COMPARITIVE EVALUATION OF LOOP MEDIATED ISOTHERMAL AMPLIFICATION IN THE DIAGNOSIS OF MYCOBACTERIUM TUBERCULOSIS WITH CULTURE AND FLUORESCENT MICROSCOPY

DISSERTATION SUBMITTED TO

In partial fulfillment of the requirement for the degree of

DOCTOR OF MEDICINE IN MICROBIOLOGY

(Branch IV) M. D. (MICROBIOLOGY)

of

THE TAMIL NADU DR. M. G. R MEDICAL UNIVERSITY

CHENNAI- 600032

DEPARTMENT OF MICROBIOLOGY

TIRUNELVELI MEDICAL COLLEGE

TIRUNELVELI- 11

APRIL 2016

BONAFIDE CERTIFICATE

This is to certify that the dissertation entitled "**COMPARITIVE EVALUATION OF LOOP MEDIATED ISOTHERMAL AMPLIFICATION IN THE DIAGNOSIS OF MYCOBACTERIUM TUBERCULOSIS WITH CULTURE AND FLUORESCENT MICROSCOPY"** submitted by **Dr. S.UMMER SHERIFF** to the Tamilnadu Dr. M.G.R Medical University, Chennai, in partial fulfillment of the requirement for the award of M.D. Degree Branch – IV (Microbiology) is a bonafide research work carried out by her under direct supervision & guidance.

Guide,

Professor And Head,

Department Of Microbiology

Tirunelveli Medical College

Head of the Department,

Department of Microbiology

Tirunelveli Medical College,

Tirunelveli.

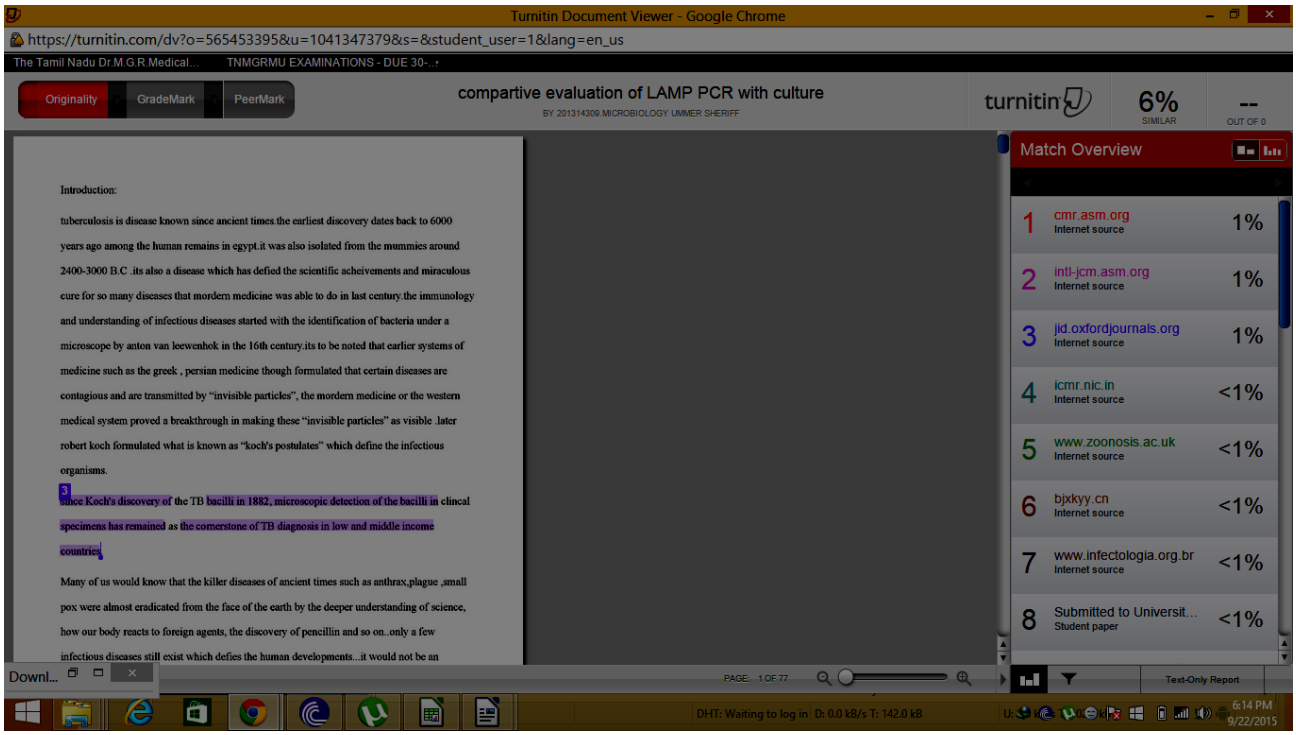
CERTIFICATE

This is to certify that the Dissertation "**COMPARITIVE EVALUATION OF LOOP MEDIATED ISOTHERMAL AMPLIFICATION IN THE DIAGNOSIS OF MYCOBACTERIUM TUBERCULOSIS WITH CULTURE AND FLUORESCENT MICROSCOPY"** presented herein by **Dr. S.UMMER SHERIFF** is an original work done in the Department of Microbiology, Tirunelveli Medical College Hospital, Tirunelveli for the award of Degree of M.D.(Branch IV) Microbiology under my guidance and supervision during the academic period of 2013-2016.

The DEAN

Tirunelveli Medical College,

Tirunelveli-627011


Turnitin Document Viewer - Google Chrome
https://turnitin.com/dv?o=565453395&u=1041347379&s=&student_user=1&lang=en_us
The Tamil Nadu Dr.M.G.R.Medical... TNMGRMU EXAMINATIONS - DUE 30-..
Originality
GradeMark
PeerMark
compartive evaluation of LAMP PCR with culture
BY 201314309.MICROBIOLOGY UMMER SHERIFF
turnitin
6%
SIMILAR
--
OUT OF 0
Introduction:
tuberculosis is disease known since ancient times.the earliest discovery dates back to 6000 years ago among the human remains in egypt.it was also isolated from the mummies around 2400-3000 B.C .its also a disease which has defied the scientific acheivements and miraculous cure for so many diseases that mordern medicine was able to do in last century.the immunology and understanding of infectious diseases started with the identification of bacteria under a microscope by anton van leewenhok in the 16th century.its to be noted that earlier systems of medicine such as the greek , persian medicine though formulated that certain diseases are contagious and are transmitted by "invisible particles", the mordern medicine or the western medical system proved a breakthrough in making these "invisible particles" as visible .later robert koch formulated what is known as "koch's postulates" which define the infectious organisms.
since Koch's discovery of the TB bacilli in 1882, microscopic detection of the bacilli in clincal specimens has remained as the cornerstone of TB diagnosis in low and middle income countries.
Many of us would know that the killer diseases of ancient times such as anthrax,plague ,small pox were almost eradicated from the face of the earth by the deeper understanding of science, how our body reacts to foreign agents, the discovery of pencillin and so on..only a few infectious diseases still exist which defies the human developments...it would not be an
Match Overview
1 cmr.asm.org Internet source 1%
2 intl-jcm.asm.org Internet source 1%
3 jid.oxfordjournals.org Internet source 1%
4 icmr.nic.in Internet source <1%
5 www.zoonosis.ac.uk Internet source <1%
6 bjxkyy.cn Internet source <1%
7 www.infectologia.org.br Internet source <1%
8 Submitted to Universit... Student paper <1%
Downl...
PAGE: 1 OF 77
Text-Only Report
DHT: Waiting to log in | D: 0.0 kB/s T: 142.0 kB
6:14 PM
9/22/2015

DECLARATION

I, **Dr.S.UMMER SHERIFF declare** that, I carried out this work on "**COMPARITIVE EVALUATION OF LOOP MEDIATED ISOTHERMAL AMPLIFICATION IN THE DIAGNOSIS OF MYCOBACTERIUM TUBERCULOSIS WITH CULTURE AND FLUORESCENT MICROSCOPY"** at the Department of Microbiology, Tirunelveli Medical College, I also declare that this bonafide work or a part of this work was not submitted by me or any others for any award, degree, or diploma to any other University, Board, either in India or abroad.

This is submitted to the Tamilnadu Dr. M.G.R. Medical University, Chennai in partial fulfillment of the rules and regulations for the

M.D Degree examination in Microbiology.

Dr. S.Ummer Sheriff

post graduate

department of microbiology

tirunelveli medical college

ACKNOWLEDGEMENT

I am grateful to The **Dean, sithy athira munawarah MD** Tirunelveli Medical College and, Tirunelveli Medical College hospital Tirunelveli for permitting me to carry out this study.

I extend my sincere thanks to **Prof.Dr.C.Revathy M.D.,** for her valuable guidance and support during the study period.

I would like to express my sincere thanks to **Prof. Dr. S. Poongodi @ Lakshmi M.D.,** for her constant guidance and encouragement given to me throughout this study.

I am highly indebted to **Prof. Dr.Ramesh Babu M.D.,** whose sincere guidance and encouragement were a source of strength for the successful completion of this study.

I also express my thanks to **Prof.Dr.Dhanapal MD** who gave me guidance ,technical inputs to conduct this study

I would like to express my sincere thanks to all my Assistant Professors **Dr.B.Cinthuja M.D., Dr. G.Velvizhi M.D., Dr. G.Sucila Thangam, M.D., Dr V.P Amudha M.D., Dr I.M Regitha M.D., Dr.S.Gowri M.D., Dr.M.Kanagapriya M.D.** for their valuable suggestions, guidance and support throughout the study.

I would like to thank all my colleagues, **Dr.T.Sankaranarayanan, Dr.P.Rajpriya, Dr.S.Suganya, and Dr.M.Jeeva, Dr.R.Shiny, Dr.R.Poornakala, Dr.V.Indhumathi and Dr.S.Prarthana**, **Dr.P.Anbumathi** for their support and cooperation rendered during the work.

I extend my thanks to all staff members, Department of Microbiology for giving full cooperation and timely help in carrying out the laboratory studies.

Special thanks are due to my Messer **V.Parthasarathy, V.Chandran, S.Pannerselvam, S.Santhi, S.Venkateshwari. M.Mali, S.Arifal Beevi, A.S.Abul Kalam, A.Kavitha, T.Jeya, K.Sindhu, K.Umayavel, Sreelakshmi** and are other supporting staffs for their services rendered.

I am greatly indebted to **Mr. Heber** for his expert guidance in statistics. Finally I thank the Almighty for without Him nothing would have been possible. I extend my thanks to all the patients for their participation and kind co-operation throughout the study period.

Finally, I extend my wholehearted thanks to my beloved parents, friends, family members,and my wife **Mrs.Fazliya syed** for their silent sacrifices, support and words of encouragement which were a source of inspiration for the successful completion of this study.

CONTENTS

Abstract:

TB is a common disease and leading causes of mortality due to communicable diseases. Research has been going to find the nature of the disease as well to find new technique that could give us a leading edge in cracking this disease in an early stage.recently a number of nucleic acid amplification assays have been employed to detect the mycobacterium tuberculosis in clinical specimens, among these loop mediated isothermal amplification posess superior reaction characteristics

in the present study we evaluate a LAMP method to identify the MTB specific IS6110 gene among 50 sputum samples collected from patients suspected of tuberculosis in tirunelveli medical college and compare them with gold standard “culture on lowenstein jenson media” for 8 weeks .the study showed the LAMP method has an excellent sensitivity of 95% and specificity of 79% which shows that this method in conjunction with smear microscopy can be a powerful tool in the early detection of TB.

Introduction:

Tuberculosis is disease known since ancient times. The earliest discovery dates back to 6000 years ago among the human remains in Egypt[1]. It was also isolated from the mummies around 2400-3000 B.C. Its also a disease which has defied the scientific achievements and miraculous cure for so many diseases that modern medicine was able to do in last century. The immunology and understanding of infectious diseases started with the identification of bacteria under a microscope by Anton Van Leewenhouk in the 16th century.its to be noted that earlier systems of medicine such as the greek, Persian medicine though formulated that certain diseases are contagious and are transmitted by “invisible particles”, the modern medicine or the western medical system proved a breakthrough in making these “invisible particles” as visible.

Since Koch's discovery of the TB bacilli in 1882, microscopic detection of the bacilli in clincal specimens has remained as the cornerstone of TB diagnosis in low and middle income countries.

Many of us would know that the killer diseases of ancient times such as anthrax, plague, small pox were almost eradicated from the face of the earth by the deeper understanding of science, how our body reacts to foreign agents, the discovery of penicillin and so on..only a few infectious diseases still exist which defies the human developments...it would not be an exaggeration to say that though effective treatments, diagnostic methods had been evolved to tackle tuberculosis it is still largely “invisible” or undetected.

The statistics by the world health organization says that one third of every human being in this world is infected with *Mycobacterium tuberculosis* [3,4]. not to say its more prevalent in

developing countries or the “third world countries”.this disparity in its geographical distribution is due to the poor living conditions, inadequate housing, poor sanitation, malnutrition and to a greater extent on the health care measures taken by the respective governments.

India is one of world's nest in tuberculosis an estimated 2 patients per second die of tuberculosis[5]. And the post independent India started its national tuberculosis program since 1962 which was evolved in to the revised national tuberculosis control program in 1992 with the help of international agencies and adopted the international strategy of DOTS-direct observed short term treatment strategy[5].

The importance of tuberculosis and the need for further research into it is obligated by its “invisible nature”.why it is said as “invisible”? Because the difficulties in the diagnosis whether clinical or non clinical. In clinical spectrum the diagnosis includes symptoms such as cough with expectoration for more than 2 weeks, evening rise in temperature, night sweats, loss of weight and appetite and radiologically in finding any cavitary lesions in the lungs. In microbiology the diagnosis includes the classical acid fast staining method, the gold standard culture on solid media. The developments in molecular biology and the development of polymerase chain reaction has made numerous strides in the field of microbiology in diagnosing infectious diseases. Scientists have gone to the extent of describing the era of molecular biology as “before PCR” and “after PCR”.

The PCR techniques enables to diagnose small quantities of the infectious material and has proved to be a good diagnostic method in terms of sensitivity, specificity and rapidness. However they are not without negatives. The negatives include its cost, and cannot differentiate

between live or dead organisms.

