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SEARCH OF MYCOBACTERIUM ANTIGEN LIKE PEPTIDES FROM PHAGE DISPLAY LIBRARY

A PROJECT REPORT

Submitted by

H.NEETHU

In partial fulfillment for the award of the degree

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IN

BIOTECHNOLOGY

KUMARAGURU COLLEGE OF TECHNOLOGY, COIMBATORE

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BONAFIDE CERTIFICATE

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DECLARATION

I , do hereby declare that this dissertation entitled "**SEARCH OF MYCOBACTERIUM ANTIGEN LIKE PEPTIDES FROM PHAGE DISPLAY LIBRARY**" submitted to Anna University ,Chennai in partial fulfillment of the requirement for the award Degree of Bachelor of Technology in Biotechnology is a record of original research work done by me under the guidance and supervision of **Dr.Sathish Mundayoor** ,Scientist G,Molecular Microbiology Division, Rajiv Gandhi Centre for Biotechnology, Trivandrum and also with the co-guidance of **Mr.Biljo.V.Joseph** .This thesis has not formed the basis for the award for any degree/Diploma/Associateship/Fellowship or other similar title to any candidate of any university.

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(H. Neethu)

DEDICATED TO MY BELOVED PARENTS

ABSTRACT

Libraries of random peptides displayed by bacteriophage can be screened to select phage expressing peptides that specifically bind antibodies, so that the peptide sequence motifs expressed by the phage can help to define the epitopes of the antibodies.It is often desirable to screen antibody-selected phage for binding of the selecting antibody in an immunoassay in order to verify the specificity of the interaction. Enzyme-linked immunosorbent assays (ELISAs) are commonly used for this purpose. However, for many antibodies, the best techniques for measuring specific, high affinity interactions are immuno-precipitation assays. Immuno-precipitation was therefore investigated as a means of measuring interactions between antibodies and phage clones selected from random peptide display libraries. linear 12-mers on pIII of M13 phage.Following the amplification ,purification and sequencing of selected phage, mixtures of antibody and phage were incubated in solution and the immune complexes were precipitated with anti-human rabbit polyvalent immunoglobins conjugated with alkaline phosphatase coated on the plate(ELISA).The aim was to quantitate the phage precipitated by determining the number of plaques produced, which would therefore be proportional to the degree of interaction between the phage and the antibody in solution. The results that this method of measuring monoclonal antibody interactions with phage selected for expression of peptides recognised by the monoclonal antibody by ELISA technique is highly specific and sensitive.

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ABBREVATIONS:

Ab	- Antibody
APS	- Ammonium persulphate
BCIP	- 5-Bromo-4-Chloro-3-Indolyl Phosphate
bp	- Basepair
BSA	- Bovine Serum Albumin
ELISA	- Emzyme Linked Immuno Sorbent Assay
ddNTP	- Deoxy ribonucleicoside triphosphate
DNA	- Deoxy ribonucleic acid
dNTP	- Di-deoxy ribonucleicoside triphosphate
EDTA	- Ethylene diamine tetra acetic acid
g	- Gram
hr	- Hour
IPTG	- Isopropyl β-D-1-thiogalactopyranoside
Kb	- Kilo Base
KDa	- Kilo Dalton
LB	- Luria-Bertani
µg	- Micro gram
mg	- milli gram
mM	- Milli Molar
ml	- Milli litre
µl	- Micro litre
min	- minute
M	- Molar
M.bovis	- Mycobacterium bovis
MTB	- Mycobacterium tuberculosis

OD	- Optical Density
PBS	- Phosphate Buffer Saline
PEG/NACL	-Poly Ethylene Glycol /Sodium Chloride
PFU	-Plaque Forming Unit
PCR	- Polymerase Chain Reaction
rpm	- rotation per minute
TAE	- Tris Acetic acid EDTA
TB	- Tuberculosis
TBE	- Tris Borate EDTA
TBST	-Tris Buffered Saline+Tween
Tet	- Tetracyline
X-gal	- (5-Bromo-4-chloro-3-indolyl-β -D-galactoside)

INTRODUCTION

INTRODUCTION

"Evolution appears to work in bursts of activity. A species may survive for a very long time, even millions of years, with relatively little change, then suddenly, seemingly overnight, a variant species springs from it. But the evolution of deadly pathogens like the tubercle bacillus is a continuous process.Man,with his deadly power defends over the alarming disease for their better survival."

In an age when we believe that we have the tools to conquer most diseases, the ancient scourge of tuberculosis (TB) still causes 2 million deaths a year worldwide—more than any other single infectious organism—reminding us that we still have a long way to go. Even equipped with drugs to treat TB effectively, we haven't managed to eradicate this deadly infection.

Tuberculosis (TB) is a contagious or infectious disease. It is spread from person-to-person. A person is often infected by inhaling the germs. Recently, antibiotic-resistant strains of tuberculosis have appeared. Effective and rapid methods for the detection and drug susceptibility testing of TB, that are suitable for implementation in low income countries, which bear the highest TB burden, are urgently needed for effective identification, treatment and control of the disease.

By the 17th century, anatomical and pathological descriptions of tuberculosis began to appear in the medical literature. In 1720, Benjamin

Marten, an English physician, was the first to suspect that tuberculosis could be caused by "minute leaving creatures" and that by coming into contact with a consumptive an individual could contract the disease.

In a landmark study, the French army physician Jean-Antoine Villemin demonstrated in 1865 that tuberculosis could be transmitted from humans to animals and hypothesized that a specific organism caused the disease. It was not until 1882, however, that Robert Koch convincingly demonstrated that M. tuberculosis was the cause of tuberculosis.

Genetic studies suggest that M. tuberculosis has been present for at least 15,000 years. Evidence of tuberculosis in humans dates back to 2400-3400 B.C where mummies have been shown to have evidence of disease in their spines. The acid-fast stain is a laboratory test that determines if a sample of tissue, blood, or other body substance is infected with the bacteria that causes tuberculosis and other illnesses.

1.1Mycobacterium tuberculosis

Tuberculosis is caused by a group of organisms called Mycobacterium Complex. The *Mycobacterium tuberculosis* complex of organisms consists of the following species:

- *Mycobacterium tuberculosis*
- *Mycobacterium africanum*
- *Mycobacterium bovis*
- *Mycobacterium microtii*

Tuberculosis usually appears as a lung (pulmonary) infection. However, it may infect other organs in the body. *M. tuberculosis* is an obligate pathogen and can infect a wide variety of animals. Man is the principal host. *Mycobacterium tuberculosis* (MTB). Tuberculosis (TB) may be regarded in two categories: active disease or latent infection. The most common form of active TB is lung disease, but it may invade other organs, so-called "extrapulmonary TB."

The Nontuberculous mycobacteria (NTM) refers to all the species in the family of mycobacteria that may cause human disease, but do not cause tuberculosis (TB). Another one causes leprosy. Still others cause infections that are called atypical mycobacterial infections. They aren't "typical" because they don't cause tuberculosis.

Mycobacterium tuberculosis is an **obligate aerobe**. The bacterium is a **facultative intracellular parasite**, usually of macrophages, and has a slow generation time, 15-20 hours, a physiological characteristic that may contribute to its virulence. Two media are used to grow MTB Middlebrook's medium which is an agar based medium and Lowenstein-Jensen medium which is an egg based medium. MTB colonies are small and buff colored when grown on either medium. Both types of media contain inhibitors to keep contaminants from out-growing MT. It takes 4-6 weeks to get visual colonies on either type of media.

General characters:

- Slender, straight or slightly curved bacillus, non-motile, non-encapsulated and does not form spores

- Aerobic, rods are 2-4 micrometers in length and 0.2-0.5 um in width
- Slow growing- divides every 18-24 hr.
- Acid fast bacillus (AFB)
- Resistant to drying and chemical disinfectants
- Sensitive to heat (Pasteurization) and UV light

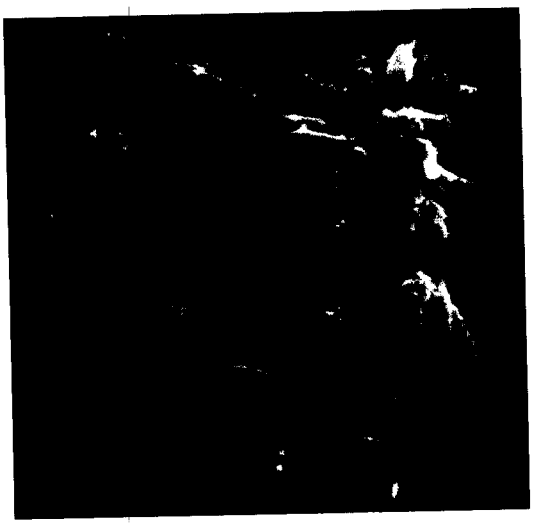


Fig 1.1: *Mycobacterium tuberculosis*

Although TB can be treated, cured, and can be prevented if persons at risk take certain drugs, scientists have never come close to wiping it out. Post-genomic tools are continuously generating information on mycobacteria. The fatty acid biosynthetic pathway, especially mycolic acid (targeted by many drugs), has been extensively modified in *M. tuberculosis* by horizontal gene transfer as shown by phylogenetic analysis of the *M. tuberculosis* genome with 58 complete bacterial genomes.

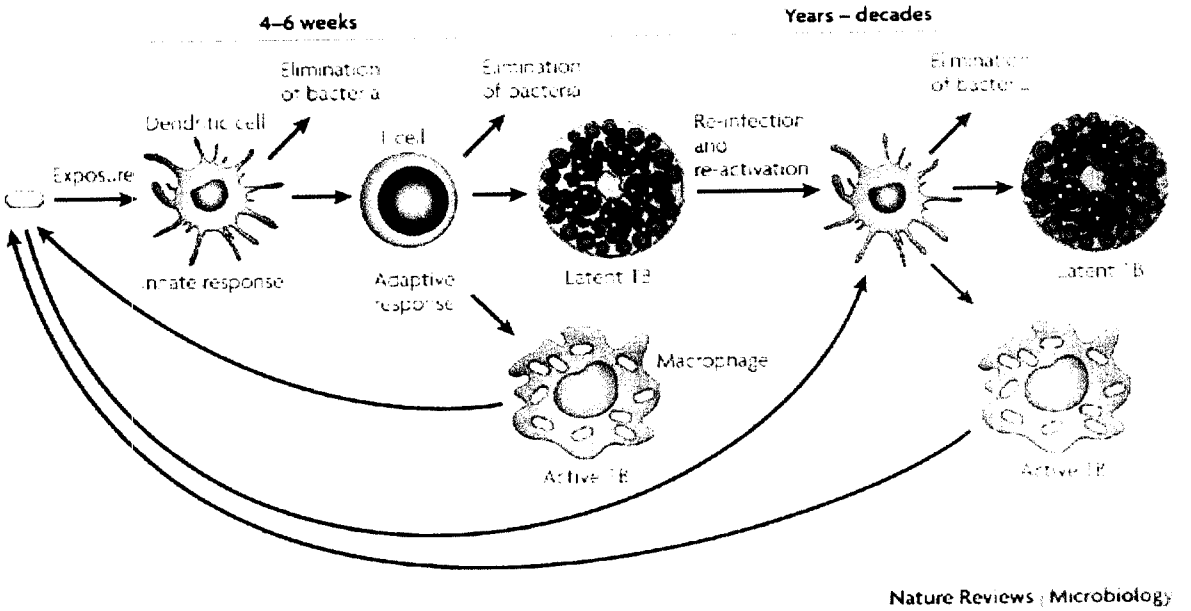


Fig 1.2:Various Stages in TB infection

Macrophages are key effector cells in mycobacterial killing.Dendritic cells engulf bacteria, or bacterial components, circulate to the draining lymph nodes and prime T cells, which then return to the lungs to orchestrate control of the infection. T cells enhance the antibacterial activity of macrophages by releasing cytokines, such as interferon-γ,

1.2BCG Vaccine

Bacille Calmette Guerin (BCG) is the current vaccine for tuberculosis. It was first used in 1921. BCG is the only vaccine available today for protection against tuberculosis. It is most effective in protecting children from the disease. BCG was first used as a vaccine to protect humans against tuberculosis in 1921. After about thirteen years the strain was seen to be less virulent for animals such as cows and guinea pigs. During these thirteen

years many undefined genetic changes occurred to change the original stain of *M. bovis*. This altered organism was called BCG. In addition to the loss of virulence, other changes to BCG were noted. These included a pronounced change in the appearance of colonies grown in the laboratory. Colonies of *M. bovis* have a rough granular appearance whereas colonies of BCG are moist and smooth.

Since the advent of anti-tuberculosis (anti-TB) medications in the 1940s, the treatment of drug-susceptible TB has become highly effective if administered and taken properly.In most cases, a treatment program for drug-susceptible TB involves taking two or four medications for a period of time ranging from six to nine months. Medications may include:

- Isoniazid
- Rifampin
- Pyrazinamide
- Ethambutol
- Streptomycin

For example, live vaccines like BCG may be needed to mimic natural disease. Subunit vaccines that contain the plasmid cannot replicate may be safer.

"Clear and certain medical knowledge, namely that the BCG-vaccine is not only ineffective but also harmful, was kept quiet for 27 years (in Germany) while the vaccine continued to be used and children were being harmed by it....almost 500 (estimated) cases of vaccine damage

per year."--The Decline of Tuberculosis despite "Protective" Vaccination by Dr. Gerhard Buchwald M.D..

1.3 Epidemology of TB

Treating these patients can be a long and expensive task. If there are problems with patients not taking their medicines, it may be necessary to arrange supervision either in a hospital or at home with a nurse. This programme is known as DOTS (Direct Observed Therapy Short course) and is recommended by the World Health Organisation (WHO).

