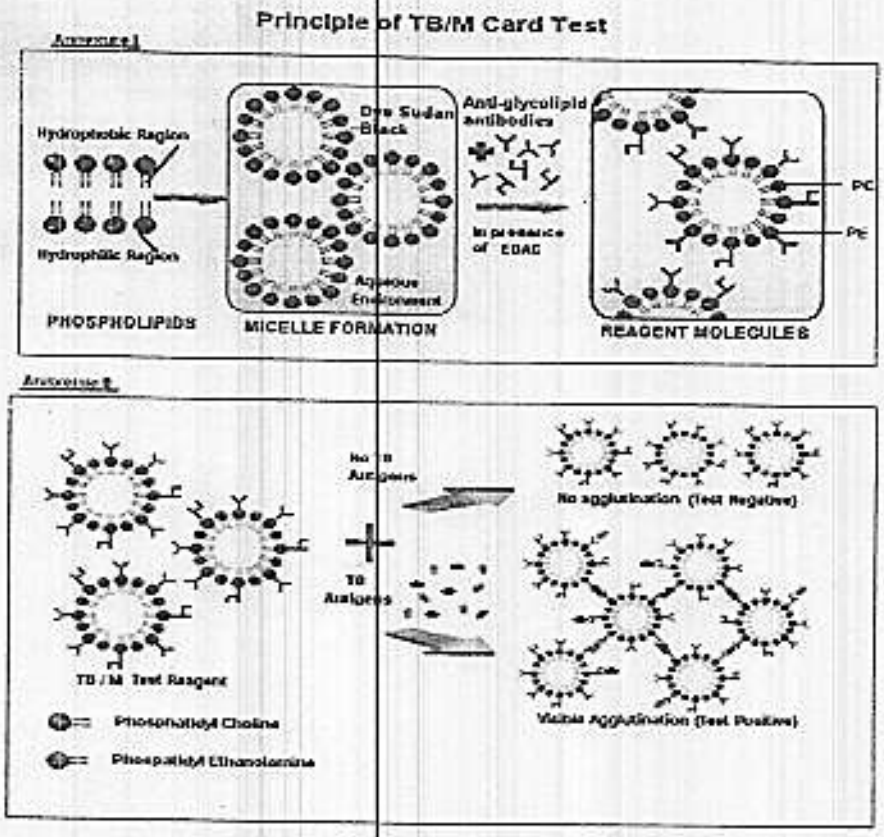

Fig. 3 Schematic representation of principle of TB antigen detection (TB/M card test) kit.

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