The PCR technique originally developed by Kary Mullis in 1983 was based on amplifying a single gene or a few gene to many fold times , thereby enabling to identify them. He used a polymerase enzyme taq polymerase isolated from the thermophilic bacterium thermus aquaticus. Infact the process is named after that enzyme. Since then the process has been used in a wide variety of applications such as gene cloning, DNA fingerprinting and to identify infectious organisms. The method relies on the thermal cycling and enzymatic replication of the DNA. Primers which are short DNA fragments which are complementary to the target gene along with the polymerase enzyme make this possible.

It is to be noted that PCR is not without errors, the polymerase enzyme can mutate the PCR fragments made and hence affect their specificity.

The technique has been evolved into many variations and one of the variations is loop mediated isothermal amplification. This LAMP technique employs a Bst polymerase extracted from bacillus stearothermophilus. Incontrast to the conventional PCR which uses thermocycling, this technique could be done in a constant temperature. This is made possible by using a polymerase enzyme with a high strand displacement activity in addition to the replication activity. Typically 4 different sets of primers are used to amplify six different areas of the target gene which adds to their specificity. A large number of copies are made and their diagnosis is done with naked eye due to the increased precipitation of magnesium pyrophosphate which is a byproduct of the amplification. It can also be detected via photometry .dyes such as a SYBR green can be used to intercalate the amplified DNA and hence by measuring the colour change its possible to quantitate the reaction as well.

By eliminating the use of costly thermocyclers this technique promises a great and low cost alternative in diagnosing infectious diseases. The laboratory diagnostic method include Ziehl-Neelson acid fast staining which is routinely used in all the primary health care centers in India. However the sensitivity of the test is only 30%, thereby missing a huge chunk of the positive patients. Culture on the other hand is the “gold standard” but it needs sophisticated centers with biosafety levels and the result cannot be achieved unless 8 weeks which is huge time for which the treatment cannot be delayed. The skin test can determine the immune status of the individual and cannot determine if he's suffering from active diseases or had previous immunization, nevertheless its useful in screening purposes. The RNTCP has achieved great lengths in combating tuberculosis in India, nevertheless the gaps in diagnosing the infection still remains a hurdle.

In India 40% of population are infected with tuberculosis with a lifetime risk of 100% even in the absence of HIV. The emergence of HIV has made the TB/HIV a dreadful combination as HIV itself is not a killer disease while it lowers the immunity, thereby making tuberculosis an opportunistic infection and thus the rates of Tuberculosis in patients infected with HIV has soared. One more thing which is of great concern is the emergence of the “super bugs”, which is the multidrug resistance Tuberculosis which has higher mortality and morbidity. The RNTCP has launched PCR based techniques in detecting the above two clauses which is CBNAAT which is cartridge based nucleic acid amplification techniques. This has been accepted worldwide to detect *Mycobacterium tuberculosis. But* their role in screening *Mycobacterium tuberculosis* and detecting it in patients in outpatient setting has seen limitations owing to the higher costs and still needs further evaluation and research regarding the same. The present

study aims to find the efficacy of this newer technique the “loop mediated isothermal amplification” in the detection of *Mycobacterium tuberculosis* in clinical specimens. as tuberculosis remains as one of the diseases which needs a definitive control strategy numerous research works are on the way especially in the light of MDR TB and its association with HIV.

Aims and objective:

aim of the study:

To find a low cost, rapid and efficient microbiological diagnostic method for the detection of *Mycobacterium tuberculosis* from clinical specimens that could be implemented without much training and even in a rural setting

objective:

to evaluate the sensitivity, specificity of loop mediated isothermal amplification in sputum samples collected from both smear positive, smear negative specimens in a blinded study and compare it with a gold standard technique -culture in solid lowenstein jenson media.

Review of literature:

Epidemiology:

According to ICMR bulletin dated august 2002,

about one third of the worlds population is infected with tuberculosis.

Around 10 million people acquire tuberculosis annually and 3 million people die of the disease each year.

Mortality for tuberculosis is 25% of all avoidable deaths.

95% of all TB cases and 98% of death due to TB are in developing countries which includes India, and among them 75% are in economically active age group.

In India around 2 million people acquire the disease annually and of that 1 million people die of it each year.

This means one patient dies of TB every minute.

To compound this problem, is the emergence of HIV.

India has 3.5 million HIV patients of which 1.8 million patients have TB. Around 60% of HIV patients acquire active TB during their life time[7] .

Its worth to mention the emergence of the super bugs MDR and XDR and their coincidence with HIV is nevertheless associated with higher fatality rates.

Terminology:

TB incidence: number of new cases of active TB /year

TB prevalence: number of people living with active TB

the revised national tuberculosis control programme was launched in india in 1997.

Definition:

TB relapse: if the patient becomes ill after finishing the first course of treatments

Treatment failure: some improvement or total failure to respond to treatment

Default: patients who havent finished the first course of treatments

all the above three categories can develop drug resistance and need drug susceptibility testing .

Exposure to TB depends upon the following factors:

1) number of active cases in a community

2) duration of their infection

3) number and nature of interaction between the active case and contacts in a given time.

The above facts were derived from Tbfacts.org

in a study by Dr.William W Stead in the annals of internal medicine say that " person who react after exposure of tuberculosis fall into three types.the reaction here is tuberculin sensitivity test.

1)patients who have positive TST and have a high risk of tuberculosis , they need prophylaxis

2) patients where there is no screening done before exposure and are positive to TST should be treated as like above said

3) people who are positive to TST but the risk is too low..they dont need prophylaxis"

corresponding to the topic I am quoting another study which is worth when considering the screening in epidemics and health care workers[8].

In a study by Peter E Sokolovo in the annals of the internal medicine finds that" systematic monitoring by tuberculin sensitivity testing is necessary to find the adequacy of respiratory isolation procedures carried out in the emergency department of a hospital during epidemics."

smoking is a known risk factor which predisposes to tuberculosis.[10]

In a study by Dr.C.Kolappan from the tuberculosis research center, ICMR published in british medical journal of thorax says that " the association between tuberculosis and smoking is a strong dose-response relationship".[11]

there's some good news.by implemeting effective diagnosis and treatment strategies a few countries have been able to bring down TB.eg: china,brazil and cambodia.[11]

Causative organism:

Scientific work suggests that *Mycobacterium tuberculosis* evolved from a common human specific pathogen about 40000 years ago. .the evolution of the TB bacilli is closed linked with migration of human beings out of africa .the bovis species evolution has been linked with the domestication of animals.[12]

The tubercle bacilli is an aerobic ,acid fast bacilli.the acid fastness is due to the peptidogylcan layer linked with the mycolic acid in the cell wall. This arrangement is one of the virulent characters of the organism as its difficult for the antibiotics to penetrate the cell wall and to the action of the antibodies..cell wall has another component , lipoarabinomannan which eases the intracellular survival of the organism.

Regarding the evolution of TB bacilli, In a study by cristina gutierrraz ,the author says that "scientists show that the TB bacilli is able to exchange parts of their genome with other bacteria , which makes them adaptable to specific hosts".

Transmission:

TB is a disease thats spread by aerosols.factors which influence the transmission are

1) Ventilation in the room

2) Number of bacilli in the droplet

3)Exposure to UV rays

primarily its a disease affecting the lungs .

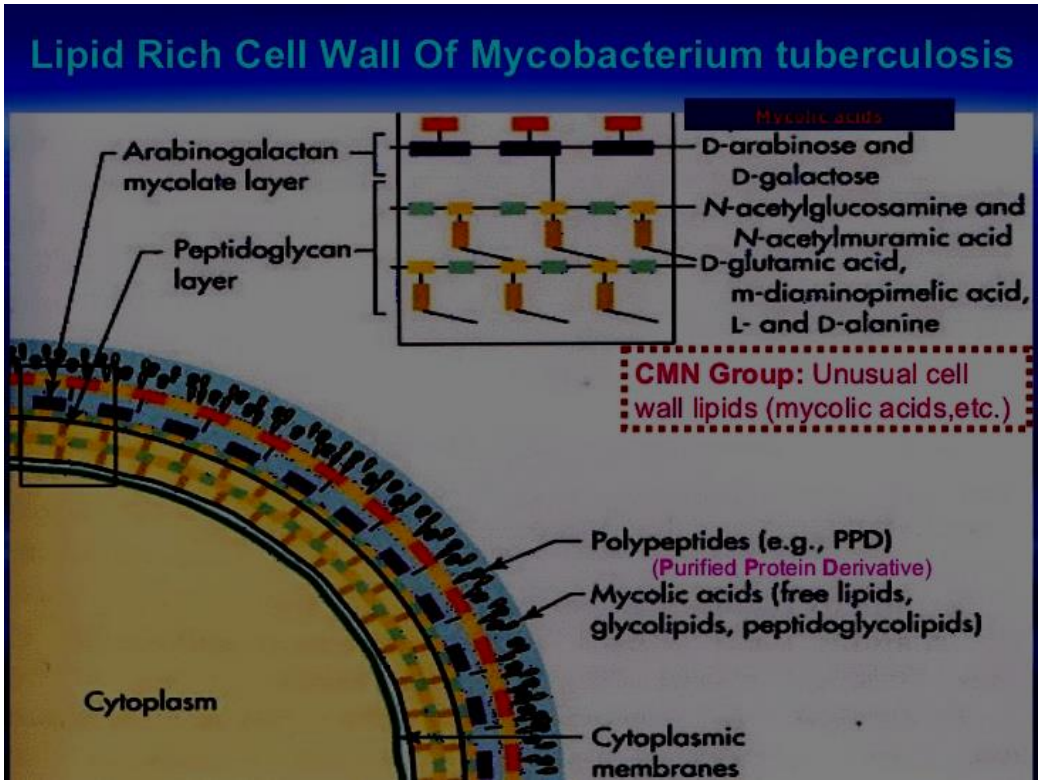
Greek physician and the father of medicine Hippocrates said phthisis, the other name for tuberculosis as "ulceration of lungs, thorax accompanies by cough, fever, and consumption of

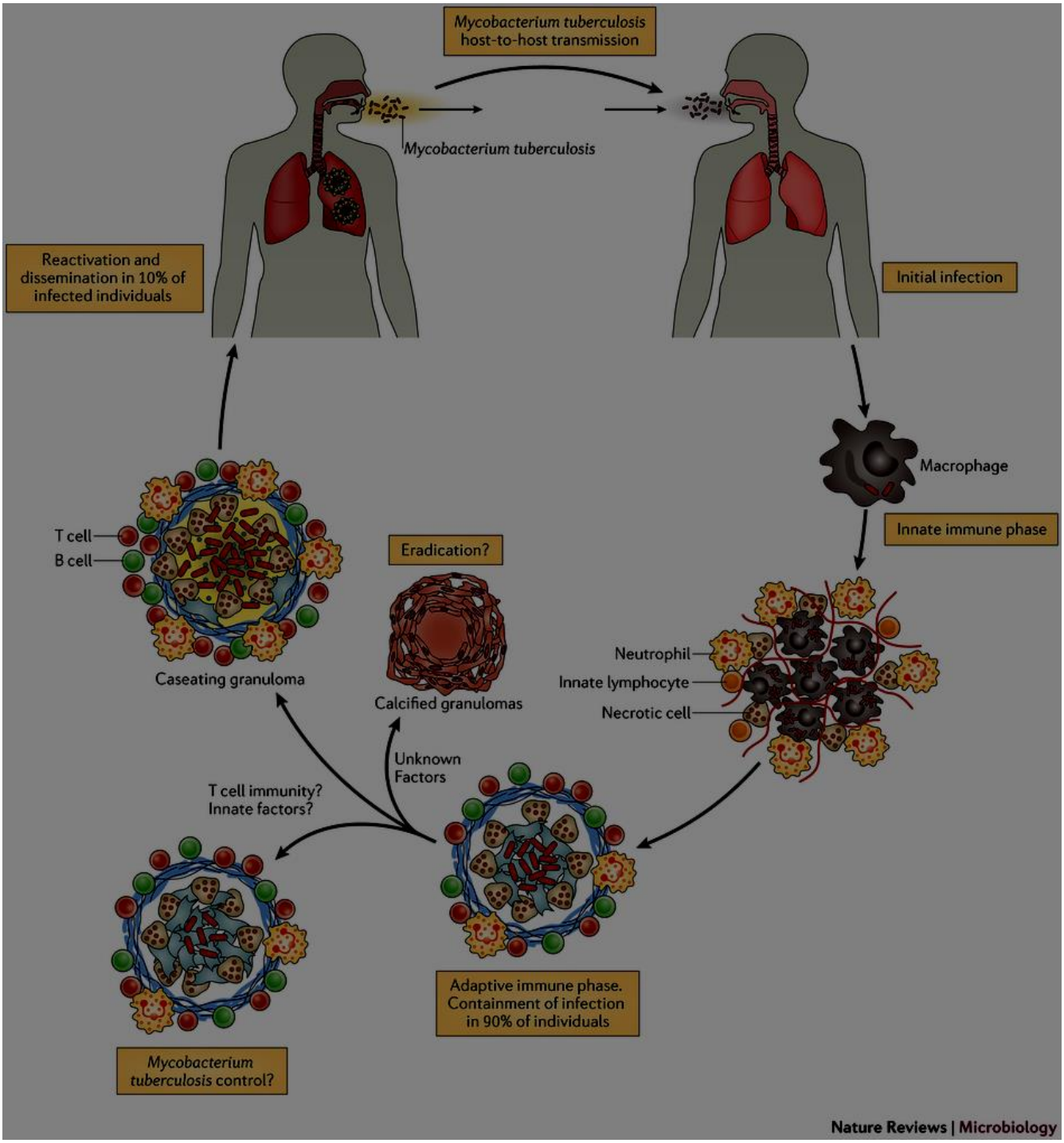
the body by pus".[13]

No wonder the disease was also called a consumption disease.

In a study by Sheela V.Shenoi et al in the journal of current HIV reports in 2013 says that "prevention of tuberculosis requires airborne infection control methods and such methods have been ignored and with grave consequences".[14]

In yet another study by Riley et al published in the American journal of epidemiology confirmed the airborne transmission and its association with quantified exposure risk.[15]





Pathophysiology:

In a study by parsons et al on the laboratory diagnostic aspects of drug resistant TB states "the main reasons for the requirement of multiple drugs to effectively cure Tb is because of the hydrophobic cell envelope surrounding members of the *Mycobacterium tuberculosis* complex that serves as a permeability barrier to many compounds; the sequestered, non replicating sub

population of TB that is affected by drugs only when the cells reemerge from dormancy; and the drug target or drug activating enzymes in TB that are altered by mutation and result in a population of drug resistant cells".[16]

The primary defense system is the airway itself. The infectious droplets when inhaled are caught in the mucus secreted by the goblet cells of the respiratory tract. the cilia present in columnar epithelium push the trapped bacilli upwards by their movement.