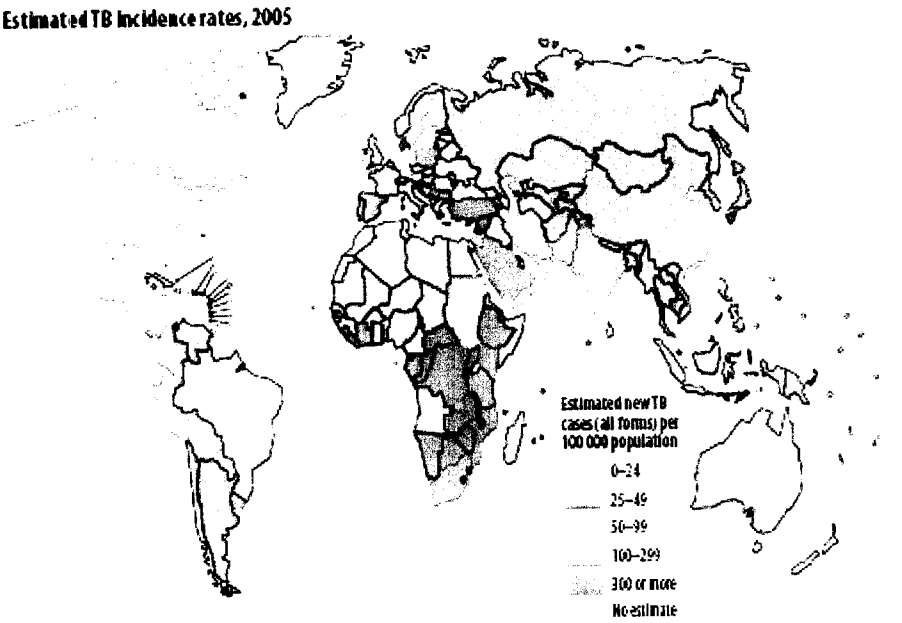


Fig 1.3:Estimated incidence of TB reported in the year 2005

Every patient who is cured stops spreading TB, and every life saved is a child, mother, or father who will go on to live a longer, TB-free life.

1.4 Drug Resistant TB

Multiple medications are often needed. It is important to take all of the doses prescribed. Often all of the TB germs cannot be destroyed with one medication. Multi-drug resistant TB means the TB germ has become resistant to at least two of the first-line medicines used to treat TB. When a person has extensively drug-resistant TB, there is the same tendency for the germs to become resistant to medications, but the germ is resistant to even more of the medications. Extensively-drug resistant TB means the TB germ has become resistant to at least three of the first-line medicines used to treat TB. These medicines are isoniazide, rifampin and fluoroqunolone Rapid TB diagnosis and drug susceptibility testing is limited by the slow growth of the causative organism, *Mycobacterium tuberculosis* (MTB).

The emergence of multidrug-resistant tuberculosis (MDR-TB) and, more recently, extensively drug-resistant TB (XDR-TB) is widely considered a serious threat to global TB control.

1.5 Diagnosis of TB

Tuberculosis is nearly always diagnosed by tuberculin skin tests, although one can also be diagnosed by chest x rays and analysis of sputum (matter from the respiratory tract) smears and cultures. The most common tuberculin skin test is the Mantoux test, which consists of injecting a small amount of protein from the TB bacillus into the forearm.

1.5.1Chest X-Ray

A chest x-ray is an x-ray of the chest, lungs, heart, large arteries, ribs, and diaphragm.

1.5.2Tuberculin Skin Test

The PPD skin test is a method used to diagnose tuberculosis. PPD stands for purified protein derivative

1.5.3 Sputum Culture Test

Routine sputum culture is a test of secretions from the lungs and bronchi (tubes that carry air to the lung) to look for bacteria that cause infection.

1.5.4 Serodiagnosis

Several new techniques have been developed to improve the diagnosis of tuberculosis, including newer radiometric methods, DNA probes, chromatography of mycolic acid, polymerase chain reaction, and serologic tests. These diagnostic approaches have had a dramatic effect on the ability to diagnose disease accurately and expeditiously .

A serologic diagnostic method was first introduced by Arloing as a technique of hemagglutination in 1898. However, it did not fulfill the clinical requirement with acceptable sensitivity and specificity in the diagnostic field of tuberculosis until 1972, when Engvall and Perlmann described a simple, highly sensitive, reproducible, and inexpensive technique of enzyme-linked immunosorbent assay (ELISA).

Many antigenic materials have been subsequently employed in the ELISA method in an attempt to improve both the sensitivity and specificity. These have included complex antigen from *Mycobacterium tuberculosis* , bacille Calmette-Guérin (BCG) socinate , purified protein derivative (PPD)

antigen , Antigen 5,6, and *Mycobacterium* glycolipids. The overall sensitivity of these methods ranges from 45 to 95%, and their specificity ranges from 90 to 100%, respectively. Antigen 60 is one of the best-known antigens used in the TB ELISA method. According to the latest literature reviewed, both sensitivity and specificity are approximately 80% in serodiagnosis.

1.6 M13 phages-A Boon for TB diagnosis:

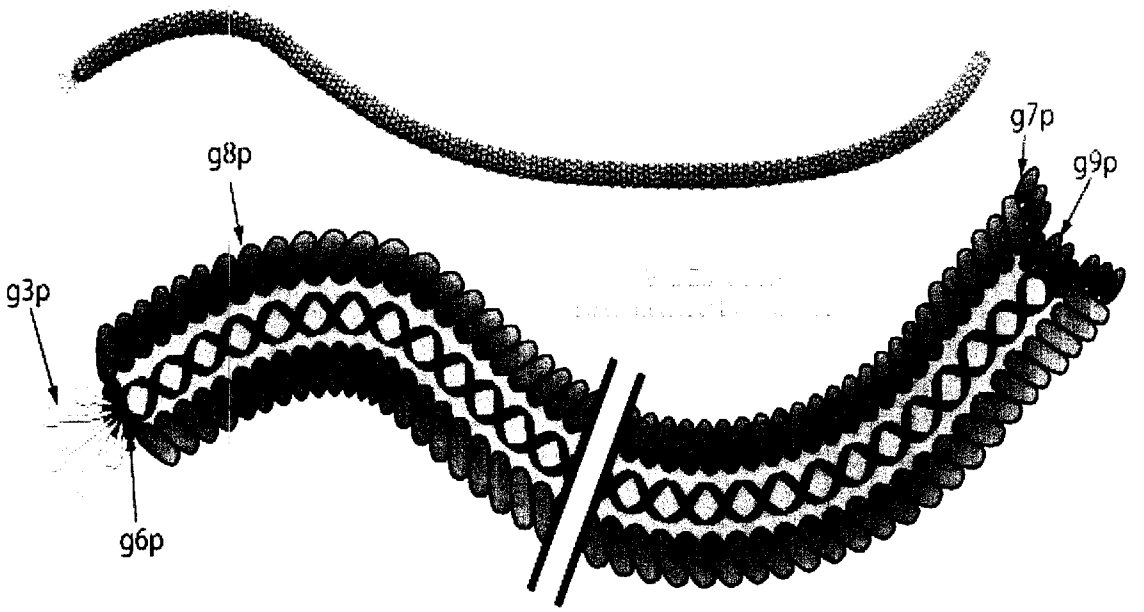


Fig 1.4: M 13 phage Genome

1.6.1Genome:

Circular, single-stranded DNA of 4.5 to 8kb encoding for 4 to 10 proteins. Replication occurs via dsDNA intermediate and rolling circle.

1.6.2 Gene expression:

Each gene is transcribed by host cellular machinery, via a specific promoter. Some genes ends by a transcription terminator.

1.6.3 M13 infection and replication

M13 is a filamentous *bacteriophage* which infects *E. coli* host. The M13 genome has the following characteristics:

- Circular *single-stranded* DNA
- 6400 base pairs long
- The genome codes for a total of 10 genes (named using Roman numerals I through X)

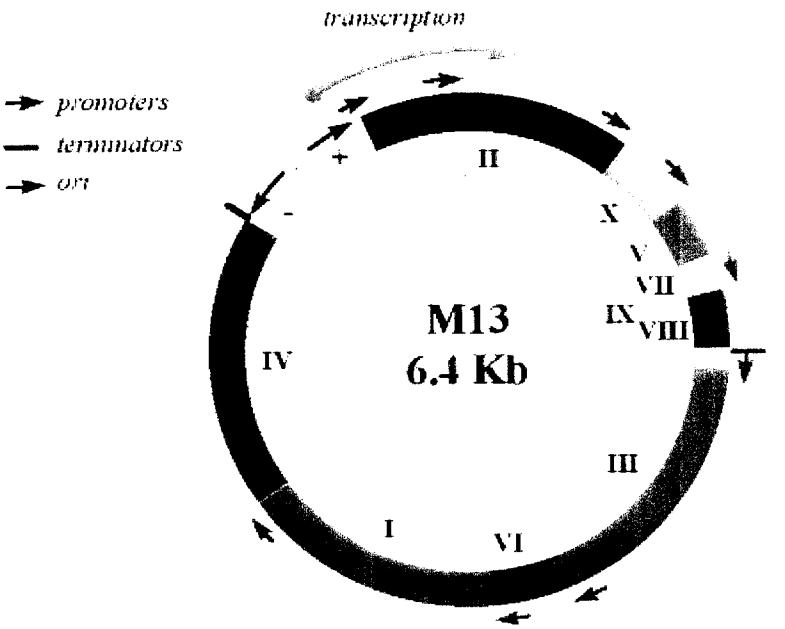


Fig 1.5: Provides information on M13 Genes and Proteins

- *Gene VIII* codes for the major structural protein of the bacteriophage particles
- *Gene III* codes for the minor coat protein

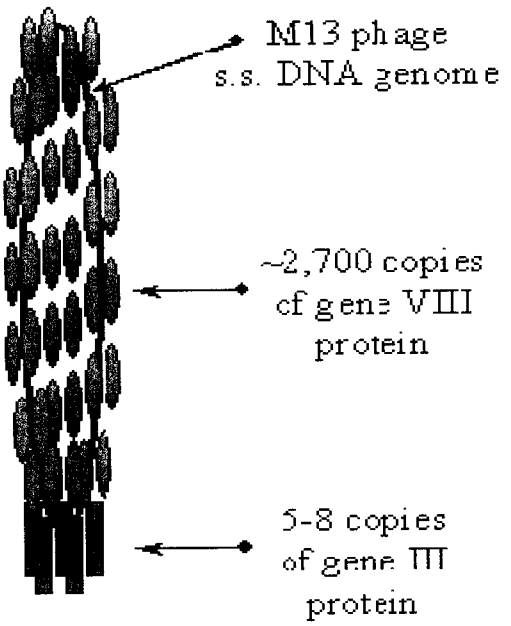


Fig 1.6. M13 Single stranded Genome

- The gene VIII protein forms a tubular array of approx. 2,700 identical subunits surrounding the viral genome
- Approximately five to eight copies of the gene III protein are located at the ends of the filamentous phage (i.e. genome plus gene VIII assembly)
- Allows binding to bacterial "sex" pilus
    - Pilus is a bacterial surface structure of *E. coli* which harbor the "F factor" extrachromosomal element

1.6.4 Infection

- Single strand genome (designated '+' strand) attached to pilus enters host cell
    - Major coat protein (gene VIII) stripped off
    - Minor coat protein (gene III) remains attached
- Host components convert single strand (+) genome to double stranded circular DNA (called the replicative or "RF" form)
- Transcription begins
    - Series of promoters
        - Provides a gradient of transcription such that gene nearest the two transcription terminators are transcribed the most
    - Two terminators
        - One at the end of gene VIII
        - One at the end of gene IV
    - Transcription of all 10 genes proceeds in same direction

1.6.5 Amplification of viral genome

- Gene II protein introduces 'nick' in (+) strand
- Pol I extends the (+) strand using *strand displacement* (and the '-' strand as template)
- After one trip around the genome the gene II protein nicks again to release a completed (linear) '+' genome
    - Linear (+) genome is circularized
- During first 15-20 minutes of DNA replication the progeny (+) strands are converted to double stranded (RF) form

- These serve as additional templates for further transcription

- Gene V protein builds up
  - This is a *single stranded DNA binding protein*
  - Prevents conversion of single (+) strand to the RF form
- Now get a buildup of circular single stranded (+) DNA (M13 genome)

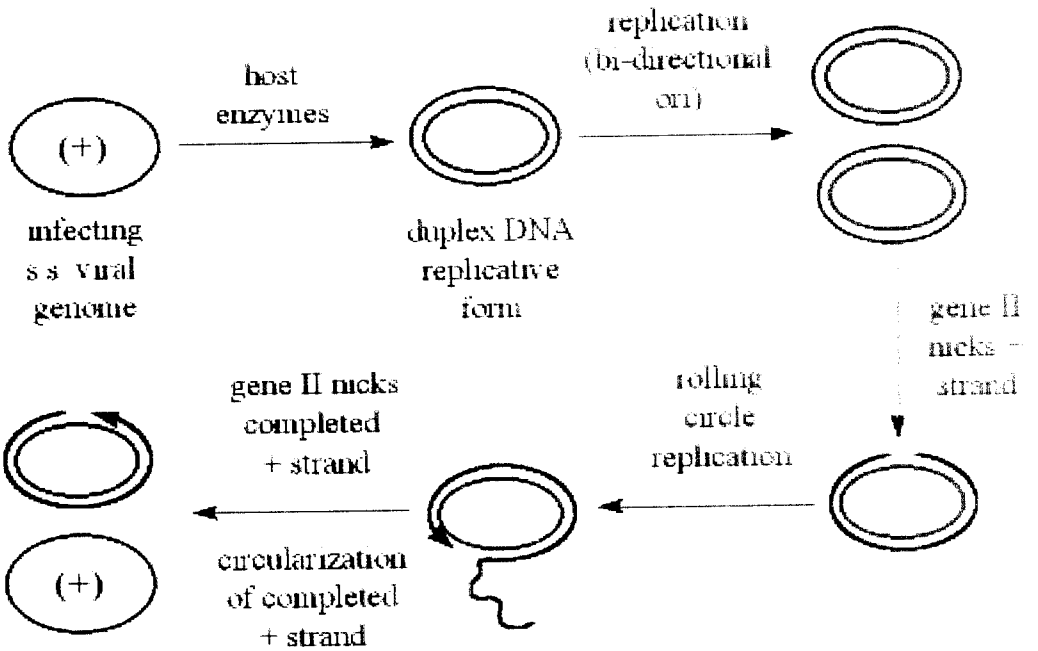


Fig 1.7: M 13-Rolling circle mode of Replication

1.7 Phage packaging

- Major coat protein (Gene VIII) present in *E. coli* membrane
- M13 (+) genome, covered in ss binding protein - Gene V protein, move to cell membrane
- Gene V protein stripped off and the major coat protein (Gene VIII) covers phage DNA as it is extruded out
  - Packaging process is therefore *not linked to any size constraint* of the M13 genome