Those bacilli which escape this primary defense mechanism are engulfed by the alveolar macrophages which are present in the lungs. This is the second life of defense. Macrophages are cells which need no previous exposure to get sensitized. The action of macrophages starts a chain of events which result in either containing the infection or the bacilli able to withstand this defense too. At last to be an active disease all depends on this delicate balance, the immunity of the body and the invading bacteria.

Inside the macrophages the bacteria continue to multiply, the macrophages began to release a few immune markers like cytokines and proteolytic enzymes. This attracts the T lymphocytes and marks the cell mediated immunity. This forms the third step of defense.

The end result of this cell mediated immunity is destruction of the lesion (granuloma) into a necrosis, in some cases, the lesion is replaced with fibrosis, while in some cases where the immune system is weak the lesion progresses further to primary progressive tuberculosis.

The TB bacilli is contained within the lesion, which may be activated later when the host immune defenses are weak..this dormant lesion is called latent tuberculosis.

In some cases when the lesion erodes into a blood vessel, the bacteria is carried to distance sites producing extra pulmonary tuberculosis. If it enters the lymphatic system, it reaches the adjacent lymph nodes producing soft cheese like necrosis called caseating necrosis.

Clinical features:

Depends on the stage of tuberculosis

1) **Latent Tuberculosis**: MTB can be confined, but difficult to eradicate. Patients with latent TB will not have any clinical signs and symptoms. Neither they are infectious. But the bacilli is present in them for long time.in case of immunodeficiency as in case of AIDS,diabetes, steroid therapy etc the disease may get reactivated .its more common in elderly patients.

in a study by kiwanuka et al and lama et al says "coinfection with TB has been associated with an increased replication of HIV-1 and the development of TB disease during HIV infection involves both a reactivation of latent TB and increasingly progressive primary TB"[17,18]

2) **Primary pulmonary tuberculosis:**

This is usually non symptomatic. Only diagnostic tests can detect the disease. However the following findings may be detected in case a patient comes with primary disease:

Lymphadenopathy, pleural effusion, dyspnoea, fever.

3) **Primary Progressive Tuberculosis**:

Active disease develops in 5-10% of exposed cases. The clinical features are non specific during early stages.The C/F include: malaise, fatigue, loss of weight, evening rise in temperature associated with night sweating. Wasting is due to altered metabolism, loss of appetite. Clubbing due to insufficient oxygenation. The cough is first dry later becomes productive. If any blood vessel becomes ruptured, hemoptysis may occur. Blood tests show anemia and increased leukocyte count.

4) **Extra Pulmonary Tuberculosis**:

It occurs in around 20% of immunodeficient cases. The commonest forms include, TB

meningitis, TB spine, miliary TB and lymphatic TB.

In a study by S.K .Sharma and Mohan et al

“diagnosing extra pulmonary tuberculosis is very difficult. The recent advances in imaging studies can locate the site of infection .with the help of biopsy its possible to isolate the bacilli from the lesion”.[19]

In a study by Sayantan Rat et al

“The main event in causing miliary TB is the massive lympho-hematogenous spread of the bacilli from a focus to multiple organs. The main organs involve liver, spleen and bone marrow. The predilection to these organs is because of many phagocytes in their sinusoidal wall. Miliary TB can be due to

1) Simultaneous reactivation of multiple loci either from primary disease or latent disease

2) reinfection

The pathogenecity is because of the insufficient T cell response. in this disease, the recruitment of Teff cells fails to provide an adequate level of immunity because of homing of regulatory Treg cells which inhibit teff cells”[20]

In a study by bourdin trunz et al in the lancet edition of April 2006 studied the effect of BCG vaccination in the prevention of TB meningitis and miliary tuberculosis and find that “BCG vaccination is a good cost effective intervention against severe forms of TB such as TB meningitis and miliary TB and it should be retained in high incidence countries as a strategy to supplement the chemotherapy of active tuberculosis”.[21]

Multi Drug Resistant Tuberculosis:

Standard anti tuberculosis has been used for many years since the advent of those medicines ,

however the resistance to them have emerged and is now global..there has been resistance at least one drug in all the countries with tuberculosis.

In a study by dorman et al and gandhi NR et al published in lancet states “recently multi drug resistant TB and extensive drug resistant TB have had devastating effects on populations of HIV infected individuals in developing countries, who ironically had good access to HIV care but dies from some untreatable form of TB”.[22]

MDR TB, there is resistance to isoniazid and rifampicin, the first line drugs.

The most common cause of MDR TB is faulty treatment. This type of TB can be cured by second line drugs. But these drugs are not easily available. Moreover the medicines in the second line had to be taken at least for two years consecutively which can reduce patient compliance as well severe side effects. Its to be noted that when starting the primary therapy of first line of drugs patient education is important creating awareness regarding faulting treatment which the can complicate this disease further.

More worse than MDR TB is the XDR TB or extreme drug resistant TB which has high mortality rates.

In the study about half a million people worldwide developed MDR TB in 2013.

HIV and TB:

Statistics of association between HIV and TB have been described above in the statistics. Its noteworthy to mention that the people living with HIV can develop active tuberculosis 26 to 31 times more than normal population. Also Africa “the dark continent” which has has been subject to numerous disease since the origins of mankind is no exception to this association. In 2013 there were 1.1 million cases of TB among HIV patients , 78% of which were from Africa. This may be linked with poor healthcare, AIDS denial ism among many politicians, poverty

and malnutrition. The origins of AIDS, HIV have been subject to numerous conspiracy theories and well disputed by reputed scientists all over the world and the disappearance of over a 100 microbiologists in the last decade alone, the treatment for AIDS has been disputed among many main stream scientists.

In studies by klautau GB et al and Murray JF et al in 2005 "HIV mediated immunosuppression impairs granuloma formation, resulting in both ineffective containment of *Mycobacterium tuberculosis* bacilli and diminished formation of pulmonary cavities"

in a study by poprawski D in 2000 "the above mentioned effects cause extra pulmonary disease in HIV-TB subjects".

In further studies by pitchenik AE et al, lee MP et al and colebunders R et al "the HIV-TB manifests with atypical chest findings, greater involvement of lower lobes of the lung and lower concentration of bacteria in sputum".

"The world health organization recommends a 12 component approach of collaborative TB-HIV activities".

In a study by NR Gandhi et al in Lancet says that "MDR tuberculosis is more prevalent than previously realized in this setting. XDR tuberculosis has been transmitted to HIV co-infected patients and is associated with high mortality. These observations warrant urgent intervention and threaten the success of treatment programmes for tuberculosis and HIV".[22]

In another study by Diperri giovani et al in lancet says that "In an investigation of a nosocomial outbreak of tuberculosis, 18 HIV-infected inpatients were found to have been exposed to *Mycobacterium tuberculosis*; active tuberculosis developed in 8, 7 within 60 days of diagnosis of the index case. The patients with lower total lymphocyte and CD4 lymphocyte

counts were more likely to get the disease than were those with higher counts. A low score on multiple antigen skin testing was also associated with the development of active tuberculosis. 4 of the 18 patients had a positive tuberculin skin test before exposure to *M tuberculosis*; none of them subsequently got the disease"[23]

In a study by karp et al on co infection with HIV and tropical infectious diseases states "much of the deadliness of tuberculosis epidemics, especially in sub-Saharan Africa has to do with the virulent synergy between HIV and tuberculosis".[24]

According to AIDS epidemic update 2009 and WHO global TB control update 2009 "out of the 9.27 million incident cases of TB in 2007,an estimated 1.37 million were HIV positive ;79% of these HIV positive cases were in the African region and 11% were in south east Asia region".[25]

In a study by Parsons et al in clinical microbiology reviews "an estimated 1.3 million deaths occurred among incident TB cases who were HIV positive. Infection with HIV 1 increases the risk of reactivating latent TB infection by 80 to 100 fold and HIV patients acquire new TB infections have higher rates of TB progression. Tuberculosis can occur at all points in the immunosuppressive spectrum of HIV disease with variable presentations and particularly in high burden countries TB may be the first presentation of HIV disease".[26]

According to a study by mukadi et al on tuberculosis fatality rates in high hiv prevalence populations in sub Saharan Africa "fatality rates are higher for HIV-TB co infected patients who are on anti-TB treatment but not on ART than for treated TB patients who are HIV negative".[2\7]

In a study by ackah et al on response to treatment, mortality and cd4 lymphocyte counts in HIV infected patients with tb in abidjan states that "highest death rates occur in co infected patients

with lowest CD4 counts".[28]

According to world health organization

"Latent tuberculosis infection (LTBI) is a state of persistent immune response to stimulation by *Mycobacterium tuberculosis* antigens without evidence of clinically manifested active TB. A direct measurement tool for M. tuberculosis infection in humans is currently unavailable. One-third of the world's population is estimated to have LTBI: they do not have active TB disease but may develop it in the near or remote future, a process called TB reactivation. The lifetime risk of reactivation for a person with documented LTBI is estimated to be 5–10%, with the majority developing TB disease within the first five years after initial infection. However, the risk is considerably higher in the presence of predisposing factors"

In a study by farba karam et al,

"The identification and treatment of latent TB infection in HIV positive individuals is one of the main recommendations developed by the World Health Organization in order to reduce morbidity and mortality in HIV patients living in high TB prevalence areas . Tuberculin skin testing has been shown to be highly inadequate for identifying LTBI in HIV infected individuals, as the anergy to skin testing may lead to false negative results . More sensitive and specific diagnostic tools are therefore urgently required . the study showed that, EC-ELISPOT(enzyme linked immuno spot) assay appears more sensitive than the TST in HIV infected individuals, but this sensitivity appears impaired in those with severe immuno-suppression."[29]

Lab Diagnosis :

Research in new diagnostics, treatment and prophylaxis for tuberculosis is happening in a fast pace. The global plan to stop TB formulated a plan to make these new modalities available to resource less countries and high TB prevalent areas. This organization involved in the adoption, introduction and implemention of these new techniques.

In study by harries AD in 2001 “Up to 20% of all the TB patients in sub Saharan Africa, whom the treatment is initiated die within a year and 2/3 rd of these deaths occur in first 2 months”.[30]

Isn’t this pathetic? This study reveals that the delay in detection of TB due to inadequate diagnostic techniques resulting in patients presenting in advanced stage of the disease.

In a study by Pai M on novel and improved technologies for tuberculosis diagnosis in the journal of clinical chest medicine states that “the WHO stop TB partnership's new diagnostics working group and foundation for innovative new diagnostics (FIND) have classified tools for the diagnosis of TB as the following:

1) WHO endorsed Tools

2) tools in late stage development or evaluation

3) tools in early stage development.”[31]

in a study by lange C et al and pai M et al states that “considerable effort and resources have been invested in developing novel diagnostics and improving existing ones”.[32,31]

In studies by madebo T et al &liam CK et al “patients in many countries with high TB burden do not receive diagnosis for 3-6 months”.[33,34]

the world health organization said “although the treatment success rates are improving every year, case detection rates are increasing more slowly”.

In the journal of clinical microbiology review linda M parsons et al states “the global awakening to TB's devastating impact on vulnerable populations has been brought about in many countries by its deadly synergy with HIV. In response WHO has provided several key guidance documents for national TB control and laboratory programs for the use of LED microscopes to improve the sensitivity of a turnaround time of front line assay, sputum smear microscopy, and for improving the accuracy of TB detection and drug susceptibility testing through the use of liquid culture and molecular line probe assays”[26]

In a study by madhukar pai in the new tools and emerging technologies for diagnosis of tuberculosis states that “despite this enormous burden (high prevalence of TB) case detection rates are low posing major hurdles for TB control”.[31]

In the African continent alone, less than 50% of cases are being detected by the currently adopted DOTS programmes. There are new challenges to this like drug resistance and HIV co infection.

In studies by perkins MD et al & WHO global tuberculosis control report 2010 states that “the dual specters of TB and AIDS have drawn recent attention to the lack of suitable diagnosis for TB”[35]

In study by study by Keeler E et al on reducing the global burden of tuberculosis states that “TB case detection is the first hurdle towards tackling the TB epidemic”.[36]

In studies by abe C et al, beavis et al, down et al states that “to address the need for rapid and

sensitive identification of *Mycobacterium tuberculosis* and other mycobacteria, various genotyping methods for routine diagnosis have been introduced during the past decade".[37,38,39]

In a study by fisher M on diagnosis of MDR TB states that

"Theres a long standing need for new, rapid, accurate and convenient tests for TB diagnosis and drug resistance is currently being addressed by several agencies and group"[40]

In a study by perkins et al in the topic admitting defeat in a journal states that "conventional tests for detecting drug resistance are slow, complicated, expensive and difficult to perform in field conditions"[35]

in a study by Moore DF et al & campos M et al states that "a highly sensitive rule in test can significantly improve the case detection whereas a highly specific rule out test can reduce the turnaround time and the duration of respiratory isolation as well as unnecessary administration of potentially toxic drugs".[41,42]

In a study by lemaire J et al on advances in the diagnosis of tuberculosis states "despite the recent advances in TB diagnosis, cost and accessibility continue to be the major limiting factors in the effort to eradicated tuberculosis".[43]

In a study by catharina C.Boehme et al states that "improved technologies that can abbreviate the diagnostic process and facilitate early diagnosis can save patients time and money, decrease morbidity, improve treatment outcomes, and interrupt the transmission of the disease".[44]

In a study by perkins in the international journal of tuberculosis and lung diseases states that "

such technologies need to be more sensitive, faster in yielding results and simpler to use than microscopy"[35]

in a study by Pai M et al in the national medical journal of india states “better efforts to control TB require faster and more accurate diagnostic tests”.[31]

According to WHO any new technique for diagnosing TB should have the following things to be considered:

- should be evidence based. Not only should the test be effective in controlled trials but also should perform well in actual field conditions
- the program readiness: the implementation of any technique requires change in national policy and treatment guidelines.
- Turnaround time: results should be faster than existing ones
- cost effective
- quality control measures strictly adhered
- biosafety precautions adopted
- adequate staff training

The currently available diagnostic techniques at a glance

1) Sputum microscopy: its 125 years old technique and remains the most common method used in the diagnosis of TB.