- Length of the filamentous phage is determined by size of the DNA in the genome
- Inserts of up 42 Kb have been introduced into M13 genome and packaged (7x genome size)

- ~8 copies of the Gene III protein are attached at the end of the extruded genome

M 13 bacteriophages have the potential to become useful tools in the diagnosis of TB, as they are specific for mycobacteria and only replicate in, and hence detect, viable cells. The ability of phages to rapidly multiply within the bacteria, the specificity of phages for particular bacteria, and the ability of phages to increase in number during the infection process make phages excellent potential diagnostic and therapeutic agents for fighting bacterial disease. However, temperate phages are of little use in phage diagnostics and therapy. a vaccine is needed to prevent infection that could not be given to infants. *M. tuberculosis* in clinical specimens need to be robust and sensitive. To maximize the sensitivity of bacteriophages for the detection of *M. tuberculosis*, it was necessary to optimize phage infection and replication. Here an investigation of infection of *M. tuberculosis* by bacteriophage M13 was carried out to identify specific peptide that interacts with the tuberculosis sera. Initial studies were done by infecting the mycobacterium with M13phages in the presence of antibiotic tetracycline. Phage display is a selection technique in which a protein or peptide is expressed as a fusion with the coat protein on the surface of the virion. In this experiment identification of the peptide was by an in-vitro amplification process called biopanning. The phage display used here is based on a combinatorial library of peptides 12-mers fused to a minor coat

protein(pIII)of M13 phage. The M13 coat protein mediates infectivity by binding to the F-pilus of the recipient bacterium.

Panning is carried out by incubating a library of phage-displayed peptides in a plate coated with the target(pooled TB sera)It was necessary to investigate the time taken for the phage DNA to enter a host bacterium and the time at which multiplication inside the host bacteria commenced (latent phase of infection). This is followed by washing away the unbound phages and eluting the specifically bound phages. The eluted phages are then amplified and purified inorder to enrich the pool of binding sequences. Bound phages may be detected by serially diluting and plating samples in LB+tetracycline along with top agar containing the infected bacteria. Repeated cycles of infection and lysis cause clear areas (plaques) to form in the bacterial lawn which can be visualized after overnight incubation at 37c.From which the titre value can be calculated to carry on with the next rounds of panning. Final step of panning will include the elimination of exogenous phages, which means that all bound phages that were eluted from the TB sera were added to the pooled Hep B,Hep C,normal sera were removed. Only the unbound phages were collected to remove all the unspecific proteins. Unbound phages may be detected by plating samples in LB+tetracycline along with top agar containing the infected bacteria. Repeated cycles of infection and lysis cause clear areas (plaques) to form in the bacterial lawn which can be visualized after overnight incubation at 37c.From which the titre value can be calculated to carry on with the next step of phage amplication ,purifivation &sequencing. The final objective of the study was to investigate the use of the optimized unbound phages for the diagnosis of tuberculosis.

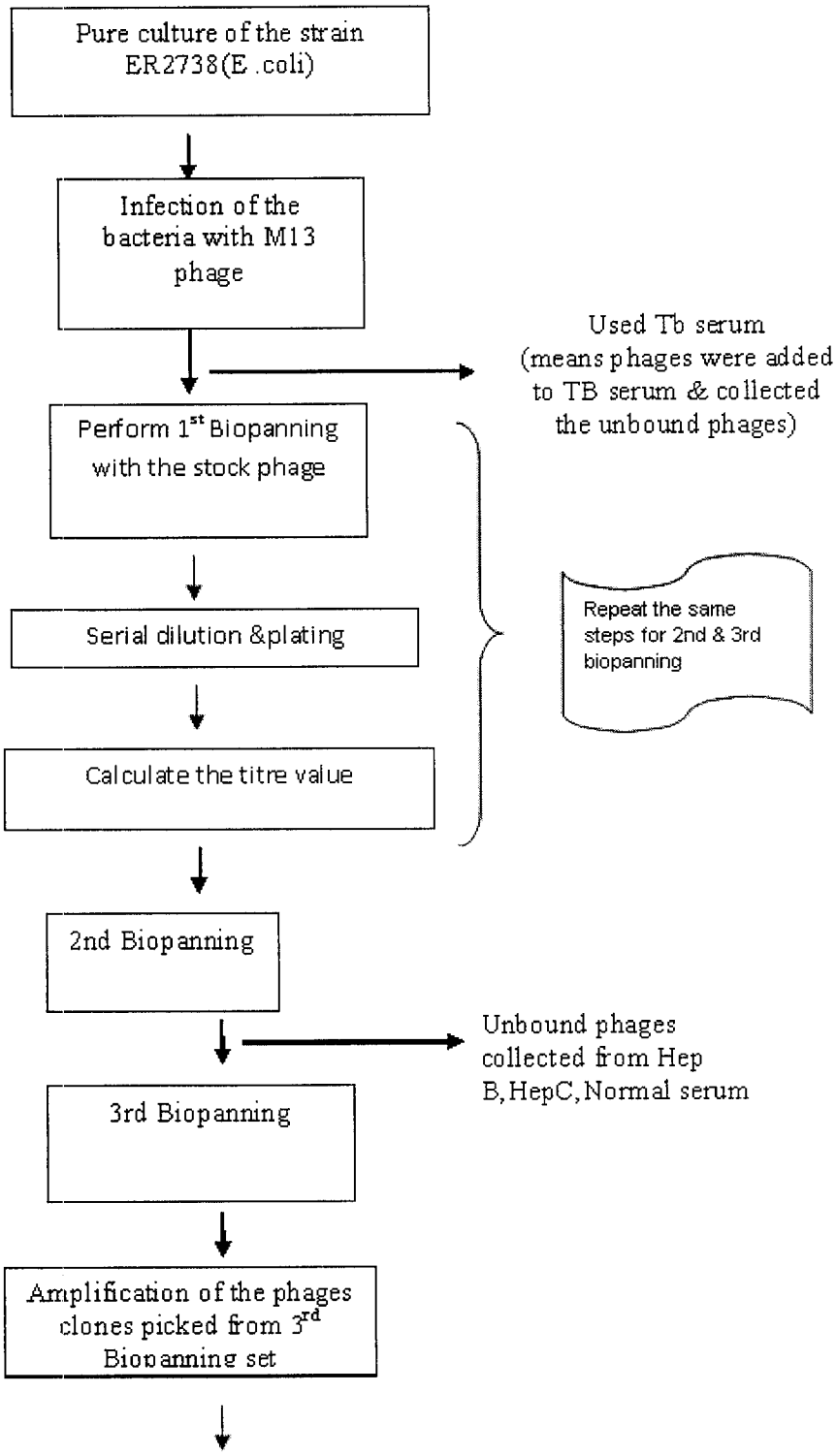
1.8 Objective:

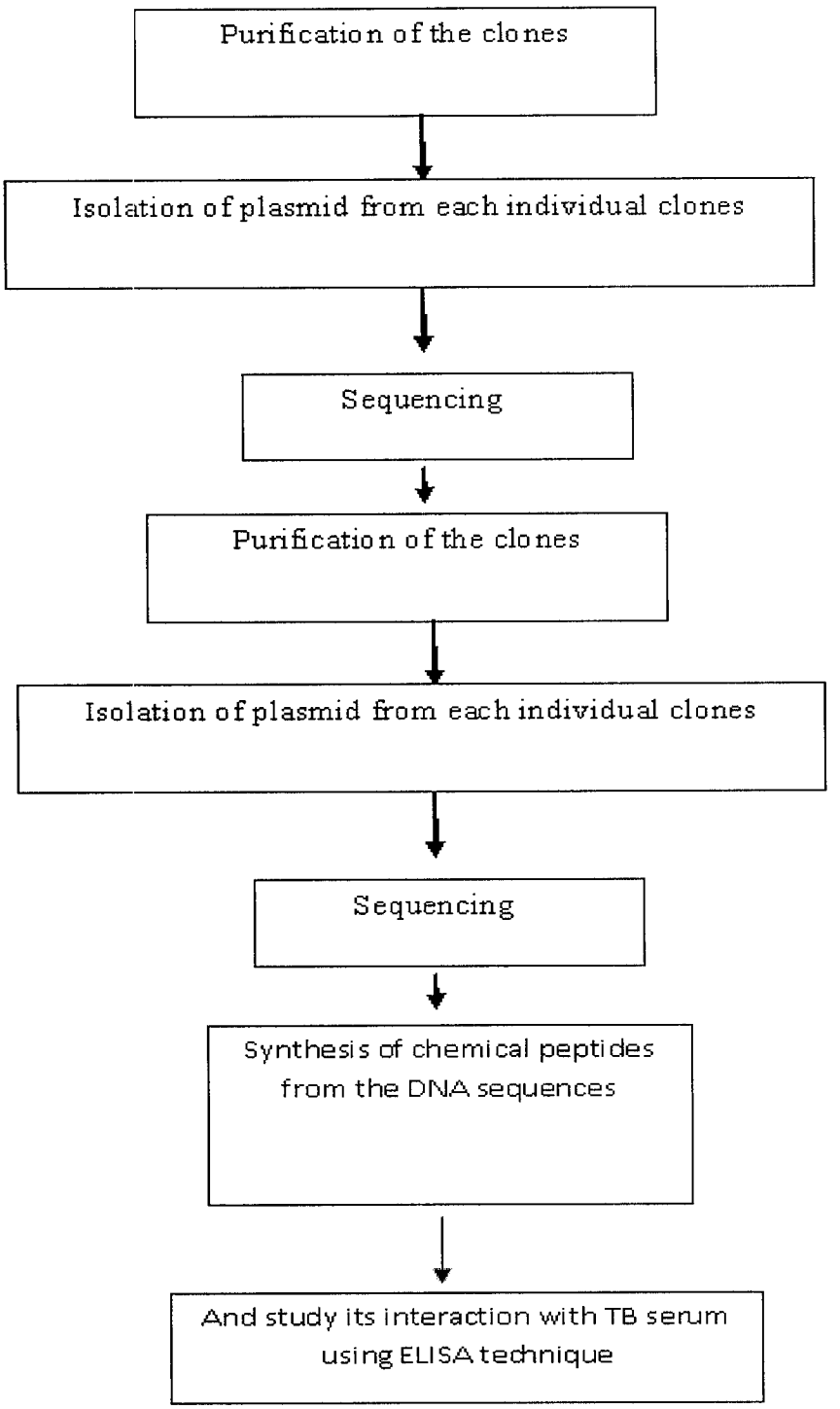
i) To infect the strain ER2738 with M13 phage to produce phage clones

ii) Amplification and purification of phage clones

iii) Chemical Synthesization of the single stranded DNA and its analysis using ELISA technique.

FLOW CHART OF EPITOPE MAPPING – A DIAGNOSTIC TOOL FOR DETECTION OF M TB




Purification of the clones
Isolation of plasmid from each individual clones
Sequencing
Purification of the clones
Isolation of plasmid from each individual clones
Sequencing
Synthesis of chemical peptides from the DNA sequences
And study its interaction with TB serum using ELISA technique

LITERATURE REVIEW

Tuberculosis(TB) is an infectious disease with a high morbidity and mortality rate around the world (Chintu *et al.*, 1995).The extreme susceptibility to *M.tuberculosis* infection is reflected by the fact that inhalation of the a few bacilli is enough to cause the infection (CDC 1996).TB is spread by aerosol droplets expelled by people with the active disease of the lungs when they cough ,sneeze, speak or spit . These infectious droplets are 0.5 to 5μm in diameter and about 40,000 can be produced by a single sneeze (Cole *et al.*, 2003).People with prolonged ,frequent , or intense contact are at highest risk of becoming infected ,with an estimated 22% infection rate .A person with untreated ,active tuberculosis can infect 10- 15 other people per year(WHO 2004).

Tuberculosis is classified as one of the granulomatous inflammatory conditions Macrophages ; T lymphocytes ; B lymphocytes and fibroblasts are among the cells that aggregate to form a granuloma, with lymphocytes surrounding the infected macrophages. The granuloma functions not only to prevent dissemination of the mycobacteria ,but also provides a local environment for communication of cells of of the immune system .Within the granuloma , T lymphocytes ($CD4^+$) secrete cytokines such as interferon gamma ,which activates macrophages to destroy the bacteria with which they are infected (Kaufmann et al .,2001). T lymphocytes (CD8+) can also directly kill infected cells (Houben et al., 2002).

TB infection begins when the mycobacteria reaches the pulmonary alveoli, where they invade and replicate within alveolar macrophages .The primary site of infection in the lungs is called the GHON focus. Bacteria are picked up by dendritic cells, which do not allow replication, although these cells can transport the bacilli to local lymph nodes .Further spread is through the bloodstream to the more distant tissues and organs where secondary TB lesions can develop in lung apexes, peripheral lymph nodes , kidneys, brain and bone (Herrmann et al., 2005). Simple diagnostic assays that are rapid, inexpensive and do not require highly trained personnel or a complex technological infrastructure are essential for global control of tuberculosis (Di perri et al., 1996).