In a study by geojith george et al states that “notwithstanding the advent of novel diagnostic techniques ,smear microscopy remains the most practical test available in resource limited settings, where majority of the TB is present”.[45]

Its rapid and requires little training to the technicians and no bio safety levels are required.

In a study by steingart KR on fluorescence vs conventional sputum smear microscopy for TB states that “microscopy is rapid, specific and inexpensive but has low sensitivity”[46]

it depends on the bacterial load in the specimens and detects around 50% of all active cases. sensitivity is very low (20%) in children and HIV patients. The cons include inability to detect resistance to drugs.

In a study by mathew P et al in journal of clinical microbiology and expert review of molecular diagnostics 2010 states that “while cheap and relatively easy to perform, the more than 125 year old smear microscopy method has only modest sensitivity (35-80%) and cannot differentiate between drug sensitive and drug resistant *Mycobacterium tuberculosis*”[47]

In a study by dye et al on evolution of TB control and prospects of reducing TB incidence report “the human immunodeficiency virus epidemic has further diminished the utility of routine microscopy and smear negative TB has arisen as a particular problem in sub Saharan Africa”.[48]

The patient has to come twice to the clinic. Once to give the specimen and second to collect the results. This means the transportation costs and the loss of pay.

In studies by foulds J et al in the new tools for diagnosis of tuberculosis and perkins MD et al in international journal of tuberculosis and lung diseases the limitations of conventional methods of diagnosis including smear microscopy have been explained.[49&35]

In parsons et al on clinical microbiology reviews states “in high incidence countries, TB control relies on passive case finding among individuals self presenting to healthcare facilities, followed by either diagnosis based on clinical symptoms or lab diagnosis using sputum smear

microscopy. Serial sputum specimens are required which means that the people are asked to make repeated visits to the health care center for specimen delivery and collection results. For many patients, the costs of repeated visits to health care facilities are prohibitive and patient dropout is a significant problem"[26]

in a study by squire sb et al in 2005 "in places where HIV is prevalent, the proportion of cases detected by smear microscopy is 20-30%.to address this issue multiple sputum examinations was done due to which a relatively large number of patients do not complete the testing".[50]

in a study by steingart et al published in lancet "sensitivity of sputum smear microscopy has been reported to vary from 20-80% often depending on the diligence with which specimens are collected ,smears are made and stained smears are examined".[46]

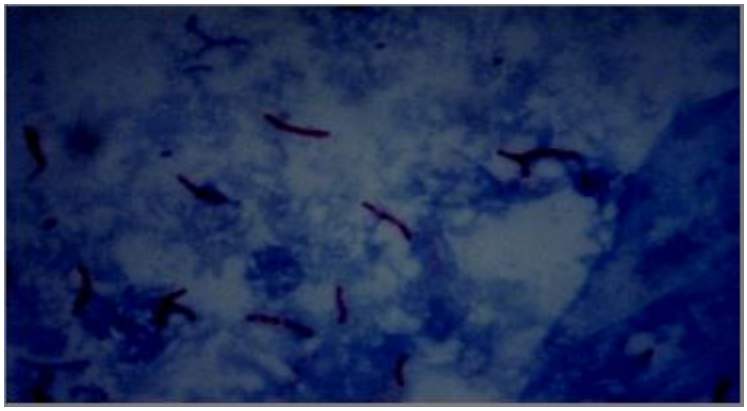
In a study by getahun et al published in lancet states "Tb smear microscopy is highly insensitive for HIV co infected individuals and for children due to the reduced pulmonary bacillary loads in these patients".[51]

In study by harries ad et al in 2003 "more than a third of patients with smear negative TB in Malawi needed more than 6 visits to a health care center for treatment to be initiated"[52]

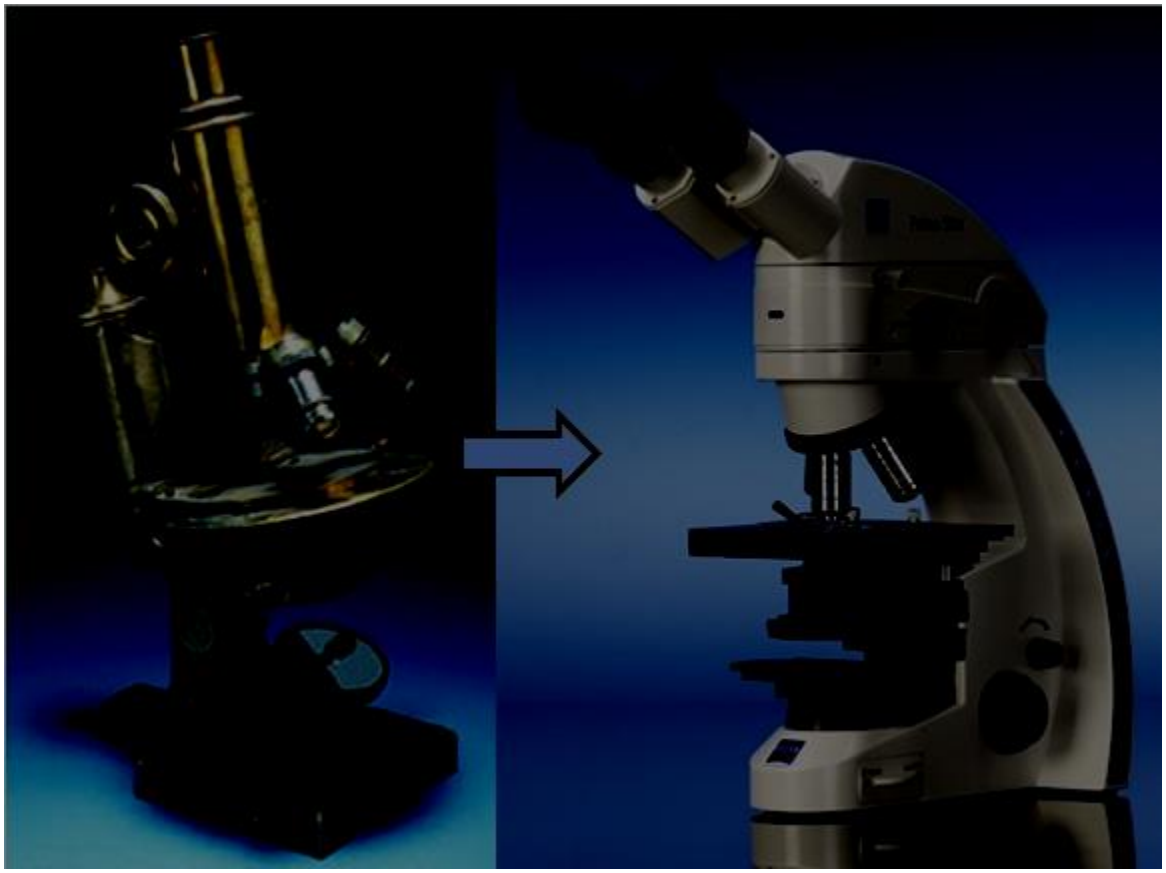
this is due to the difficulties in confirming smear negative TB, underlines the need the new and simple, yet rapid and effective diagnostic methods to address such cases.

In parsons et al clinical microbiology reviews states "in resource poor countries many smear microscopy labs are single room and insufficiently staffed with poor maintenance of microscopes. These labs don't have consistent clean water supply and electricity. There are few opportunities for the training of staff and little staff capacity to handle high volume workloads.

Quality assurance programs including quality control and external quality assessments are often lacking. This a critical need for new, sensitive, easy and paid point to care diagnostics and also for investments in laboratory infrastructure, quality assurance programs and well trained staff".[26]



In a



study by monkongdee et al states “the who recommendation is two rather than three sputum examinations be used was generated largely for non -HIV patients. However this approach was evaluated in HIV infected patients in southeast Asia and the incremental yield of microscopy was found to rapidly diminish to 2% after the second smear.[53]

According to who laboratory policy in 2007 “for TB detection two sputum smear are to be collected and examined”.

The above policy was drafted based on a meta analysis of 37 studies which quanitified the incremental yields of serial sputum specimens.

In a study by mase at al in 2007 states “85.8% of Tb cases were detected with the first sputum specimen. With second sputum specimen, the average incremental yield was 11.9%, while the incremental yield of third specimen, when the first two were negative was 3.1%”[54]

in a study by bonnet et al in 2007 conducted in Kenya shows “decreasing the number of smears examined for detection of TB leads to reduction of patient visits to clinic and lab workload”.[55]

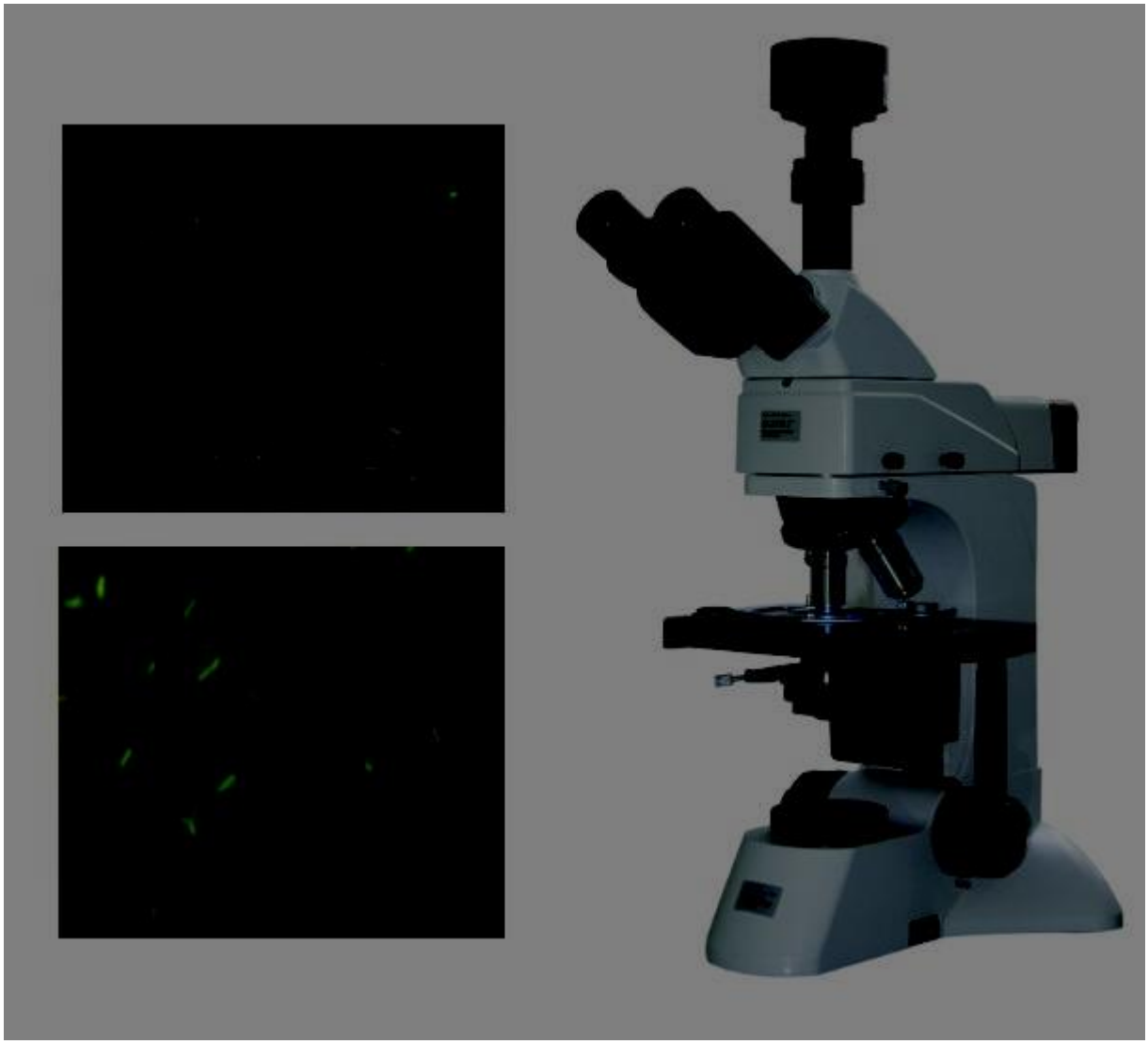
It is to be mentioned that the above reasons are major drawbacks of multiple smear examinations and prevent patient from taking from collecting the results thereby diagnosing TB.

In a study by aziz et al tells “screening of TB patients from three to two specimens should be recommended only in settings with a well established lab network and a fully functional EQA program for smear microscopy including on site evaluation and follow up training for problem laboratories”.[56]

Its a well known phenomenon that most of the labs in our country don’t have quality control

assessments.

In a report by world health organization-strategic and technical advisory group for Tuberculosis



(STAG-TB) dated 2009 recommends “conventional fluorescence microscopy be replaced by LED microscopy in a phased manner.”

in a study by steingarta kr et al “a meta analytical study of 45 studies show that the sensitivity advantage of fluorescence microscopy over conventional microscopy is greater by 10%”.[46]

also sputum processing increases the chance of finding TB bacilli my microscopy by 13-33%.

Tb Culture:

It is the gold standard test. Most sensitive method and accommodates DST and speciation. Takes about 2-8 weeks since the TB bacilli is a slow growing organism having a slow generation time of 14-15 hours. Standard method is using solid LJ media.

WHO STOP TB report 2008 “some national TB programmes in developing countries have no functioning TB culture facility at all. In others its done only in national reference labs and high grade hospitals. Few countries have the capacity for DST”.

Because of the shortcomings, automated liquid systems have been developed.

In a study by william -bouyer n et al “these systems detect the O2 production, CO2

consumption by radiometry, calorimtery, pressure sensors and decrease the time to detection by half".[57]

Disadvantage if this system includes contamination especially when inexperienced and difficult to examine the colony morphology.

In a study by van kampen sc "the liquid culture systems suffer from important shortcomings as they are prone to contamination, human resource intensive, costly".[58]

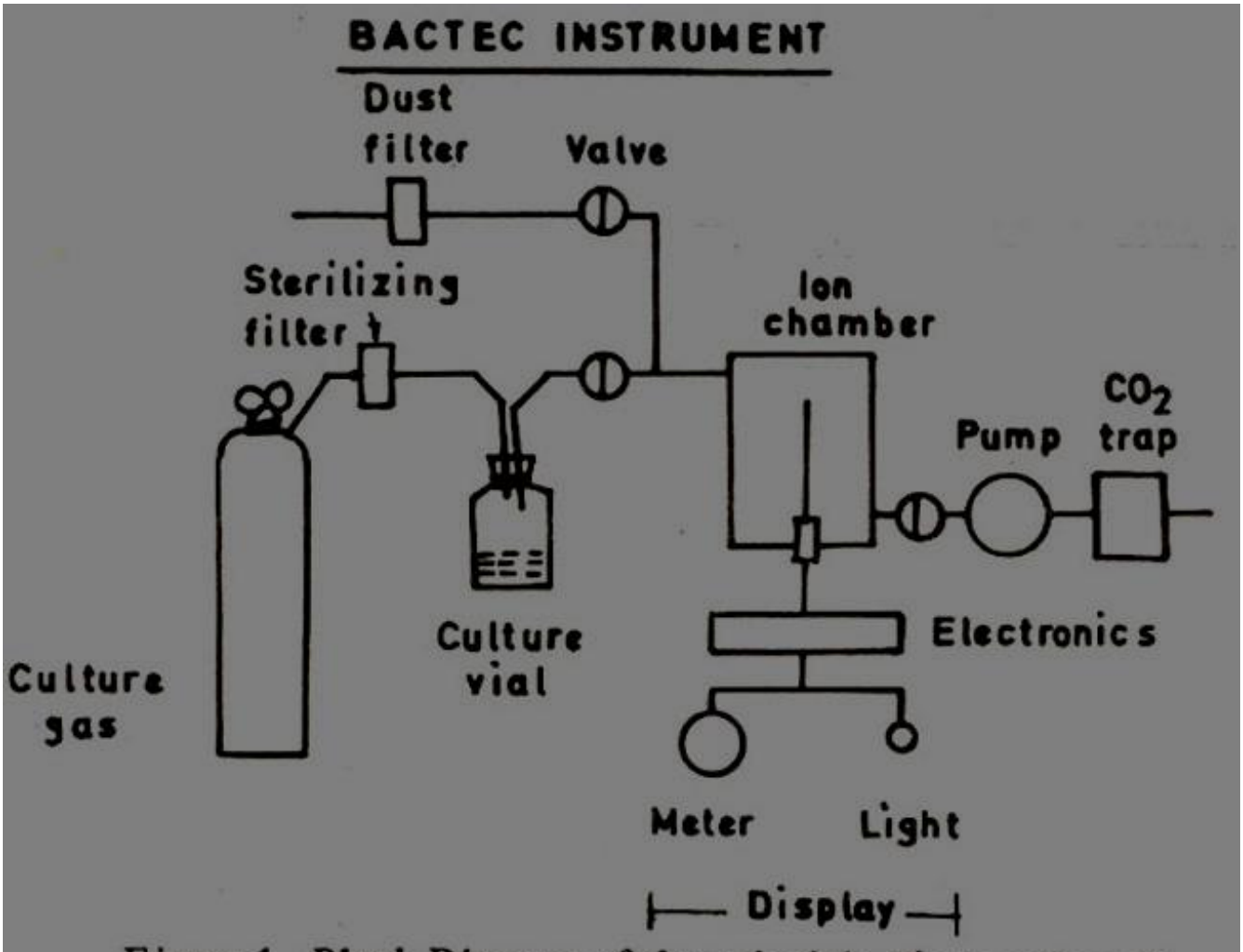


Figure 1. Block Diagram of the principle of operation of Bactec unit.

In studies by kocagoz t etal &baylan o et al "solid media called TK uses a colorimetric method

to find the bacterial growth. Its possible to discriminate and differentiate non tuberculous bacteria in this method. Its cheap and rapid. Detection time is 10 days less than conventional LJ media".[59&60]

In study by farnia p et al "a number of redox reagents have been developed to find the bacterial growth in early cultures". This needs further evaluation.[61]

MODS-microscopic observation drug susceptibility:

manual technique in which the colonies of the culture are examined under a microscope, in this case an inverted microscope is used.

Advantages: DST could be done, inexpensive and could be done in one week time.

Inverted microscope

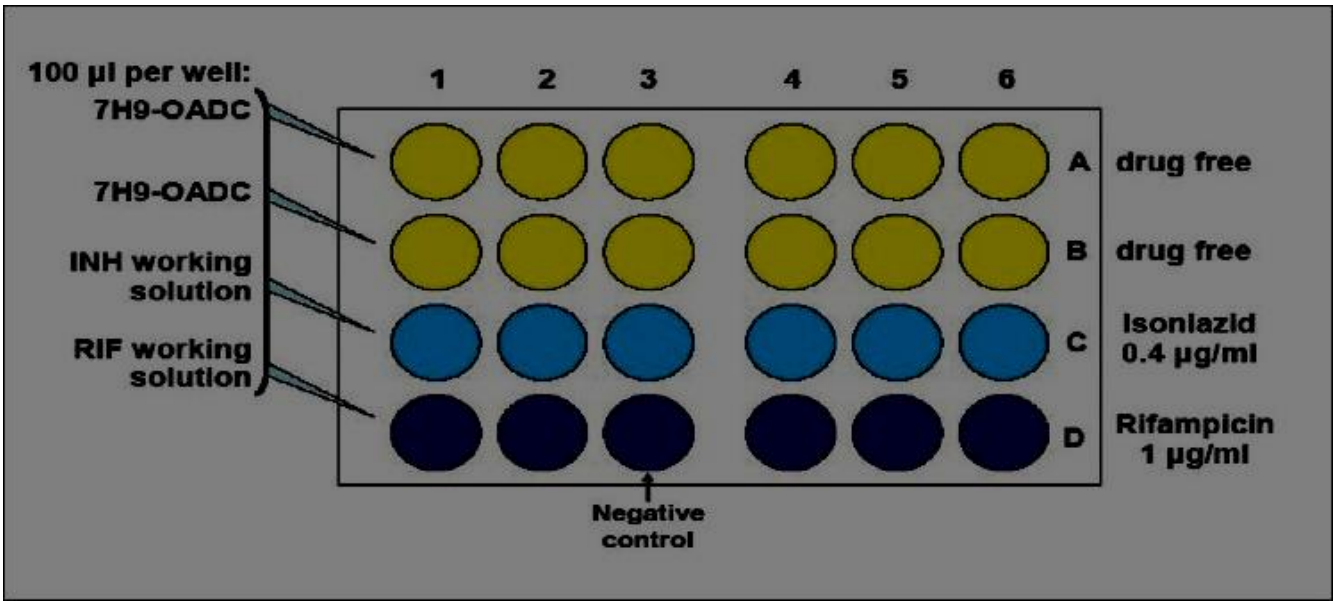
Cons: the technique need training to technicians, bio safety level 2 precautions and difficulty in differentiating between NTM and MTB.

In a study by Moore DA et al "by adding Anti TB antibiotics to adjacent wells and examining for comparative growth, MODS can be applied for early detection of DST as well".[62]

In a recent study on a large scale carried out in Peru by Moore DA et al "MODS detected 94% of 1908 culture positive cases whereas LJ medium detected only 87%".[62]

the average detection time in this method was only 8 days.

This seems to be a promising too.



Septi check AFB method:

In the ICMR bulletin dated 2002 “it is non radiometric method has the unique advantage of simultaneous detection of MTB, NTM and contaminants”.[63]

In a study done in USA by isenberg HD et al shows “the system gives a better culture result compared with other methods including BACTEC 460 TB”.[64]

Radiometric BACTEC 460 TB method:

14C labeled palmitic acid in 7H12 medium is used. When MTB grows 14C is generated which is measure by the system. The system also identifies MTB as well as can be used for DST.

In a study done in TRC, chennai by Venkatraman Et al shows "rate of isolation of positive cultures was significantly faster with the BACTEC method with 87% of the positives obtained by 7days and 96% by 14 days.DST could be done in 8 days".[65]

MGIT 960 mycobacteria detection method:

In this method, O2 utilization by MTB is determined by a fluorescent dye in the medium. Its an automated system.

In a study carried out in usa by tortoli et al “this system

exhibits a potential for rapid, accurate and cost effective detection of MTB especially in high volume settings”[66]

MB/Bact system:

Its a non radiometric methods based on the colorimetric detection of Co2.

In a study by rohner et al concludes “this systems is a good alternative to BACTEC 460 despite some disadvantages like contamination”.[67]

ESP culture system 2:

automated system based on the detection of pressure changes in the media in a study by woods et al " its a reliable, less labour alternative to BACTEC , however it should not be used as a stand alone system".[68]

Phage based test:

underlying principle: the ability of phages to infect bacteria has been used.

A lytic mycobacteriophage D29 infects the TB bacilli and killed with a virucide.this procedure only kills the virus and not the bacteria inside, later the cells are plated on a lawn of *Mycobacterium smegmatis* producing plaques indicating the disease(TB). The test could be done in days.

In studies by albay a et al, butt t et al "this test detects 29%-87% of smear positive cases and 13-78% of smear negative cases in 2 days".[69,70]

In a study by alcaide F et al "the capacity of D29 phage to replicate in nontuberculous mycobacteria has not impaired clinical specificity which remained high (99.1%) even in a study in which 30% of all culture isolates were non tuberculous mycobacteria.[71]

This technique requires necessary infrastructure .

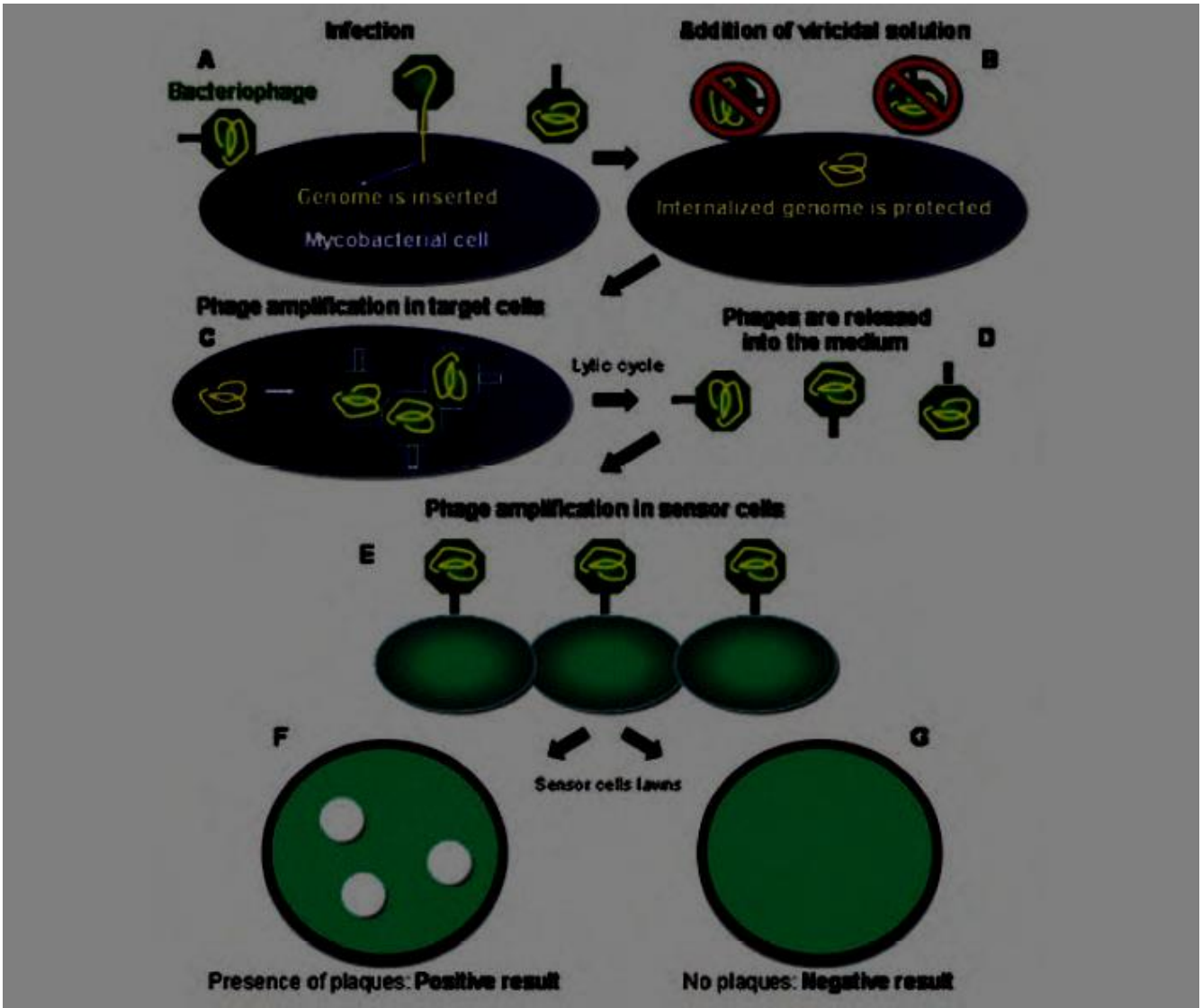


Figure 5. Detection of *M. tuberculosis* by PhaB assay. A. Phages are added to the decontaminated clinical sample. Phages will infect viable mycobacterial cells present in the solution. B. Ferrous ammonium sulphate is added (viricidal solution) to destroy all non-infecting phages. C. Phages begin to replicate leading to cell lysis and release of the new phage particles into the medium (D). E. This solution is added onto a lawn of sensor cells (*M. smegmatis*) to produce plaques (F). In case the sample was negative, no plaques will be present (G).

Immunodiagnosis

In studies by gennaro ML et al, daniel TM et al ,bothamley GH et al, "immune based tests for detection of antigen, antibody and immune complexed have been reviewed".[70,71,72]

There's no such test available yet to replace smear microscopy and culture.

The problem is these test aim at detecting the humoral response rather than the cell mediated immunity which plays a prominent role in TB.

The above mentioned studies have analyzed the reason for failure of such tests.

In studies by Laal S et al "the Tb is characterized by a wide spectrum from latent to active and in each stage different set of antigens are expressed, and even in a single stage the antigens are not the same, for eg: its different from cavitary lesions and the disease with non cavitary lesion"[73]

Hence any serological test should encompass to detect all these antigenic responses. Previously crude antigens were used, which led to poor specificity.

In review by Madhukar Pai et al "the development of recombinant proteins specific to MTB has partially overcome this problem".[74]

Many serological tests employ a single antigen and hence the detection of disease during all stages is less. In the above mentioned studies "the development of a cocktail of antigens specific to each stage of the disease has partially addressed this problem".