Several new techniques have been developed to improve the diagnosis of TB including newer radiometric methods, DNA probes, and serologic tests. These diagnostic approaches have had a dramatic effect on the ability to diagnose disease accurately and expeditiously. (Kent et al., 1985). In the recent years there has been considerable focus on the discovery and characterization of proteins derived from Mycobacterium tuberculosis and the intensive search for immunologically active molecules has led to the identification of a number of candidate antigens for the use in vaccine development or for diagnostic purpose (Wadhawan et al., 1991).

2.1. History of Tuberculosis

Over recorded history, the burden of Mycobacterium tuberculosis had increased and described as "White Plague" (Dubose *et al.*, 1952).TB was almost unknown within the Sub-Saharan Africa till 1908 and New Guinea till 1920,until the exploration of these areas by Europeans (Cummins et al., 1920). In 1993,the gravity of the situation led the WHO to declare tuberculosis a "Global Emergency". Tuberculosis caused by emergence of resistant strain is much more difficult and expensive to cure and treat to the control of the disease. Another important factor promoting spread of tuberculosis is co infection with another deadly pathogen, the HIV virus .Tuberculosis is one of the leading causes of death among HIV positive people accounting for about 11% AIDS deaths worldwide(Corbett *et al* .,2003).

However, real scientific understanding of TB did not begin until 1882,when Robert Koch (March 24 ,1882) identified Mycobacterium tuberculosis as the causative agent of the disease *M.tuberculosis* as the causative agent of the disease. *M.tuberculosis* along with *M.bovis,M.africanum,M.microtii* all cause the disease known as tuberculosis (TB) and are members of the tuberculosis species complex. *M.bovis* and *M.africanum* are very rare in causing disease in immunocompetent people,and *M.microtii* is not usually pathogenic, although it is possible that the prevalence of *M.microtii* infections has been underestimated (Niemann *et al.*, 1999).

Other pathogenic mycobacteria are known, such as *M.leprae, M.avium* and *M.kansasii*. The last two are part of the group defined as Non-Tuberculous Mycobacteria(NTM).Non tuberculous bacteria are mycobacteria that are not part of the M. tuberculosis complex and do not cause leprosy , but do cause pulmonary diseases resembling tuberculosis (Griffith *et al.*, 2000).

2.2. Mycobacterium tuberculosis:-General characteristics

Mycobacterium tuberculosis H37Rv was first isolated in 1905, as remained pathogenic and is the most widely used strain in tuberculosis research (CDC., 2006) .

Their cell wall is rich in mycolic acids and lipids making them resistant to normal staining procedure. They are closer to gram positive organism based on phylogenetic analysis of 16S rRNA sequence (Pitulle *et al.*, 1992) and stain faintly positive for gram stain due to presence of peptidoglycan in the cell walls. They are referred to as acid fast bacilli due to their resistance of destaining with acid alcohol following staining with carbol fuchsin (Zielh – Neelson staining). *M.tuberculosis* gives a weak positive response to gram stain; but it is phylogenetically more closely related to gram positive eubacteria (Kent *et al.*,1985). Mycobacterium is classified as neither gram positive nor gram negative due to the lack of typical cell wall structure representative of either class(Prescott *et al.*, 1996).

The mycobacterial cell wall is rich in immunoactive macromolecules many of which are involved in the pathology of

tuberculosis (TB), a disease that has killed millions in the past and that continues to do so at present .(Lecueur *et al.*, 1989, Phalipon, *et al.*, 1999) TB has been studied not only because of its medical importance but also because it is considered a very interesting system that can be used to provide an understanding of the host-pathogen relationship. In particular, TB is a useful model with which to study how pathogens evade host responses. Although the phenomenon is complex, many pieces of evidence suggest that mycobacteria can manipulate host responses, usually to their own benefit (Rastogi *et al.,* 1991). As in the case of other pathogens, the first encounter between mycobacteria and the host cells is through surface-to-surface contact. This involves different types of receptors on the part of the host cells and a variety of ligands that are exposed on the surface of the mycobacteria. (Stead et *al.*, 1987, Ehlers *et al.*, 1998)Many of these ligands are complex sugars, and they function to activate various cellular responses in the host.

2.3. Multiple Drug Resistence (MDR)

During 1990 and 1991, four outbreaks of multidrug-resistant tuberculosis (MDR-TB), involving nearly 200 cases, were investigated by CDC and local health departments in Florida and New York City (CDC 1998-1991).

Virtually all M. tuberculosis isolates from the MDR-TB outbreak cases were resistant to both isoniazid (INH) and rifampin (RIF); some were resistant to other drugs, including ethambutol (EMB), strepto- mycin (SM), ethionamide, kanamycin (KM), and

rifabutin. Data for susceptibility to pyrazinamide (PZA) are incomplete, but for those isolates in which PZA testing has been completed, most were susceptible to this drug.

2.4. Identification and Evaluation of persons exposed to tuberculosis

Several factors should be considered in the management of persons exposed to TB (i.e., contacts):

a) the likelihood that the contact is newly infected with M. tuberculosis;

b) the likelihood that the infecting strain of M. tuberculosis is multidrug resistant;

c) the estimated likelihood that the contact, if infected, will develop active TB (Clark et al., 2004).

2.4.1. Positive Tuberculin test:

Because contacts of persons with infectious TB are at high risk for tuberculous infection, contacts should be rapidly identified and evaluated when a case of infectious TB (i.e., TB involving the respiratory tract or oral cavity) is identified (American Thoracic Society/CDC1983).Contacts who are not known or likely to be immunosuppressed and who have a documented prior positive tuberculin skin test (Mantoux test with 5 tuberculin units of purified protein derivative {PPD} are probably not newly infected. These persons need no further evaluation for the current TB exposure unless

they have symptoms suggestive of active TB; however, they should be evaluated for preventive therapy on the basis of the prior positive tuberculin skin-test result (Folgueira et al., 1996). Contacts who are not known or likely to be immunosuppressed and who have no history of a positive tuberculin skin-test reaction should receive a tuberculin skin test and, if indicated, chest radiograph and sputum examination. Those who have tuberculin skin-test reactions with greater than or equal to 5 mm in duration should be considered probably newly infected with M. tuberculosis and should be evaluated for preventive therapy, after active TB is excluded (Bastein et al., 1987).

2.4.2.Negative Tuberculin test:

High-risk contacts who have negative tuberculin skin-test reactions (<5 mm in duration) and who are not anergic should receive follow-up skin tests 12 weeks after their exposure to TB has ended. These initially tuberculin-negative, high- risk contacts should be considered possibly newly infected. They should receive a chest radiograph and be considered for preventive therapy until follow-up testing is complete if a) there is evidence of TB contagion among other contacts with comparable exposure (i.e., tuberculin positivity or active TB), or b) the contact is a child, HIV infected, or immunosuppressed for other reasons. Preventive therapy can be discontinued if follow-up tuberculin skin-test results are negative. (Elliott et al., 1993)

Contacts who are known or likely to be HIV infected, or markedly immunosuppressed for other reasons, should be evaluated

for anergy at the time of tuberculin skin testing. Immunosuppressed persons who have a prior positive tuberculin skin test may be at risk for re-infection with M. tuberculosis; these persons should also be evaluated with a tuberculin skin test and a test for anergy. Those who are not anergic can be further evaluated in the same way as contacts who are not known or likely to be immunosuppressed. In all situations, regardless of tuberculin skin-test results, a diagnostic evaluation should be performed if symptoms suggestive of active TB are present.

Several factors should be considered in this assessment:

a) the infectiousness of the possible source MDR-TB case

b) the closeness and intensity of the MDR-TB exposure,

c) the contact's likelihood of exposure to persons with drug-susceptible TB .

Exposure during cough-inducing procedures (e.g., bronchoscopy, endotracheal intubation, sputum induction, administration of aerosol therapy), which may greatly enhance TB transmission, is also more likely to result in infection.

2.4.3. Other diseases associated with TB:

The most potent factor that increases the probability that a person infected with M. tuberculosis will develop active TB is immunodeficiency, such as that caused by coinfection with HIV .Other immunocompromising conditions, including treatment with

immunosuppressive medications, renal failure, and diabetes mellitus, also increase the risk for progression to active disease, but to a considerably lesser extent than HIV infection (Selwyn *et al.,* 1980 and Rieder *et al.,* 1989).

Recentness of infection also contributes to the risk of developing active TB. In immunocompetent persons, the risk of developing active TB is highest within the first 2 years after infection, after which the risk declines markedly. This is probably different for HIV- infected persons, who have a progressive decline in cell-mediated immunity and may remain at high risk for an indefinite period or may even have an increasing risk as the immunosuppression progresses. Finally, the age of the contact needs to be considered.

Children ages less than or equal to 5 years and persons greater than or equal to 60 years both have high TB disease attack rates and shorter incubation periods (Comstock *et al.*, 1974).

2.4. 4.Pathogenesis and prevention of TB

To reduce the risk of active TB in persons newly infected with M. tuberculosis, the American Thoracic Society/CDC and the Advisory Council for the Elimination of Tuberculosis recommend INH preventive therapy (CDC 1990). For HIV-infected persons, the higher disease attack rate and the shorter incubation period associated with newly acquired tuberculosis infection and the high mortality rate associated with TB disease reinforce the rationale for the use of preventive therapy. In HIV-infected persons who become newly

infected with M. tuberculosis, the use of drug therapy might be considered treatment of incubating or subclinical disease.

When the infecting strain of M. tuberculosis is susceptible to INH and patients adhere to the drug regimen, INH is highly effective for preventing active TB. In a wide variety of controlled studies, persons who were prescribed 12 months of INH preventive therapy had a 30%-93% reduction in the rate of active disease; the variation in effectiveness was almost entirely due to variation in patient adherence to the prescribed regimen (Zeidberg et al., 1963)

RIF is recommended as an alternative to INH when the infecting strain of M. tuberculosis is resistant to INH but susceptible to RIF. When the infecting strain is multidrug resistant (i.e., resistant to both INH and RIF), treatment options are problematic because no studies have demonstrated the effectiveness of preventive therapy for persons infected with such strains of M. tuberculosis.

2.5. Potential Alternative Regimens of Preventive Therapy

The alternative regimens of preventive therapy suggested in this document do not represent approval from the Food and Drug Administration (FDA) or approved labeling for the particular products or indications in question. In the United States, the only drug approved by the FDA for TB preventive therapy is INH. Alternative drugs should be used for preventive therapy only when susceptibility to the drugs has been demonstrated by testing the M. tuberculosis isolate of the presumed source case.

2.6. Existing strategies for TB Diagnosis

Various strategies have been used to search for immunodominant proteins, including biochemical fractionation,serological assays and expression of genomic libraries of M.tuberculosis (Alderson *et al.*, 2000).

The most promising results for serodiagnosis of TB were obtained with the use of the 38-kDa PhoS protein of M.tuberculosis which provide high specificity (Daniels, 1996) However the specificity with this antigen varied from 45 to 80% for different cohorts. This was poorly recognized by serum antibodies from HIV-infected TB patients (Meola *et al.*, 1995).

Sera from patients with an infectious disease are known to often contain a large number of different antibodies, generated by the host immune system, that are directed against the infectious agent. The degree of immune activation may be monitered by measuring the serum concentration of these antibodies. Our goal is to exploit the combinatorial library as a valuable tool for the discovery of small molecules as diagnostic agent that bind to disease specific antibodies. (Cockburn *et al.*, 1980)

2.7. Introduction to phages

The first bacteriophage found to infect the mycobacteria was isolated in 1947.
(Redmond et al., 1966) and over 250 mycobacteriophages (phages) have since been identified (McNerney., 1999). Although phages were utilized for typing isolates of tuberculosis (Redmond *et al.*, 1966), they were not previously used in routine diagnosis. In recent years the potential of mycobacteriophages has been reexamined following the application of luciferase reporter phages to drug susceptibility testing in 1993 (Dubos *et al.*, 1993)

Bacteriophages are viruses that infect bacteria. Phages are either virulent (lytic) or temperate and use the host bacterium as a factory for their own replication. Since their independent discovery by Twort and D'Herelle, research on phage has enabled major fundamental and technological advances that have been essential for the emergence of modern molecular biology (Sechi *et al.*, 1997).
The biologic phenomena known as "bacteriophages" were discovered during the mid-1910s. Phage-therapy experiments during the 1920s and 1930s yielded inconclusive results (mostly owing to a lack of knowledge about phages high specificity)

2.7.1. Phage display principles

In phage display, a heterologous peptide or protein is displayed on the surface of the phage through transcriptional fusion with a coat protein gene (Smith. 1985) which is accomplished by the incorporation of the nucleotide sequence encoding the protein to be displayed into a phage or phagemid genome as a fusion to a gene