Lipoarabinomannan assay:

Its a heat stable glycolipid present in the cell wall of mycobacteria.

Instead of searching for an antibody response specific to an antigen, this test aims at finding the specific antigen using a capture ELISA method

This method has demonstrated some success. Further evaluation needed.

Interferon gamma release assays (IGRAs):

Its a diagnostic immune response Tb test. The standard Mantoux or tuberculin sensitivity test has many limitations. This new tests measures the cell mediated immunity in response to MTB

specific antigens which are not present in BCG and NTM there by specific to MTB. Two tests are currently available. The quantiferon TB gold test and the T spot TB test. The best thing is prior BCG vaccination does not give a positive test, thereby useful in screening.

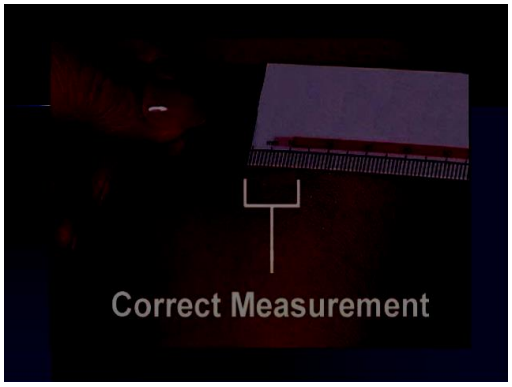
This test is not without limitations. There's no evidence to find which case with progress to active disease based on this test.

MPB64 skin patch test:

MPB 64 is a Mtb specific protein.

In research by Nakamura et al in japan in 1998 “the test was capable of eliciting a distinct response from individuals with active but not latent TB , the sensitivity was 98% and specificity was 100%”.[75]

An aqueous solution containing MPB64 is applied to the gauze and over the forearm and measurement made.



also it could be used in lateral flow assays in differentiating Mtb from NTM

the exact mechanism is unclear.

Molecular methods:

Nucleic acid amplification tests(NAT) are designed to amplify regions specific to MTB complex. They are available as commercial kits. In USA amplicor and MTD are FDA approved.

In studies by Piersimoni C et al & Hugget Jf et al "the 3 widely used methods are PCR, transcription mediated amplification(TMA) and strand displacement amplification. These methods show excellent speed and specificity, sensitivity approaching but not equaling culture".[76,77]

In house NAT are predominantly used in research settings.

In studies by Brodie et al, Pai M et al "the literature of NAT has been extensively reviewed"

In meta analytical studies by Flores L et al, Pai M et al "the results demonstrate that AT have high specificity for both pulmonary and extra pulmonary TB".[78&31]

In review by Madhukar Pai et al in the journal of expert review on molecular diagnosis "high specificity and positive predictive value confer clear advantages in terms of the tests ability to rule in TB. A positive NAT in a patient with a high pretest probability is fairly confirmatory of TB, particularly in smear positive cases".[79]

It also says "the sensitivity of NAT is lower and highly variable across studies. In many studies, sensitivity estimates have been lower in paucibacillary forms of TB. Therefore a negative test

does not rule out TB".

A negative NAAT with a positive smear can be non tuberculous mycobacteria.

Disadvantages: Cannot differentiate between live and dead bacilli as the DNA can be amplified in both cases.

In a meta analytical study by Flores et al on the accuracy of in house PCR tests "the use of IS6110 as an amplification target and the use of nested PCR were associated with higher accuracy"[79]

in the ICMR bulletin dated august 2002 "IS6110 is specific for MTB and is present upto 20 times in its genome, thus offering multiple targets for amplification".[63]

the author Madhukar Pai et al in this review concludes that "in general, the concerns about accuracy, reliability and requirement for necessary infrastructure reduce their applicability in resource limited settings".[74]

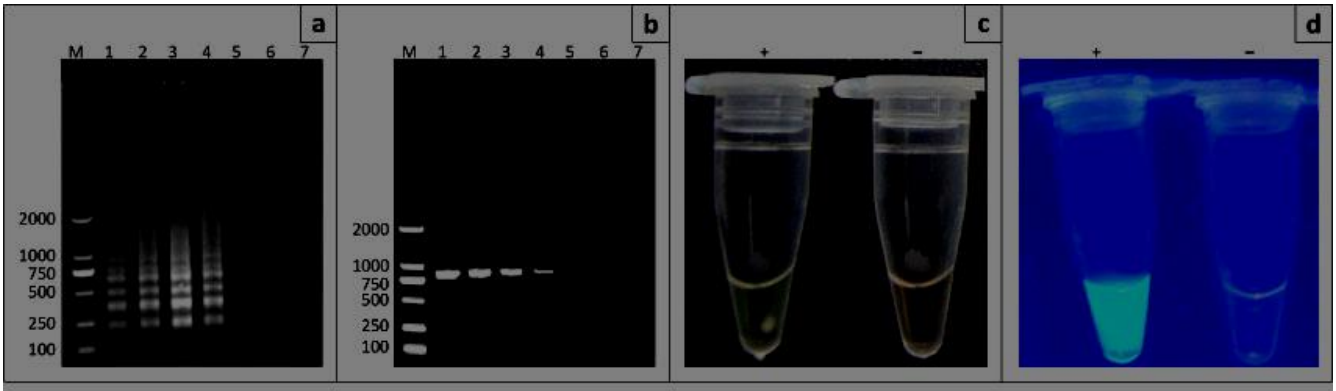
In a study by Suffys P "even in established molecular labs, the performance of NAT is highly variable, implying the need for more robustness".[80]

One such promising technology is the LAMP, loop mediated isothermal amplification. It was invented by Eiken chemicals, japan.

In study by Iwamoto T et al "the advantages of this technology is that it uses 6 specifically designed primers and a single polymerase with strand displacement activity, requires no thermocycler, its a closed system needing no bio safety levels and gives a visual readout by naked eye".[81]

In study by Boehme C et al "preliminary data suggests that this test can be done in a benchtop by technicians with no molecular training detecting all smear positive specimens and half of the smear negative specimens".[82]

The inventor Eiken in its website claims that “the amplification efficiency is high and DNA can be amplified 10^9 -10^{10} times in 15-60 mins”.[83]

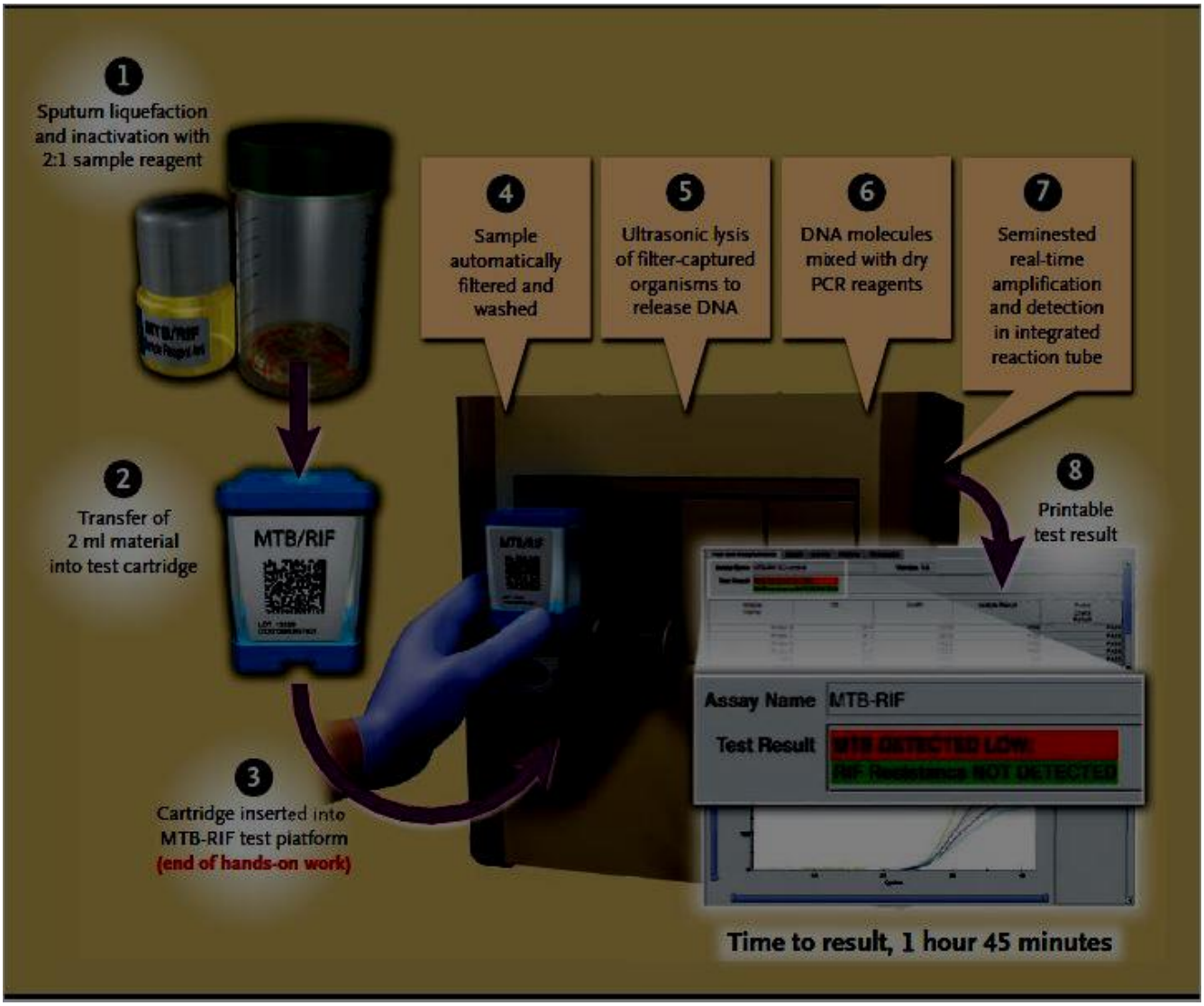


Lane M, DNA marker; Lanes 1–6, 1 × 10^6 CFU/mL, 1 × 10^5 CFU/mL, 1 × 10^4 CFU/mL, 1 × 10^3 CFU/mL, 1 × 10^2 CFU/mL and 1 × 10^1 CFU/mL of *Haemophilus parasuis* used as a reaction template, respectively; Lane 7, negative control; +, positive reaction; –, negative reaction.

FIGURE 1: Detection of (a) *inf*B loop-mediated isothermal amplification (LAMP) and (b) polymerase chain reaction products, as well as visual detection of *inf*B-LAMP products under (c) daylight and (d) ultraviolet light.

Gene x pert:

This test detects both MTB and rifampicin resistance. This is a hemi nested PCR



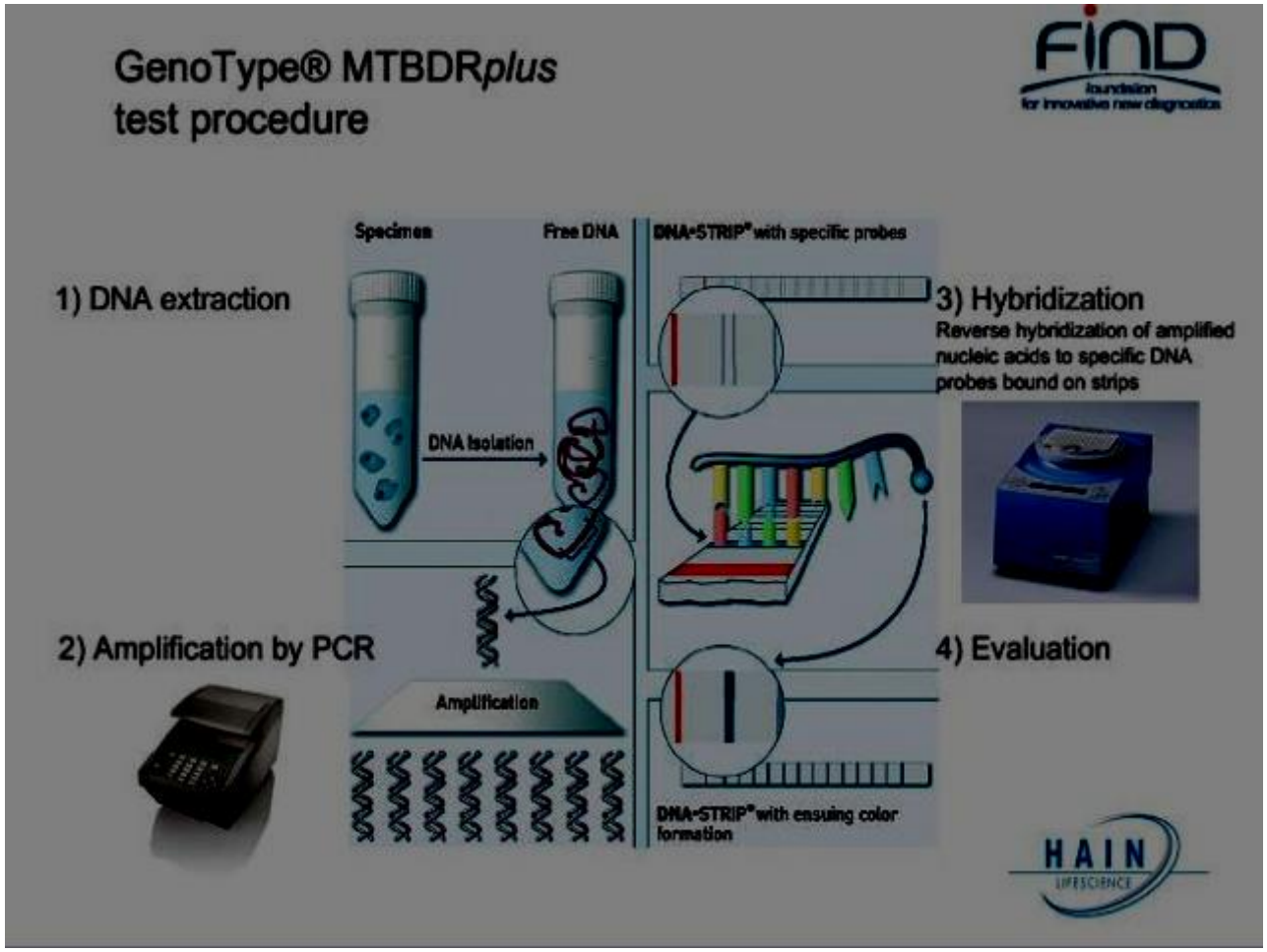
In a study by Helb D, Blakemore R “in this test PCR amplification of five overlapping probes that are complementary to the entire 81 base pair RIF resistance determining region of the MTB rpo B gene and subsequently probes this region for mutations that are associated with Rifampicin resistance”.

Disadvantages include high cost. In a review by Mark D Perkins et al “FIND(foundation for innovative and new technologies) is working with cepheid to develop a real time PCR assay for

TB on its gene xpert platform that automates sputum processing, DNA extraction, gene amplification and target detection into a single, hands free test".[35]

Line probe assays :

in review by Madhukar Pai et al says "line probe assays are are strip based tests that use PCR and reverse hybridization methods for the rapid detection of mutations associated with drug resistance and detection of MTB complex".[74]



In a meta analytical study Morgan et al "the line probe assay has high sensitivity and specificity when culture isolates are used, less when directly applied on clinical specimens"[84]

These tests are expensive and need sophisticated labs.

Materials and methodology:

This is a comparative evaluation of three techniques , fluorescence microscopy ,culture using solid media and LAMP PCR.

This study is done in tirunelveli medical college hospital over a period of four months from june 2015 to september 2015

Sample selection: 50 samples randomly collected from the district tuberculosis centre, from patients suspected of tuberculosis as referred by expert thoracic physicians in the department of thoracic medicine.

1) Fluorescence microscopy:

Principle:

Mycobacteria are acid fast.ie., they resist decolorizing even after exposure to acid. In this technique carbol fuschin is replaced with auromine -O. The mycolic acid in the cell wall has an affinity for fluorochromes. The counter stain to differentiate the background is potassium permanganate. The advantage is greater area can be examined under low power microscope thereby enabling a larger area to be examined in a less time.

The mycobacteria appear as yellow luminous rods.

Sputum smear preparation:

Label a new clean slide

using a 5mm nichrome sterile loop transfer sputum to the slide

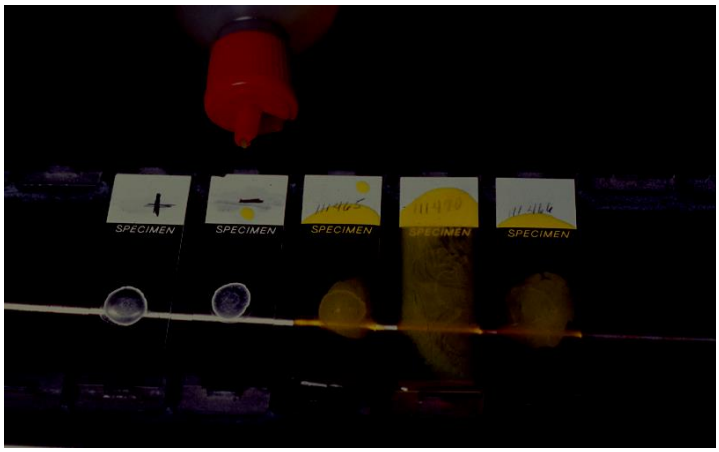
smear the slide for an area of 2-3 cm

air dry for 15 minutes

heat fix by passing over the flame 3-4 times

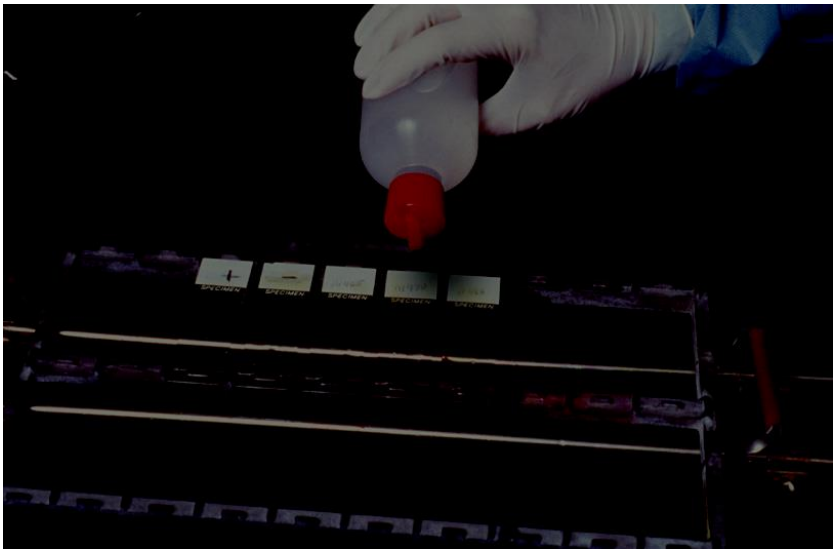
place the smeared slides on the rack

pour auramine phenol over the slides

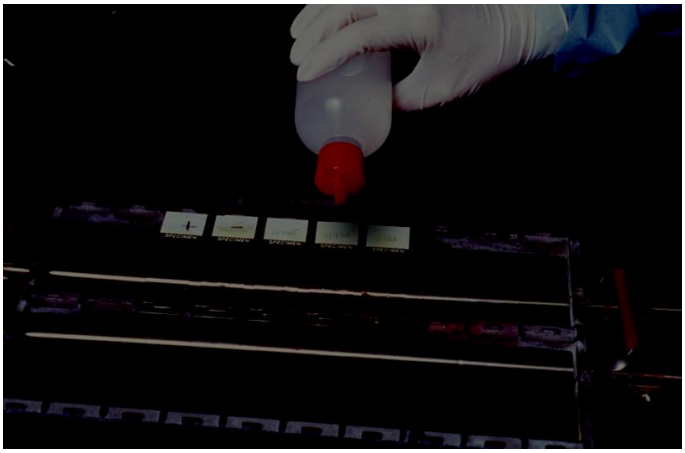


wash with running water

decolorize with acid alcohol for 2 minutes twice



counter stain with KMnO4 for 30 seconds



wash with running water and let them air dry.

The procedure was followed as per TBC India guidelines.

The following reagents were required

1) 3% phenol:

2) Auramine-phenol solution

3) acid alcohol

auramine-phenol preparation:

3% phenol 100 ml was warmed to 40ºC

0.3 gm of auramine was added and shaked for 10 minutes

The solution was filtered using whatman No.1 filter paper and stored in a dark glass bottle.

Acid Alcohol preparation

0.5 ml HCL

0.5 gm NaCl

75 ml absolute alcohol

25 ml distilled H20

4)0.1% KMnO4

2) **Culture In Lowen Stein Jenson Media:**

mucoid or mucopurulent sputum for cases suspected of pulmonary tuberculosis was collected from the district TB center, department of thoracic medicine, tirunelveli.

the specimen was labeled and the laboratory number of each specimen noted.

Strict bio safety precautions were used. N95 mask and gloves were used for sputum handling.

The specimens were decontaminated by modified petroff's method as per TBC guidelines.

Specimen should be processed as described below:

1.5 ml of sputum in universal container is transferred to centrifuge tube and double the volume of NaOH 4% is added

↓

2. the tubes are inverted and the sputum is mixed well.

↓

3. the tubes are kept in votex shaker for a minute and then in incubator at 37ºC for 15 minutes

↓

4.after 20 minutes, 15 ml of distilled water is added to the tubes

↓

5. centrifuged at 3000 x g for 15 minutes.

↓

6.supernatant fluid discorded in a disinfectant solution(bleach solution)

↓

7. another 15 ml distiller water added and centrifuged at 3000 x g for 15 minutes and the supernatant discarded.

↓

8.the pellet is inoculated in 2 slopes of LJ media.

Growth is checked weekly for 8 weeks.

Reading Of Cultures:

the colonies of *Mycobacterium tuberculosis* appear as rough, buff, like bread crumbs and appear 2- weeks after incubation

If the colony has doubtful morphology then Ziehl Neelson staining is done.if no afb is seen then the culture is discarded as contamination.

If smear positive then the following biochemical tests were done

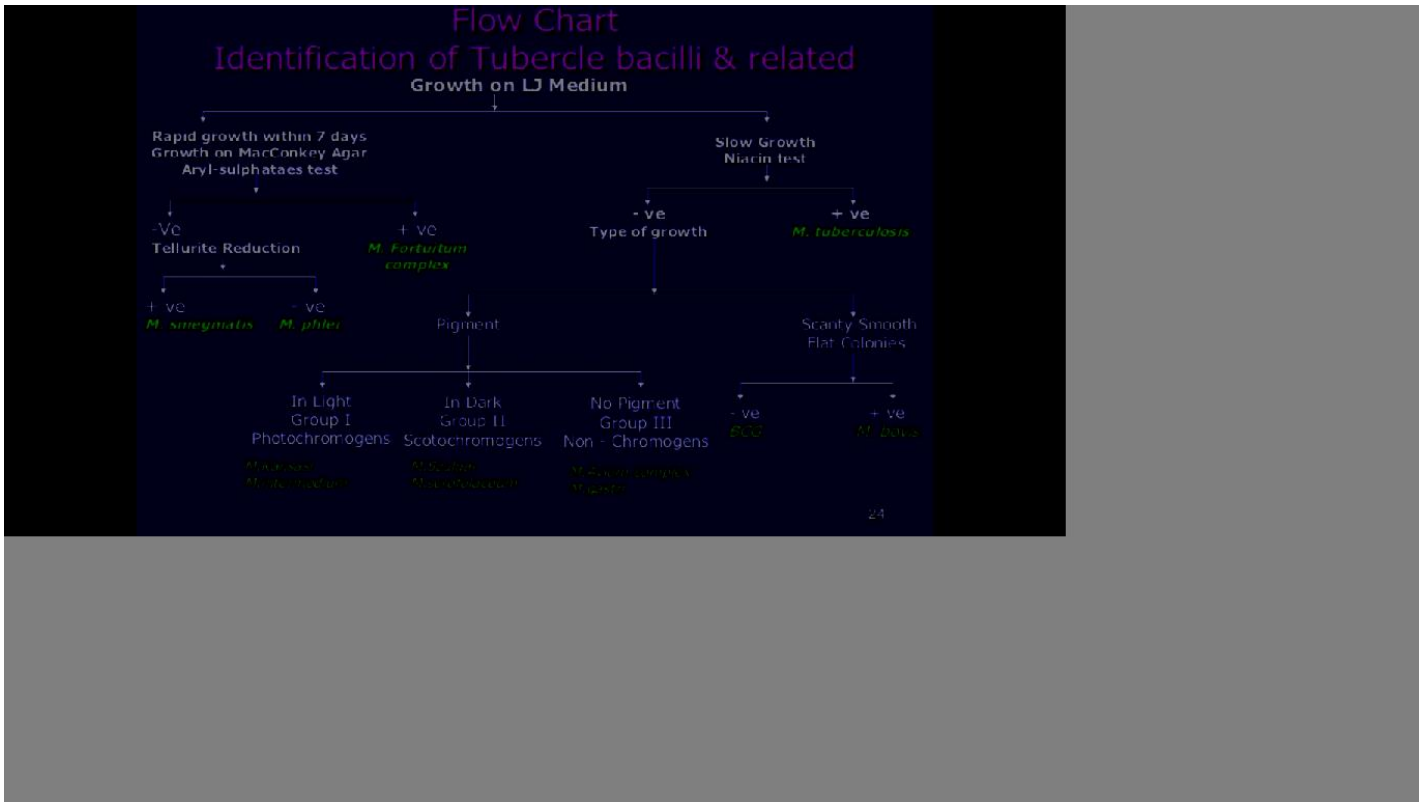
1) Niacin

2) Nitrate

if both were positive ,then *Mycobacterium tuberculosis* was confirmed

if niacin is negative then depending on the pigmentation and morphology they were grouped under BCG or *M.bovis* and non tuberculous mycobacteria.

Flow chart depicting the identification of tubercle bacilli based on growth on LJ medium



LJ media after 3 weeks incubation showing bread crumbs like colonies

centrifuge tube containing pellet

the findings were reported as per  below

Reading of culture	report
No growth	Negative
1-100 colonies	positive(number of colonies)
>100 discrete colonies	Positive 2+
Confluent growth	Positive 3+
contaminated	contaminated
<20 colonies of only NTM is one or both slopes	No growth
>20 colonies of only NTM in both slopes	Negative for M.Tb

media was prepared as per annexure 1 attached

freshly prepared lowenstein jenson medium to be inoculated before keeping in incubation

biosafety cabinet for sputum processing and inoculation

3) **LAMP PCR:**

DNA purification procedure:

1) from the pellet prepared by homogenisation and decontamination after inoculation into the LJ slopes the remainingis used.

2) add 180µl of digestion buffer and 20µl of lysozyme .votex for 10 seconds

3)incubated at 37ºC for 15 minutes

4)200µl of binding buffer and 20µl of proteinase K added.mixed well using votex

5)incubated at 56º C for 15 minutes

6) 300µl of ethanol added and mixed well by inverting several times.

7)pipette entire sample into the spincolumn.centrifuged at 10000 rpm for 1 minutes. Discard the flow through and place the column back into the same collection tube.

8) 500µl of wash buffer-1 added to the spin column.centrifuged at 12000 rpm for I minute and

the flow through discarded.place the column back into the same collection tube.

9)add 500µl of wash buffer-2 to the spin column .centrifuged at 12000 rpm for 1 minutes and the supernatant discarded.the column is placed back into the same collection tube.

10)repeat wash buffer-2 wash once

11) centrifuge the spin column with collection tube at 13000 rpm for 1 minute

12) discard collection tube and transfer the spin column into a fresh 1.5 ml fresh micro centrifuge tube.

13) add 100µl of prewarmed elution buffer to the centre of the spin column membrane

14)incubate for 2 min at room temperature and centrifuge at 13000 rpm for 1 minute.discard the spin column and store the purified DNA at -20ºC .