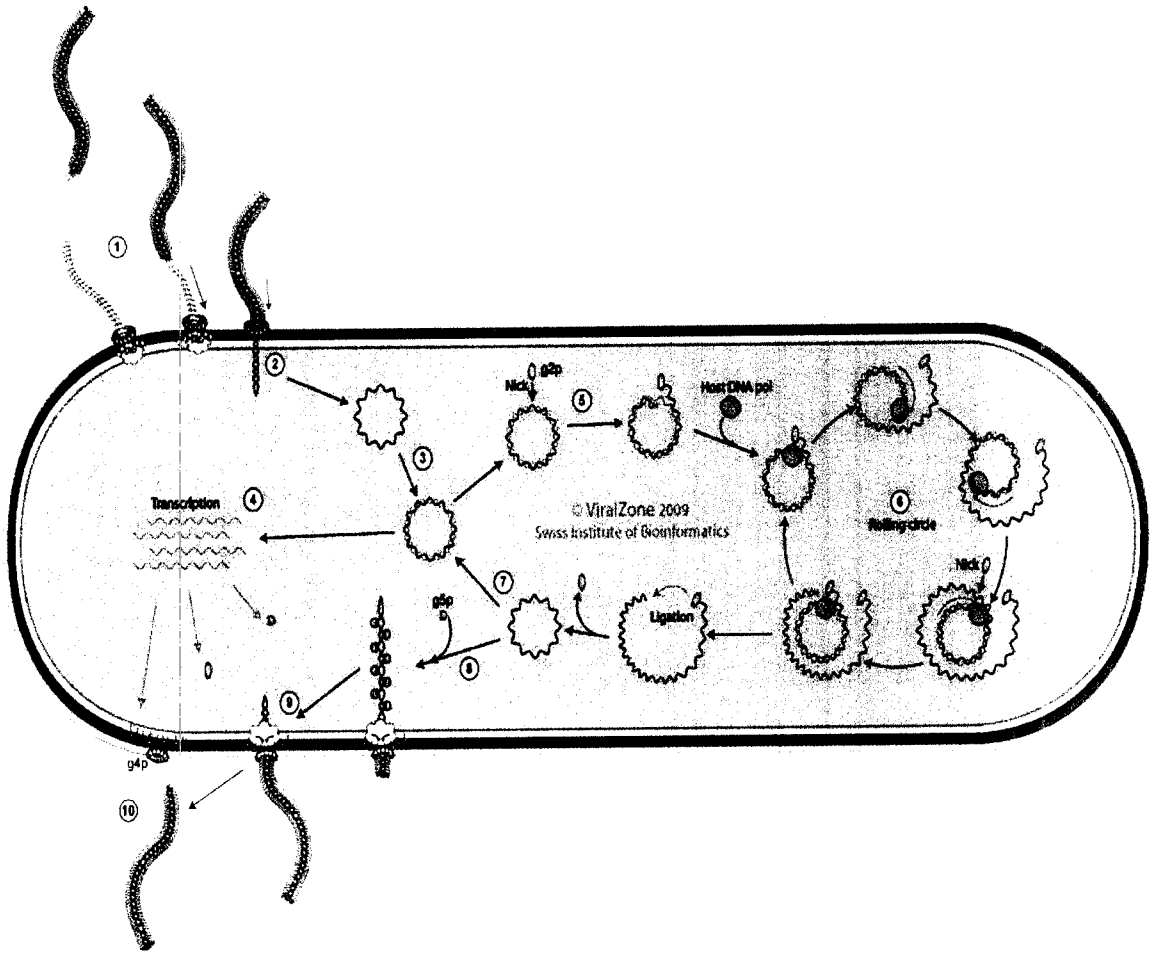


Fig2.1: Describes phage assembly and packing inside the host cell ER2738

1. Virus particle attaches to target cell by g3p protein binding of host F pilus.

2. The proteins of the capsid inject the DNA core into cell cytoplasm.
3. Host polymerase convert the (+)ssDNA viral genome into a covalently closed dsDNA called replicative form DNA RF.
4. viral genes are transcribed by host RNA polymerase.
5. Viral g2p protein nicks RF DNA strand at the origin of replication.
6. (+)strand replication occurs by rolling circle.

7. New (+)ssDNA genomes are converted into new RF molecules, and further transcription occurs.
8. When enough g5p protein is synthesized, conversion into RF dsDNA is inhibited, as neo-synthesized genomic ssDNA is covered with g5p.
9. g5p are replaced by g8p proteins to assemble the viral capsid.
10. new virions bud out from host cell.
11. Infected cell continue to divide and produces virion indefinetely.

encoding a phage coat protein. This fusion ensures that as phage particles are assembled, the protein to be displayed is presented at the surface of the mature phage, while the sequence encoding it is contained within the same phage particle. This physical link between the phenotype and genotype of the expressed protein and the replicative capacity of phage are the structural elements that underpin all phage display technology (Yaen et al ., 1997).

In brief, phage infection of the pathogenic bacterium results in the release of many phage particles that can be detected by a second (non-pathogenic), sensitive bacterial strain. Success with this approach has been seen with the detection of Mycobacterium tuberculosis (Petrenko and Wang et al., 2004).

2.7.2. Diagnosis by M13 phages–Current Mechanism

Phage display is used to select and optimize peptides or protein domains binding to virtually any protein and sometimes even non-protein targets (Hermann et al ., 2005)

A typical round of selection involves:

(*i*) target immobilization

(*ii*) reaction of the library with the target

(*iii*) removal of unbound phages through multiple washes and

(*iv*) elution of bound phages from the target followed by their amplification in bacterial cells. Several rounds of screening are performed until the increase in phage output, or binding assays performed with phage pools, indicates that the population of binding phages has been adequately enriched. The single stranded DNA is PCR amplified , Chemically synthesized and assessed by ELISA technique.

2.7.3 Advantages of Phage display technology:

- Peptide libraries displayed on filamentous phage have proven to be a powerful tool to define specific epitopes for mAbs,poly-clonal sera,or receptor molecules on various cell types.
  (Stend *et al.*, 1989)

- Phage display has exploited the physical linkage between random peptide sequence expressed on the phage and the DNA encoding that sequence. This linkage allows a rapid identification of peptide ligands. (Hill *et al.*, 1996&Johnsson *et al.*, 1999)

- Phage display is a powerful technology for engineering polypeptides that

  bind to target molecules of interest. Proteins fused to phage coat proteins

  are displayed on phage surfaces and the encoding DNA is packaged within the phage particles The encapsulated DNA simplifies mutagenesis to synthesize libraries of phage-displayed polypeptides and enables ready identification of individual variants. From a library of displayed proteins, high-affinity binding proteins can be selected through in vitro binding to an immobilized target molecule, and the sequences of the selected proteins can be rapidly deduced by DNA sequencing.

  (Hague., 2006).

MATERIALS AND METHODS

3.1 Serum

The sera used in this study are from three groups.

1) Serum from Normal people (People with out any known disease symptoms)
2) Serum from patients other than those with Tuberculosis (Hepatitis B and C)
3) Serum from Tuberculosis patients

3.1.1Isolation of Serum

The serum was isolated from the venous blood drawn from the donors with their consent. The blood was collected in a 10 mL port tube and transferred into the laboratory and incubated at 37°C for 1 hr for clotting. The clot was then disturbed using a sterile toothpick and then incubated at 4°C for 2-3 hrs. It was then centrifuged at 1500 rpm at 4°C for 10 minutes to separate the serum. The serum was collected into a 5 ml port tube and spun at 5000 rpm, 10 minute at 4°C, to pellet any debris of the lysed cells. The clear straw yellow colored serum thus obtained was then stored at –20°C with 0.01% sodium azide.

3.2 Phage Peptide Library:

Phage Display Peptide Library Kit (Ph.D. – 12 ™ ,New England Biolabs *Inc.*) was used for the rapid screening of the peptide ligands that specifically bind to the *Mycobacterium tuberculosis* specific antibodies.

The kit includes:

- Peptide 12 –mer Phage Display Library :- 100 µl, 1.5 x 10^{13}pfu/ml. Supplied in TBS with 50% glycerol. Complexity =2.7 x 10^{9} transformants.
- -28gIII sequence primer
- -96gIII sequence primer
- *E. coli* strain ER2738 host strain (Tetracycline resistant)
- Streptavidin, lyophilized
- Biotin ,10mM

3.2.1Big Dye Termination Sequencing Kit

Big Dye Termination Sequencing Kit V. 3.1 (Applied Biosystems) was used to determine the sequence of the selected phage clones.

3.3 Phage plating

LB Medium:Per liter:

10 g Bacto-Tryptone, 5 g yeast extract, 5 g NaCl. Autoclave, store at room temperature.

LB/IPTG/Xgal Plates:

LB medium + 15 g/L agar. Autoclave, cool to < 70°C, add 1 ml IPTG/ X-gal and pour. Store plates at 4°C in the dark.

Agarose Top:Per liter:

10 g Bacto-Tryptone, 5 g yeast extract, 5 g NaCl, 1 g $MgCl_2$•6H2O, 7 g agarose. Autoclave, dispense into 50 ml aliquots. Store solid at room temperature, melt in microwave as needed.

Tetracycline Stock:

20 mg/ml in Ethanol. Store at –20°C in the dark. Vortex before using.

LB-Tet Plates:

LB medium + 15 g/l Agar. Autoclave, cool to <70°C, add 1 ml Tetracycline stock and pour. Store plates at 4°C in the dark. Do not use plates if brown or black.

Blocking buffer:

0.1 M $NaHCO_3$ (pH 8.6), 5 mg/ml BSA, 0.02% NaN_3. Filter sterilize, stored at 4°C.

TBS:

50 mM Tris-HCl (pH 7.5), 150 mM NaCl. Autoclave, store at room temperature.

PEG/NaCl:

20% (w/v) polyethylene glycol–8000, 2.5 M NaCl. Autoclave, store at room temperature.

Iodide Buffer:

10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 4 M NaI. Store at room temperature in the dark.

3.3.1 Method:

A single cell colony of the host strain ER 2738 was inoculated into 3ml LB with 10 µg/mL tetracycline and aerated at 37°C overnight at 160 rpm. This culture was diluted 1:100 in 4 ml LB with 10 µg/mL tetracycline and aerated at 37°C for 3-3.30 hours. From this culture, 200µl was taken into a fresh eppendorf tube and 100 µl of the phage (diluted phages) was added to this. After mixing it was then added to 4

mL of molten top agar and vortexed. Pour the vortexed mixture into LB + tet plate. Spread overlay across the plate by tilting and rotating the plate until overlay is evenly distributed. Do not attempt to spread overlay further once it starts to set. This will produce a grainy, opaque overlay, which will make plaques difficult to see. Allow plates to cool until agar has set. Invert the plates (lid side down), and incubate 12-18 hours at 37°C. Count your plaques, and calculate the titer of pfu in the original stock.

3.4. Phage amplification

Method:

A single cell colony of the host strain ER 2738 was inoculated into 3ml LB with 10 μg/mL tetracycline and aerated at 37°C overnight at 160 rpm. Dilute the culture 1:100 in 10 mL LB with 10 μg/mL tetracycline and aerate for 2 hrs at 160 rpm. 100 μL of phage was added into this fresh culture and continued to aerate at 160 rpm. After 4 hrs of incubation, the culture was transferred to a 15 mL centrifuge tube and spun at 10,000 rpm for 15 minutes at 4 °C. The supernatant was collected in a fresh tube and spun again as before. The supernatant was then transferred to a new tube and $1/6^{th}$ volume 20% W/V PEG/NaCl was added into it and mixed well. It was then incubated at 4 °C for overnight. The mixture was then spun at 10,000 rpm for 15 minutes at 4 °C and the supernatant was discarded. The pellet was resuspended in 1 mL of TBS and $1/6^{th}$ volume 20% W/V PEG/NaCl was added into it and mixed well. It was then stored in ice for one hour. After incubation, it was spun at t 10,000 rpm for 15 minutes at 4 °C and the supernatant was discarded. The pellet was resuspended in 200 μL of TBS

3. 5. Phage titration

The phage stock was serially diluted by 10 fold serial dilutions to 10^{-12} in LB. after the dilutions were made, it was plated as given before. The plaques obtained after the incubation is counted and the titre was calculated.

3.6. Plaque lifting

This was done the similar way as the phage amplification. In this case, the phage innoculum was not the phage suspension, but the plaques from the plates. The plaques were lifted using sterile tips and put it into the one hour old diluted host strain and continued with the phage amplification steps.

3.7. Biopanning

The pooled TB sera were diluted in carbonate/bicarbonate buffer to get a concentration of 100 μg/mL and it was coated in a culture dish. It was then incubated in a humid chamber, with mild rocking for overnight at 4 °C. The plate was blocked with blocking solution for 2 hrs at 37 °C. After removing he blocking solution, plate was washed with TBST (0.1%) for two times. The phage display library was diluted to 2 mL to get a titre of 1.5 X 10^{11}/mL phages and panned over TB serum coated in the plate. After 2 hrs of incubation, the unbound phages were washed off using TBST (0.1%) and the bound phages were removed using Glycine/BSA solution. The TB serum bound phages were then eluted and amplified. The titre of the amplified phages was calculated and diluted to get the same concentration as the first biopanning (starting concentration) and this procedure was repeated for 2-3 times with an increase in the concentration of TBST in 2^{nd} &3^{rd} panning.

The TBST concentration in first, second and third biopanning were 0.1%, 0.5%, and 1% respectively. The bound phages from the TB serum were taken and panned with Hep B,Hep C and Normal serum. Here unbound phages were retrieved. The unbound phages collected from the diseased serum was serially diluted and plated to from plaques .These plaques are then amplified and purified using PEG/NaCl precipitation.

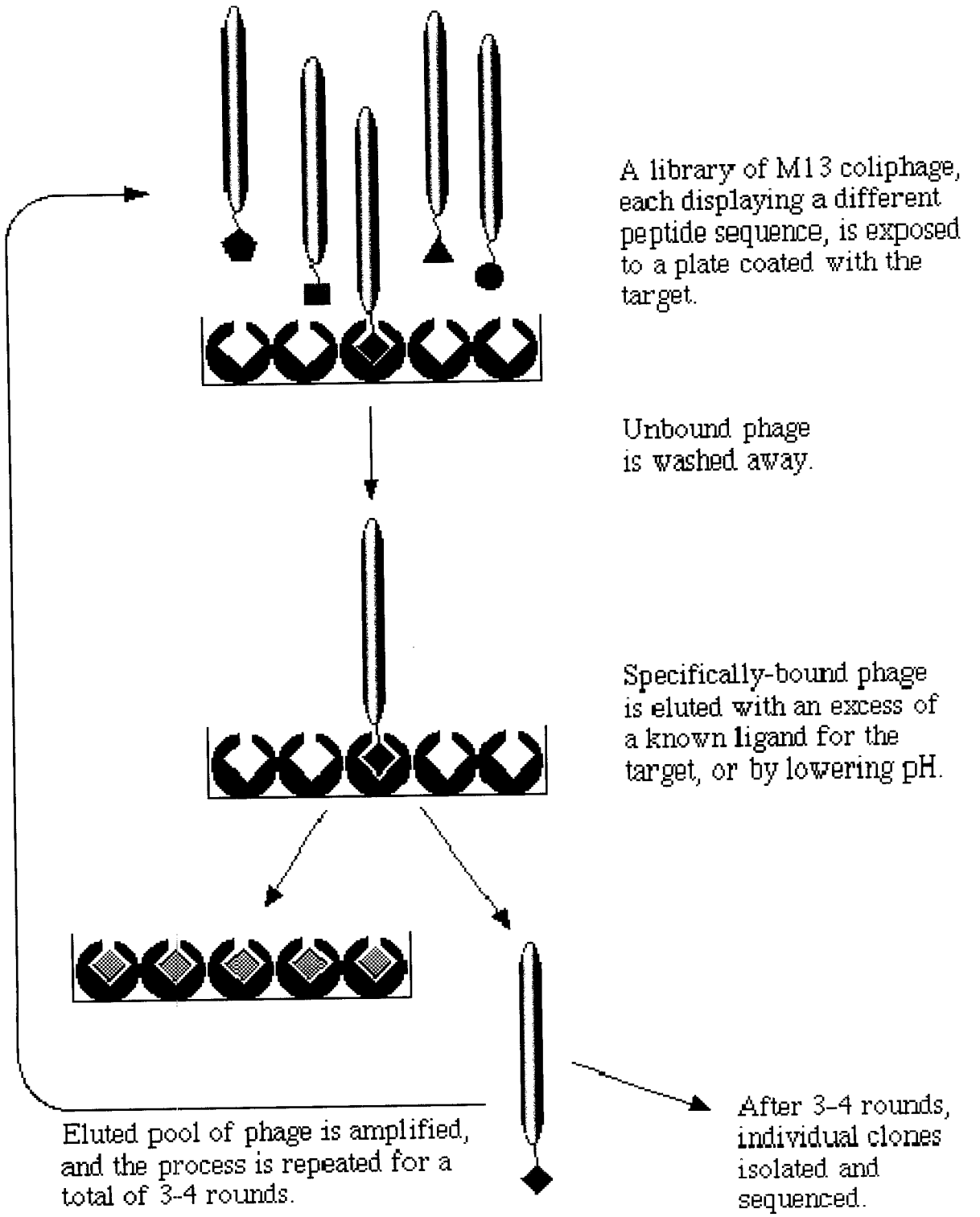


Fig 3.1 : Steps involved in Biopanning

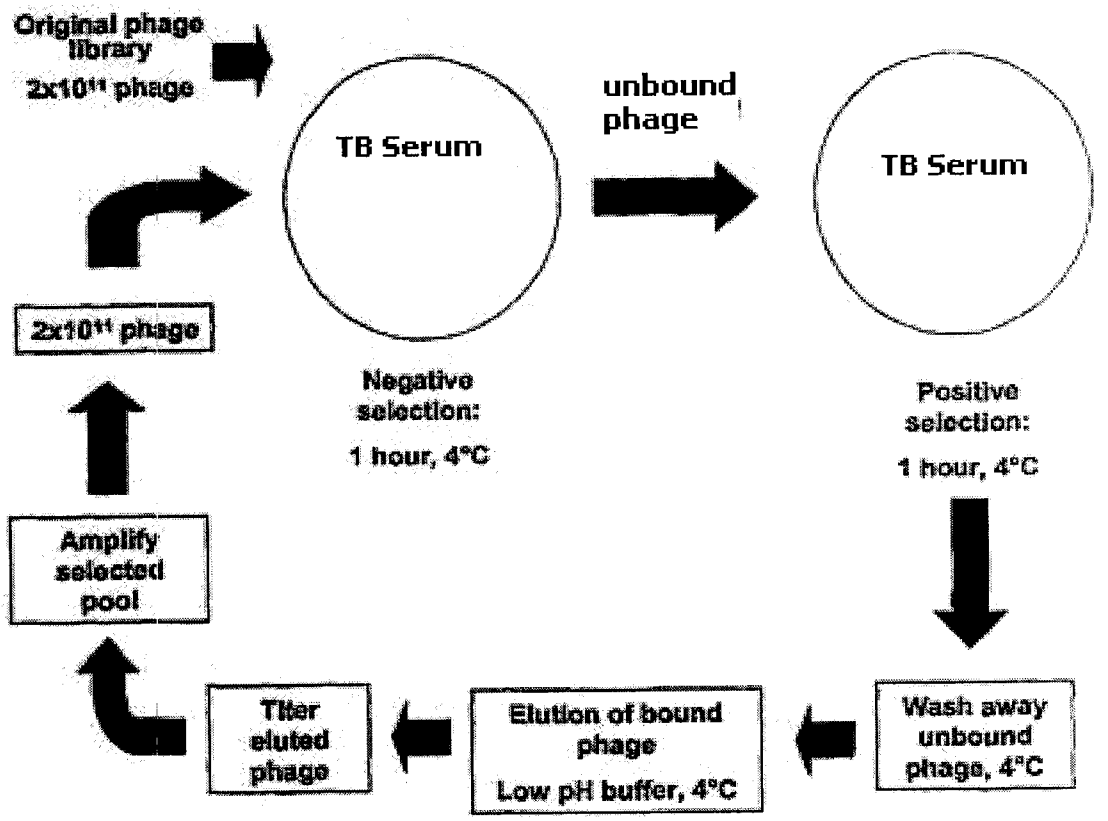


Fig 3.2: Overview of Biopanning Experiment

3.8.Single stranded DNA Isolation

Method:

An overnight culture of ER2738 in 3ml LB+Tet medium at 37°C, 180rpm was grown. This was subcultured into 20ml of LB (1% of inoculum should be used)

1. The culture was grown to the early mid- log phase
2. 100µl of the phage suspension with the culture was added and allowed to grow for 4-5hrs at 37°C, 180 rpm.
3. The culture was then transferred to a centrifuge tube and spun at

10,000rpm at 4°C,10min

4. The supernatant was transferred to a fresh tube & respunned again as the above
5. To a fresh tube containing the supernatant add $1/6^{th}$ volume of PEG/NaCl. The phages were allowed to precipitate at 4°C for overnight.
6. Spun for 15min at 10,000rpm at 4°C. Decant the supernatant, respun briefly and the residual supernatant was removed with a pipette.
7. The pellet was suspended in 1ml TBS. spun for 5min at 4°C 10,000rpm to pellet the residual cells in a microcentrifuge tube.
8. To a fresh tube containing the supernatant add $1/6^{th}$ volume of PEG/NaCl. The phages were allowed to precipitate at 4°C for at least 60min .
9. Centrifuged for 10 min at 4°C
10. The supernatant was discarded and briefly spunned again. Finally, the residual properties (supernatant)was removed with the help of micropipette.
11. At last ,Suspended the pellet in 200µl sodium iodide.

3.9. PCR Amplification of the 666 bp fragment

A 666bp fragment was amplified from the Ss DNA isolated from the phage clones using specific primers. The reaction was set as follows.

Target DNA (Phage SS DNA) : 1 µl (1ng)

10X Polymerase Buffer : 2.5 µl

2.5 mM dNTP Mix : 0.75 µl

Forward Primer	: 1 µl (50 pMoles)
Reverse Primer	: 1 µl (50 pMoles)
Taq DNA Polymerase	: 0.2 µl (1 Unit)
Water	: 18.55 µl
Total Volume	: 25 µl

PCR Conditions are given below

Initial Denaturation ($95^{O}C$)	: 5 minutes	
Denaturation ($95^{O}C$)	: 30 Seconds	35 Cycles
Annealing ($52^{O}C$)	: 30 Seconds	
Extension ($72^{O}C$)	: 2 Minutes	
Final Extension ($72^{O}C$)	: 10 Minutes	
Final Holding at 4 ^{O}C		

3.10. Agarose Gel Electrophoresis

1% agarose was melted in required volume of 1X TBE and allowed to cool to bearable warm. Ethidium bromide was added to a final concentration of 0.5µg/ml and stirred well to mix. It was then poured into the gel casting platform already adjusted with the comb. It was then allowed to solidify and the comb was removed carefully without disturbing the wells. 5µl PCR product was loaded with 1µl of 6X gel loading dye. 100bp DNA size

standard marker was also loaded. The gel was run at 3V/cm till the dye front reached the 3/4th of the gel. After washing with running tap water, the gel was viewed on a UV transilluminator and the band pattern was documented using the Quantity one software of Flour S Multi Imager Gel documentation system.

3.11. Elution of the Bands from agarose Gel

After electrophoresis, the band was eluted from the agarose gel using the GFX™ PCR Gel Band Elution Kit from GE Health care. The gel band was excised and weighed. To the volume of the gel in 'mg', an equal volume of Capture buffer in 'µl' was added and incubated at 60°C in a water bath until the gel dissolved completely. The dissolved sample was transferred to a GFX™ Column and incubated at room temperature for 1 min. the column was then spun at 12,000 rpm for 30 seconds. Column was then washed with 500µl of wash buffer for a minute and spun at 12,000 rpm for 30sec.The DNA retained in the column was then eluted using 50 µl elution buffer (1XTE) by spinning at 12,000 rpm for 1 minute. The presence and concentration of eluted DNA was conformed by agarose gel electrophoresis

3.12. Phage Sequencing

All DNA sequencing reactions were performed on ABI 310 automated sequencer (Applied Biosystems, Perkin Elmer) using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit, version 2.0(PE Biosystems). Sequencing was based on Sangers Di Deoxy Chain Termination Method. The Ready Reaction mix includes :- A Dye Terminator labelled with dichloro [R6G], C-Dye Terminator labeled with dichloro

[ROX], G-Dye Terminator labeled with dichloro [R110], T-Dye Terminator labeled with dichloro [TAMRA], Deoxynucleoside triphosphate (dATP, dCTP, dTTP, dUTP) , Amplitaq DNA polymerase FS, Mgcl2 and Tris- Hcl buffer.

The 10µl reactions were carried out with ~ 80 ng of each PCR product, 0.5 µl of Ready Reaction mix, 1µl of (5.0pmol) primer and 2µl buffer. The thermo cycling conditions were: 25 cycles of 96°C for 15seconds, 55 for 15seconds and 60°C for 4minutes with a thermal ramp of 1°C/ sec. The reaction products were cleaned up before loading into the automated sequencer. For cleaning up, the reaction product was transferred to 1.5ml tube. To this 2µl 125mM EDTA was added and incubated for 15minutes and followed by the addition of 2µl 3M Sodium Acetate (pH-4.6) and 50µl 100% ethanol. The tubes were kept for incubation for 15minutes at room temperature. The tubes were inverted slowly 2-3 times and centrifuged at 13000 rpm for 20minutes at 20°C. The supernatant was aspirated off and the pellet was washed with 250µl of 70% ethanol and centrifuged at 13000 rpm for 10minutes at 20°C. After carefully aspirating the supernatant, the pellet was air dried and suspended in Template Suppression Reagent (TSR), heat denatured and run in 310 Automated Sequencer(Applied Biosystems, Perkin Elmer) using the module SeqPOP6 1ml E.

3.13.Peptide synthesis

From the DNA sequence data obtained, peptide sequence were identified. Peptides are chemically synthesized by Solid phase synthesis scheme. The following are the steps involved:

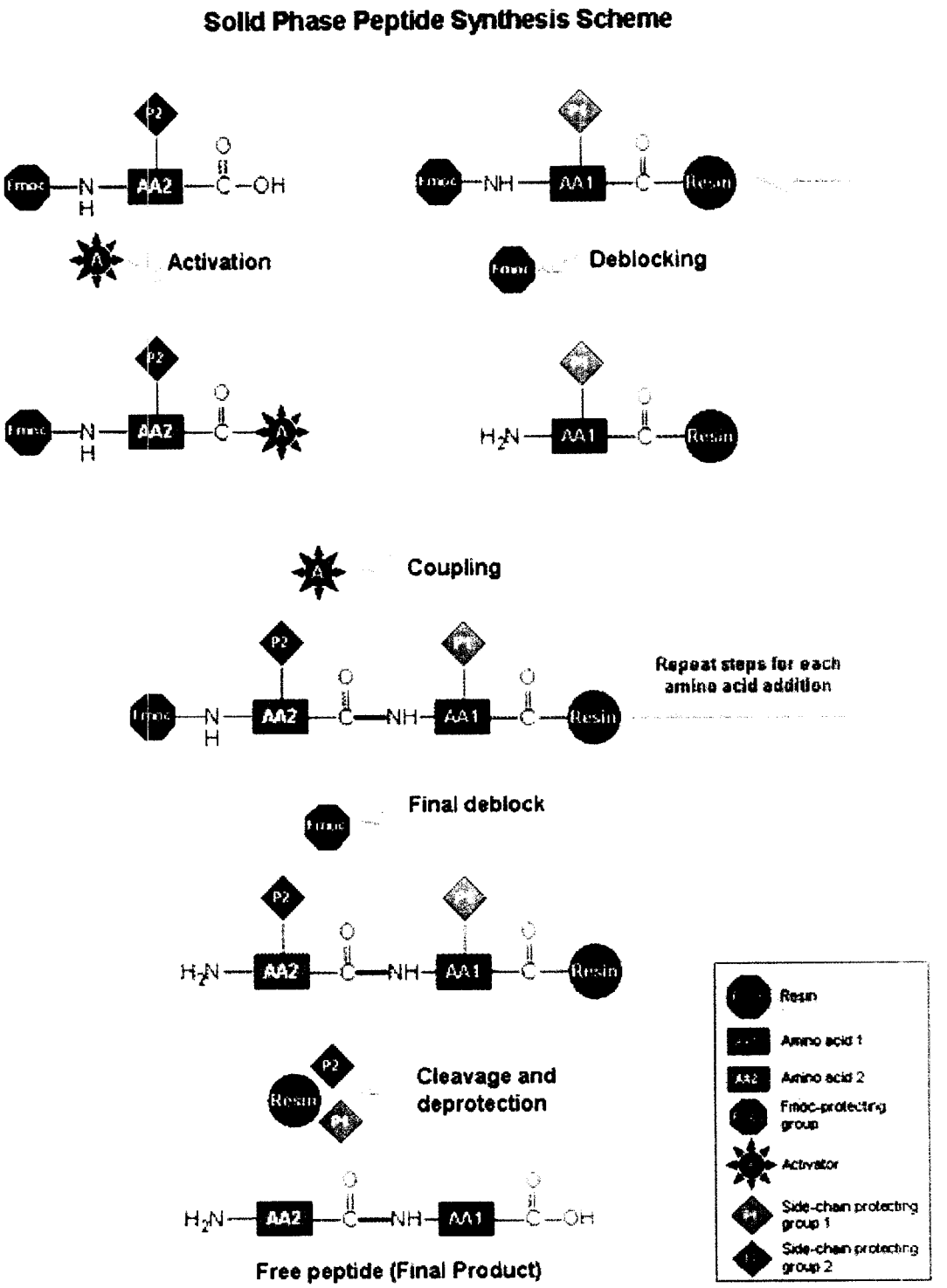


Fig 3.3: Scheme of Solid Phase Peptide Synthesis

3.14.ELISA

1. The peptide synthesized was dissolved in Carbonate/bicarbonate buffer, pH 9.6 and coated onto the maxisorp plate overnight,4°C with gentle rocking
2. Blocked the plate using blocking buffer for 3 hrs with gentle rocking at 37°C
3. Washed thrice with washing buffer
4. Diluted the TB pool serum using ELISA diluent buffer
5. Added 100µl of the diluted serum to each well with wash buffer
6. Diluted anti-human I_g conjugate 2500times and added 50µl each. Incubated for 2hrs
7. Washed the plate 5times with wash buffer
8. Prepared the substrate solution
9. Incubated at 37°C for 5 minutes -15 minutes with rocking
10. Added 25µl, 3M NaOH to stop the reaction.
11. Read the plate at 405nm

RESULTS

4.1Biopanning results for TB Serum

Table 4.1 shows the number of colonies obtained in the first biopanning when serially diluted with LB medium and incubated overnight.

4.1.1. First biopanning

Serial dilution	Plaque forming units(PFU)	Titre value
10^{-3}	301	$3x10^{6}$
10^{-4}	20	$2x10^{6}$
10^{-5}	7	$7x10^{6}$
10^{-6}	NIL	NIL

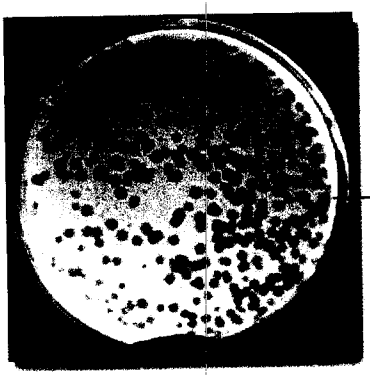


Fig 4.1 plaques in bacterial smear

RESULTS

4.1.2. Amplified first set Biopanning

Serial dilution	Plaque forming units	Titre value
10^{-10}	152	$1.52x10^{11}$
10^{-11}	20	$2.0x10^{13}$
10^{-12}	NIL	NIL
10^{-13}	NIL	NIL

4.1.3. Second Biopanning

Serial dilution	Plaque forming units	Titre value
10^{-3}	699	$6.99x10^{6}$
10^{-4}	26	$2.6x10^{6}$
10^{-5}	NIL	NIL
10^{-6}	NIL	NIL

4.1.4. Amplified second Biopanning

Serial dilution	Plaque forming units	Titre value
10^{-10}	3	$3x10^{11}$
10^{-11}	1	$1x10^{12}$
10^{-12}	NIL	NIL
10^{-13}	NIL	NIL

4.1.5. Third Biopanning

Titre value	Plaque forming units	Titre value
10^{-3}	1	$1x10^{6}$
10^{-4}	NIL	NIL
10^{-5}	NIL	NIL
10^{-6}	NIL	NIL

Plaques were calculated by using the formula given below:

PFU= Number of plaques/Dilution Factor x Volume of diluted phage added into the well/ml

Phages added during first set of Biopanning was 1.5×10^{11}

Second set of Biopanning 1.75×10^{11}

Third set of Biopanning 1.75×10^{11}

4.2. Biopanned results after treating the TB serum with Normal Sera and Hep B sera

Titre value	Plaque forming units	Titre value
10^{-7}	12	9×10^{6}
10^{-8}	6	6×10^{6}
10^{-9}	5	5×10^{6}
10^{-10}	1	1×10^{6}

Totally 24 plaques were obtained

4.3. Phage amplification

Plaques 24 in number were amplified and checked in 0.8%agarose gel electrophoresis.

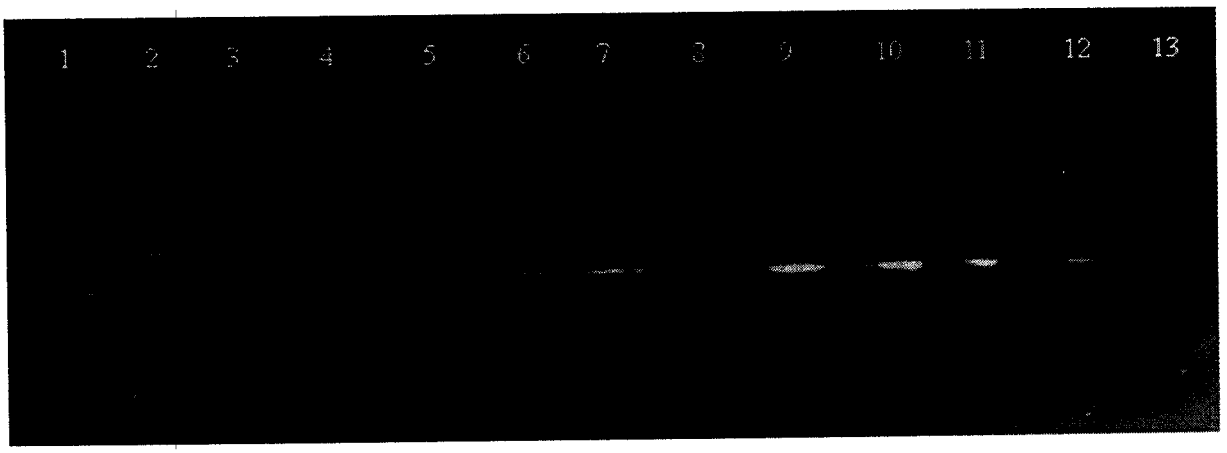


Fig 4.2:Gel picture showing bands for the samples 1-13

Lane 1 : phage sample 1

Lane 2 : 100bp marker

Lane: 3-13 : phage sample 2-12

4.4. Purification of plaques

1 µl of phage from each of the purified phages (24 in number)were taken added into LB+tet plates spreaded with 100µlof ER2738.All the 24 plaques were observed after overnight incubation at 37 °C along with X-gal.

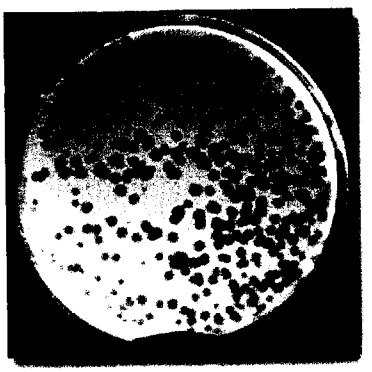


Fig 4.3:Purified plaques in X-gal

4.5. Single stranded DNA Isolation

The amplified phage clones after the third biopanning was amplified again to isolate the single stranded DNA. Gel picture showing the single stranded DNA of the phage clones separated on 0.8% agarose, stained with 0.5µg/mL Ethidium bromide.

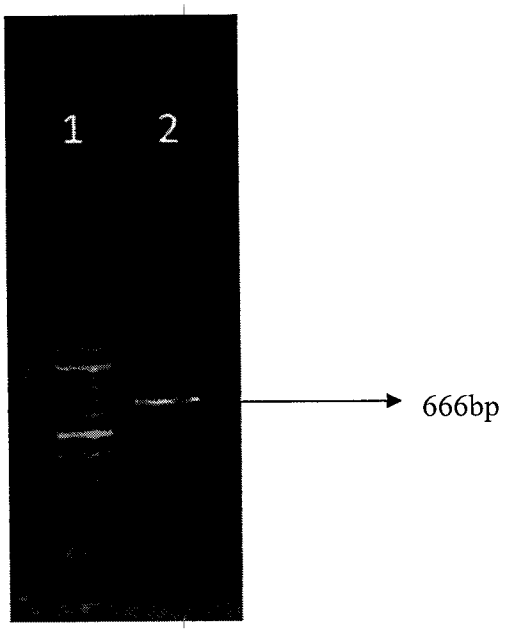


Fig4.4:Isolation of Ss DNA

Lane 1 :100bp marker

Lane2 :phage Ss DNA

4.6. PCR amplification

This DNA was used to amplify the 666 bp fragment from the phages which contained the peptide insert by PCR amplification. The amplicons were separated and the bands were cut out and purified. These bands were then subjected to sequencing

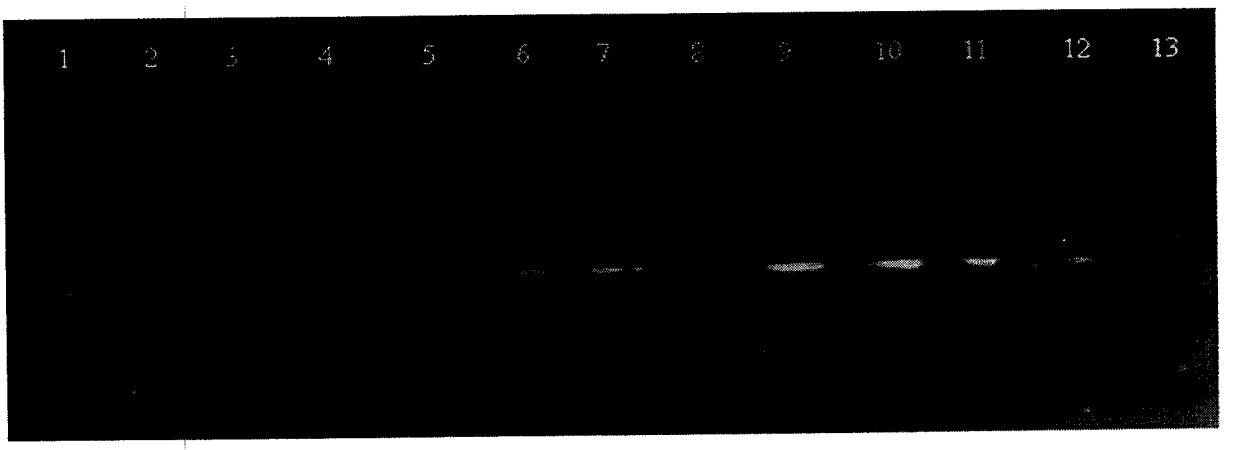


Fig 4.6:Showing PCR amplified phage

Lane 1-13 showing PCR amplified phage samples from 1-13.

4.7. Peptide Synthesis

The selected mimotopes, a structurally flexible linker (Gly-Gly-Gly-Ser)was added to the representative consensus sequences at the C-terminal ends to obtain the effective conformations of peptides. Peptides were produced on a 0.12-mmol scale by using standard fluorenylmethoxy carbonyl chemistry, as described earlier. The purity and identity of the peptides were confirmed by time of flight-electrospray mass spectrometry on a Micromass Q-T spectrometer (Waters, Milford, MA).

The concentrations of the peptides were determined by the method of Lowrys method.

The DNA sequence is then translated into a peptide sequence. The peptide sequence identified is KPLRPSIKMRSI

The picture below is a representative one for the phage sequencing. The sequence data was analyzed visually and aligned with the M13 phage sequence and the inserted peptide region was identified.

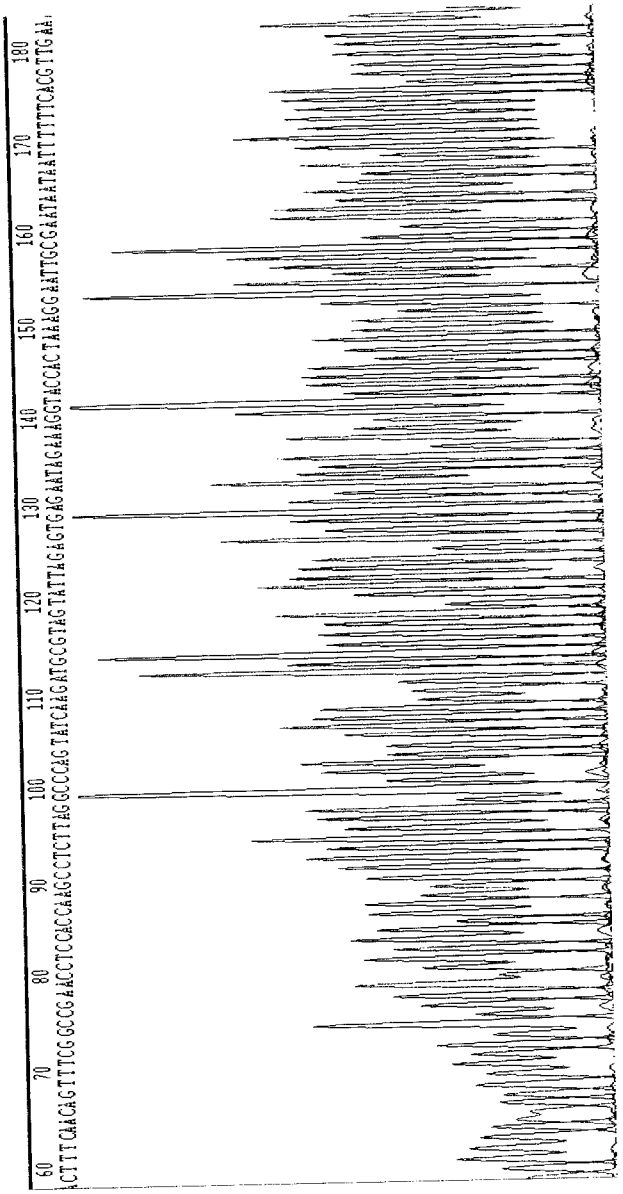


Fig 4.7:Chromatogram showing the sequence data

Chromatogram showing the sequence data and the box indicating the part of the clone, which determines the specific peptide incorporated.

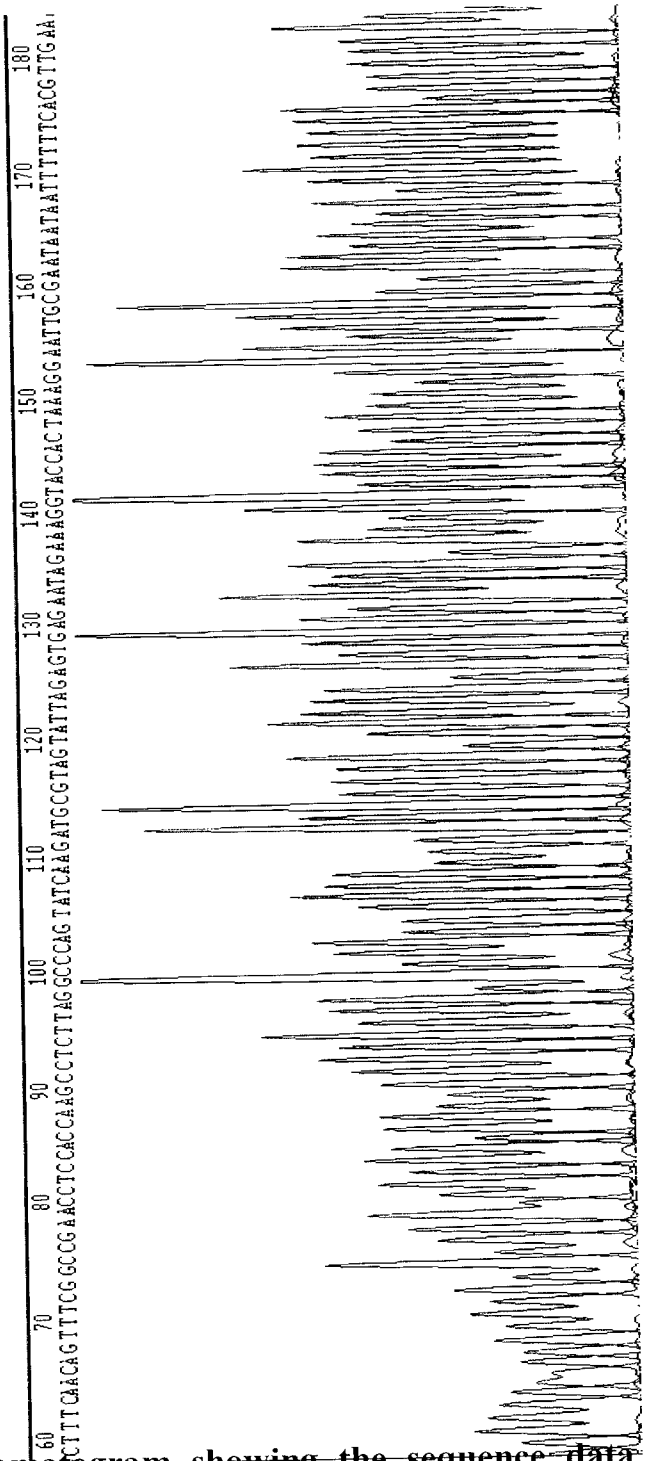


Fig 4.8: Chromatogram showing the sequence data and the box indicating the part of the clone

The identified peptide was synthesized chemically using the solid phase chemistry for peptide synthesis. This peptide was dissolved in ELSA coating buffer at a concentration of 1mg/ml and used for ELISA. The presence of peptide binding antibodies was identified using antihuman polyvalent immunoglobulin conjugated with alkaline phosphatases. The colour developed after the reaction with the substrate was stopped with NaOH and the reading was taken at 405 nm. The data obtained is shown below.

4.7. ELISA reading at 405nm.

Peptide		Blank	
TB Sera	Normal	TB Sera	Normal
0.549	0.437	0.386	0.362
0.578	0.404	0.329	0.235
0.815	0.741	0.525	0.471
0.910	0.692	0.347	0.311
1.046	0.931	0.438	0.390

Table4.1:ELISA readings obtained at 405nm

The ELISA results show that there is a difference between the reading for the TB Sera and the normal/Hepatitis sera. Here we have used three sets of sera, namely

- TB sera (Serum isolated from TB patients),

- Normal sera (Serum isolated from people who do not show any disease symptoms) and
- Hepatitis sera (Serum isolated from hepatitis B and C patients).

All the sera other than TB sera served as controls or rather it served as the method to remove all the phage clones that bind to common proteins in the sera and also those binds with the epitopes of other disease causing agents. It would have been better in this study to recruit many patients with a wide range of disease so that we could have removed all the phage clones which binds with the epitopes of all the other disease causing agents. Unfortunately, those sera were not available in the laboratory and also it was difficult to procure.

The differences observed in the values between the TB sera and the normal/Hepatitis sera are not very high. This result how ever is not enough to use this peptide as a diagnostic tool as such. But this observation could be an indication towards the use of this peptide or any other peptide to develop an ELISA method to diagnose Tuberculosis in earlier stages of infection. Much more extensive analysis and assays has to be standardized so as to get better differentiation between the tested samples and the normal/hepatitis sera. As serodiagnosis methods are very rapid and easy to perform, it can also be used for the screening for patients in an area. Also, ELISA being a very cost effective technique, this could be of great advantage in a resource poor country like India.

DISCUSSION

The present investigation explores the possibility of applying the phage display technique in the context of deriving peptide mimotopes of mycobacterial antigens. The working hypothesis is that the phage peptide mimics some of mycobacterial antigens, particularly the non-protein ones, would be useful as diagnostic agents.

Moreover, since mycobacterial antigens are known to be immunomodulators in addition to serving as diagnostic tools, such peptide mimics could be useful for therapeutic purposes, such as for the treatment of TB and cancer. This is one of the first reports in which phage display tools have been used to identify mimotopes of mycobacterial antigens. An investigation on a somewhat similar theme has been attempted earlier (Bermann et al 1995). In that investigation, antiserum against extracted mycobacterial cell surface was used to obtain peptide mimotopes. In this study we have used two approaches. One of these was a targeted approach in which involved identification epitope-specific phage sequence and the other using this peptide as a diagnostic kit for the diagnosis of TB. (Hermann et al.,2005)

In conclusion, the peptide mimotopes derived in the present study may have a wide range of applications, such as the following:

(i) they could replace corresponding antigens in diagnosticassays, leading to the development of novel combinatorial diagnosis of TB.

(ii) they may be used as immunomodulatory agents in specific contexts, such as cancer therapy;

(iii) they are proinflammatory, they could be used as adjuvants in vaccines.(Small et al., 2000).

Much more extensive analysis and assays has to be standardized so as to get better differentiation between the tested samples and the normal/hepatitis sera. As serodiagnosis methods are very rapid and easy to perform, it can also be used for the screening for patients in an area. Given the complexity of tuberculosis, it is difficult to obtain a solution in a single study; however, the information provided here could give valuable leads to future applications of the phage display to TB (Wang et al., 2004). It would be interesting to apply the phage display technique directly to TB patients. By using suitable healthy controls and TB patients, it should be possible to perform a subtractive search for new mimotopes related to *M.tuberculosis*.

CONCLUSION

CONCLUSION

New diagnostic techniques currently available are no more sensitive (and a great deal more expensive) than properly performed sputum smear and culture examination. Their usefulness in high prevalence countries, especially where there is a high prevalence of HIV co-infection, has not yet been established.

(i)Interferon-γ assay test is not affected by past BCG vaccination, and will eliminate the unnecessary treatment of patients with
BCG-related false positive results.

ii)Molecular methods have sensitivity greater than smear examination, but less than culture. False positives and false negatives occur, Cannot differentiate between live and dead bacilli. Very expensive test.

iii) Serological tests have not been adequately evaluated in high prevalence TB settings, false negative results are common in HIV infected persons, and antibody tests cannot distinguish between active TB and latent infection.

According to the experiment reviewed here, information about phage display, this technique/tool has a multipurpose utilization in molecular evolution, analysis of protein/ligand interactions and the generation of antibodies. However, the direct use of a peptide presenting by a recombinant phage displaying as a vaccine to examine its protective potential would
be simpler and much less expensive than the conventional method of peptide synthesis or recombinant protein preparation and purification for immunization. The phage displaying a pathogen specific peptide, in ER 2738 the displaying of recombinant protein or its part, representing an immunogenic mimic the natural antigen. This could lead to production of

antibodies specific to pathogen. Recombinant bacteriophage displaying a disease specific epitope can be use as a vaccine to examine the potential of an epitope of interest to prevent disease. Phage display has also the overwhelming advantage that it is has cheap and easy. It uses standard microbiological techniques that are familiar to all molecular biologists, and its key resources - phage libraries and clones - are replicable and therefore nearly cost free after their initial construction or selection. It is astonishing to contemplate that within a single tube could be fit a few hundred trillion phage particles displaying billions of different peptide structures - an abundance and diversity from which hundreds of different users with altogether different purposes in mind can select clones of great value.

APPENDICES

MEDIA AND SOLUTIONS

- **LB Medium:**

Bacto Tryptone	-10g
Yeast extract	-5g
Nacl	-5g
Distilled water	-1000ml

- **LB IPTG Xgal Plates:**

LB medium + 15gl agar

IPTG Xgal -1ml

- **Blocking buffer**

TBS	-100ml
BSA	-1.0g
NaN_3	-0.02g

Dissolve the BSA in 1x TBS and filter sterilize.

- **Carbonate /Bicarbonate buffer**

Na_2Co_3-100ml

$NaHCo_3$	-2.93g
NaN_3	-0.2g

Adjust the pH 9.6 and filter sterilize after the volume is made upto 100ml

- **Substrate buffer**

  Diethanolamine -10%

  Mgcl2 -0.5mM

- **Wash buffer**

  TBS with 0.05% Tween 20

- **ELISA Diluent Buffer**

  1xTBS

  0.05% Tween 20

  0.1% BSA

- **TE Buffer Solution, 1X**

  Tris 10.0 mM
  EDTA 1.0 mM

  Made the solution to 100ml and pH adjusted to 8

- **RNAse solution**

  RNAse -10mg/ml of the solution

- **Lysis Buffer**

SDS	-100 µl
EDTA	-20 µl
NaoH	-0.1ml

Volume made upto 1ml

- **LB Top Agar for 50ml**

Bactotryptone	- 0.5g
Yeast Extract	-0.25g
Nacl	- 0.25g
$Mgcl_2.6H_2o$	- 0.05g
Noble Agar	- 0.35g
Water	- 50ml

- **PBS**

Na_2HPO4	-1.16 g
KCl	- 0.1 g
K_3PO4	- 0.1 g
NaCl	-4 g

- **IPTG/Xgal Stock:**

IPTG
(isopropyl-β-D-
thiogalactoside) -1.25 g

Xgal
(5-Bromo-4-chloro-3-indolyl-β
-D-galactoside) - 1 g

Dissolved in DMF
(dimethyl formamide) - 25 ml

Solution can be stored at –20°C.

- **.Iodide Buffer**

Tris HCl pH 8.0	-10mM
EDTA pH 8.0	-1mM
NaI	-4M

- **TBS**

Tris 50mM	-0.605g
NaCl 150mM	-0.876g

Made upto 100ml and pH is adjusted to pH 7.5

- **TBST 0.1%**

TBS +0.1% Tween 20

- **TBST 0.5%**

TBS+0.5%Tween 20

- **50x TAE**

Tris 24.2g	
Acetic acid	-5.71ml
EDTA 0.5M	-10ml

Made upto 100ml and pH adjusted with Acetic acid

- **10xTBE**

Tris	-10.8g
Boric acid	-5.5g
EDTA 0.5M	-4ml

pH of stock should be 8.3

- **Neutralization buffer**

Nacl - 43.83g

Tris - 30.25g

pH made to 7.5 with HCl

- **PEG/Nacl for 50ml**

Nacl 7.30 -5g

PEG 8000 -10g

(500 ml distilled water) pH 7.4

- **Tris Buffer Solution**

Tris 1.0 M, -500 ml

pH 8.0 should be maintained

- **Tetracycline Stock (suspension)**

20 mg/ml in 1:1 Ethanol:Water.

Store at –20°C.Vortex before using

- **LB/IPTG/Xgal Plates:**

  1 liter LB medium + 15 g/l agar .Autoclave, cool to < 70°C, add 1 ml IPTG/X-gal Stock per liter and pour. Store plates at 4°C in the dark.

- **10% NS (Normal Serum) with 1% BSA (Bovine Serum Albumin, Fraction in TBS:**

  For 1 ml:

NS	-100 µl
BSA	-10 mg
TBS	-900 µl

  pH can vary from7.6-7.8

- **Primary antibody made up in TBS with 1% BSA:**

  For 0.1 ml

Primary antibody	-100 µl
BSA	-10 mg
TBS	-900 µl

  pH can vary from 7.6-7.8

- **Secondary biotinylated antibody made up in TBS with 1% BSA:**

(Example is of secondary biotinylated antibody used at a dilution of 1:200)

For 1 ml:

Secondary biotinylated Antibody	-5 µl
TBS	-995 µl

pH 7.6-7.8 is maintained.

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