Materials required:

pure fast bacteria; DNA mini spin purification, isothermal master mix, SYBR green Dye and primers are from helini biomolecules

isothermal master mix contains: reaction buffer, 1.6mM of dNTP mix, 0.5 M of betaine,6mM of MgSO4,8U of Bst DNA polymerase

Loop isothermal primers:

F3: GGTGAGGTCTGCTACCCA

B3: CGTGAGGGCATCGAGGT

FIP: ATCGCTGATCCGGCCACAGCGTTAGGTGCTGGTGGTCCG

BIP: CGTGGTCCTGCGGGCTTTCAGATGCACCGTCGAACG

***Mycobacterium tuberculosis* IS6110 genes**

TCAGCCGGCGGCTGGTCTCTGGCGTTGAGCGTAGTAGGCAGCCTCGAGTTCGACC

GGCGGGACGTCGCCGCAGTACTGGTAGAGGCGGCGATGGTTGAACCAGTCGACC

CAGCGCGCGGTGGCCAACTCGACATCCTCGATGGACCGCCAGGGCTTGCCGGGT

TTGATCAGCTCGGTCTTGTATAGGCCGTTGATCGTCTCGGCTAGTGCATTGTCATA

GGAGCTTCCGACCGCTCCGACCGACGGTTGGATGCCTGCCTCGGCGAGCCGCTCG

CTGAACCGGATCGATGTGTACTGAGATCCCCTATCCGTATGGTGGATAACGTCTT

TCAGGTCGAGTACGCTTTCTTGTTGGCGGGTCCAGATGGCTTGCTCGATCGCGTC

GAGGACCATGGAGGTGGCCATCGTGGAAGCGACCCGCCAGCCCAGGATCCTGCG

AGCGTAGGCGTCGGTGACAAAGGCCACGTAGGCGAACCCTGCCCAGGTCGACAC

ATAGGTGAGGTCTGCTACCCACAGCCGGTTAGGTGCTGGTGGTCCGAAGCGGCG

CTGGACGAGATCGGCGGGACGGGCTGTGGCCGGATCAGCGATCGTGGTCCTGCG

GGCTTTGCCGCGGGTGGTCCCGGACAGGCCGAGTTTGGTCATCAGCCGTTCGACG

GTGCATCTGGCCACCTCGATGCCCTCACGGTTCAGGGTTAGCCACACTTTGCGGG

CACCGTAAACACCGTAGTTGGCGGCGTGGACGCGGCTGATGTGCTCCTTGAGTTC

GCCATCGCGCAGCTCGCGGCGGCTGGGCTCCCGGTTGATGTGGTCGTAGTAGGTC

GATGGGGCGATCGGCACACCCAGCTCGGTCAGCTGTGTGCAGATCGACTCGACA

CCCCACCGCAA

Reaction Mix

components	Volume
Loop Isothermal Master Mix	10µl
Primer Mix	5µl

Purified DNA	5µl
Final volume	20µl

Negative control and Positive control are included in every run.

Centrifuged and incubated at 65°C for 1hour.

Visualization

1µl of 10X SYBR Green dye added to each PCR tube and gently mixed by vortex and placed over UV transilluminator and illumination are compared with Negative control and interpreted.

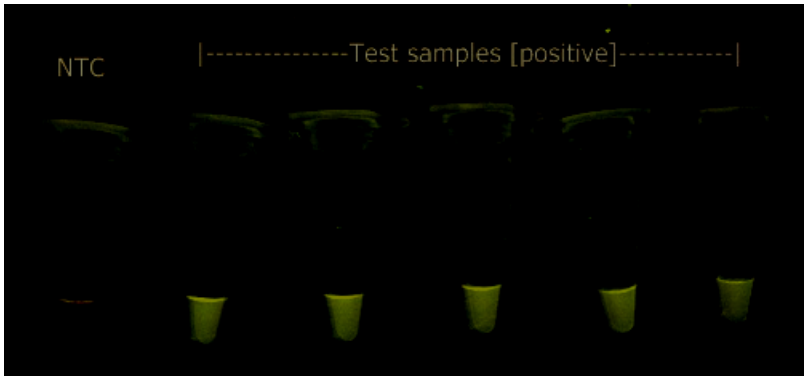
MTB Lamp Primer:

GGTGAGGTCTGCTACCCA

CGTGAGGGCATCGAGGT

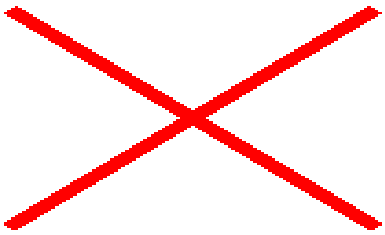
ATCGCTGATCCGGCCACAGCGTTAGGTGCTGGTGGTCCG

CGTGGTCCTGCGGGCTTTCAGATGCACCGTCGAACG



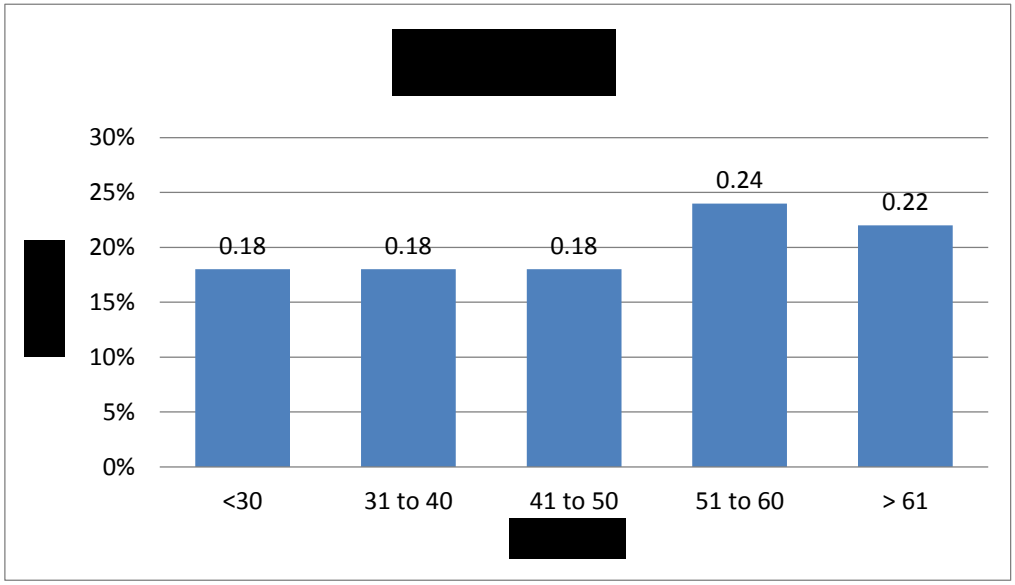
Results:

table 1- age wise distribution:



graph 1

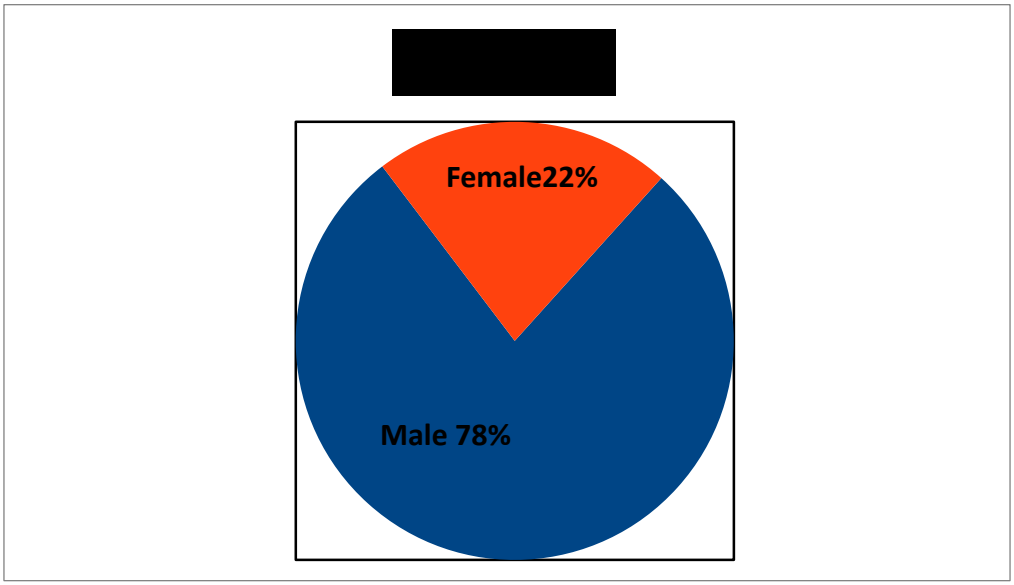
x



in the present study 18% of the study population was below the age of 30, 18% between 31-40, another 18% between 41-50, 24% of the cases were between 51-60 and 22% of the cases were more than 61 years of age.

Table 2-Gender wise distribution:

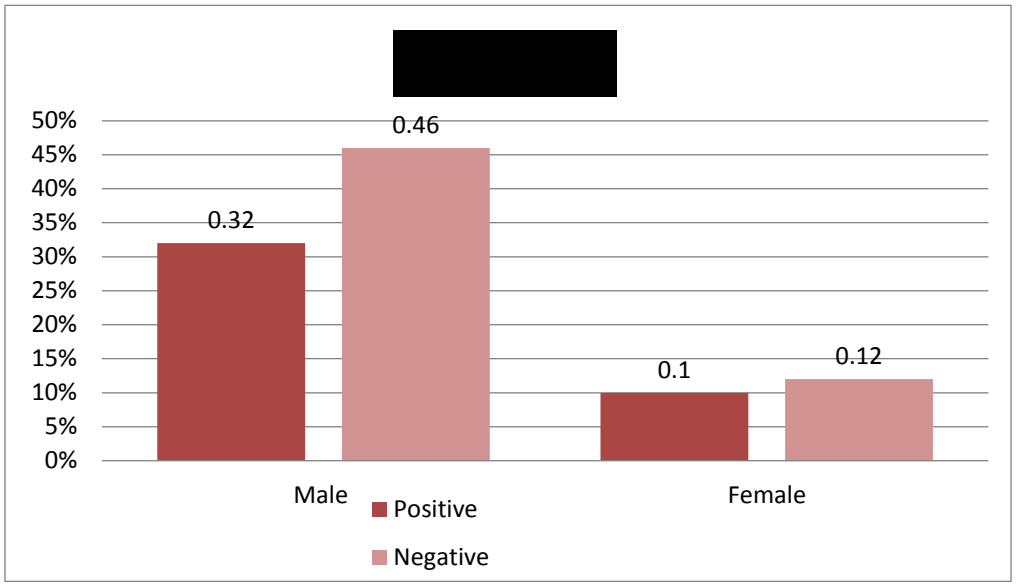
graph 2:



in the present study among 50 samples collected , 39 (78%) were males and 11(22%) were females.

table 3-Culture results:

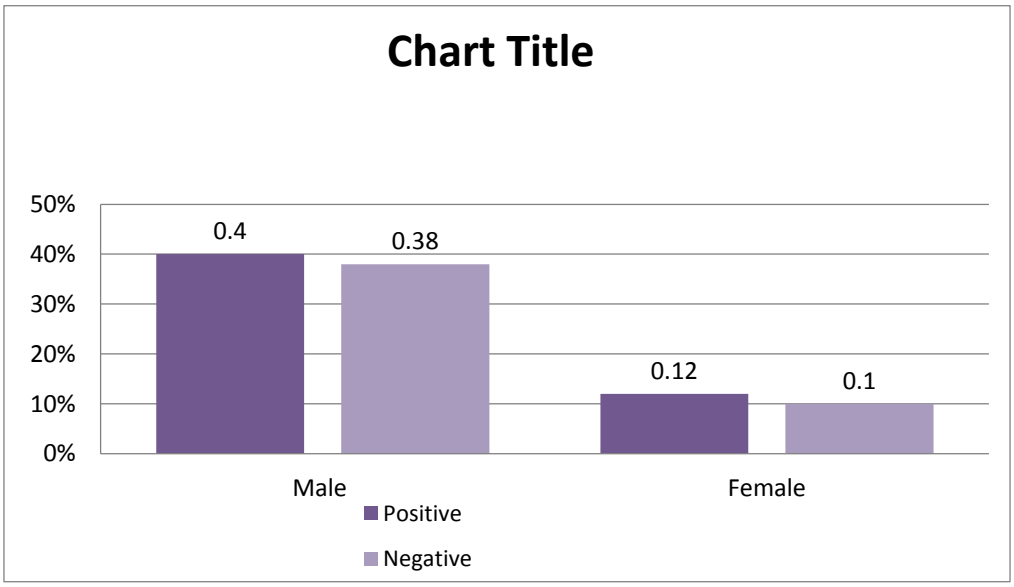
graph 3:



in the present study culture was positive in 42% of the subjects and negative in 58%.

table 4-PCR results:

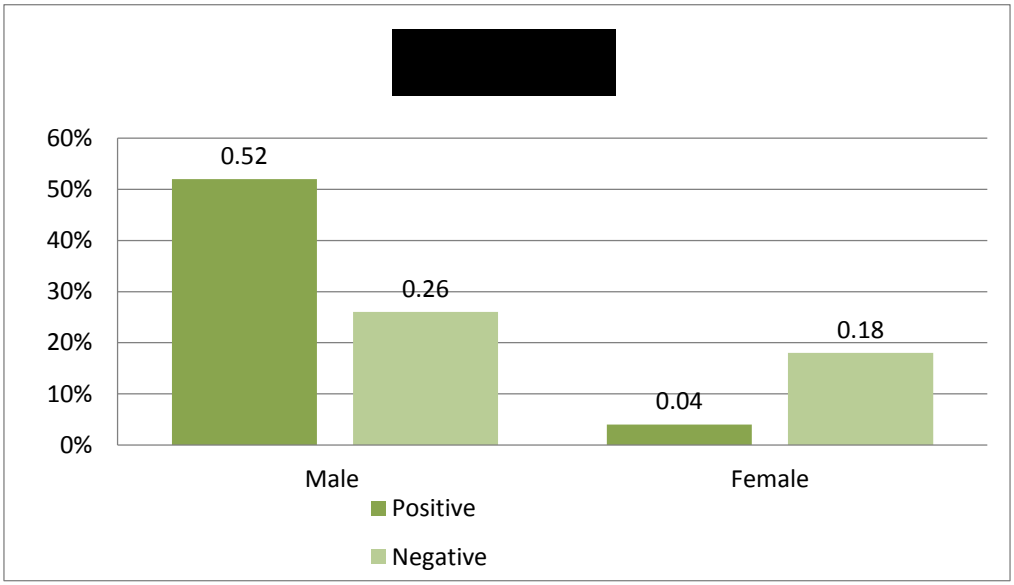
graph 4:



LAMP PCR was positive in 52% of the study population and negative in 48% cases.

Table 5-Smear results:

graph 5:



by fluorescence microscopy was positive in 56% of the subjects were as negative in the rest 44%.

this is the sample graph for reference:

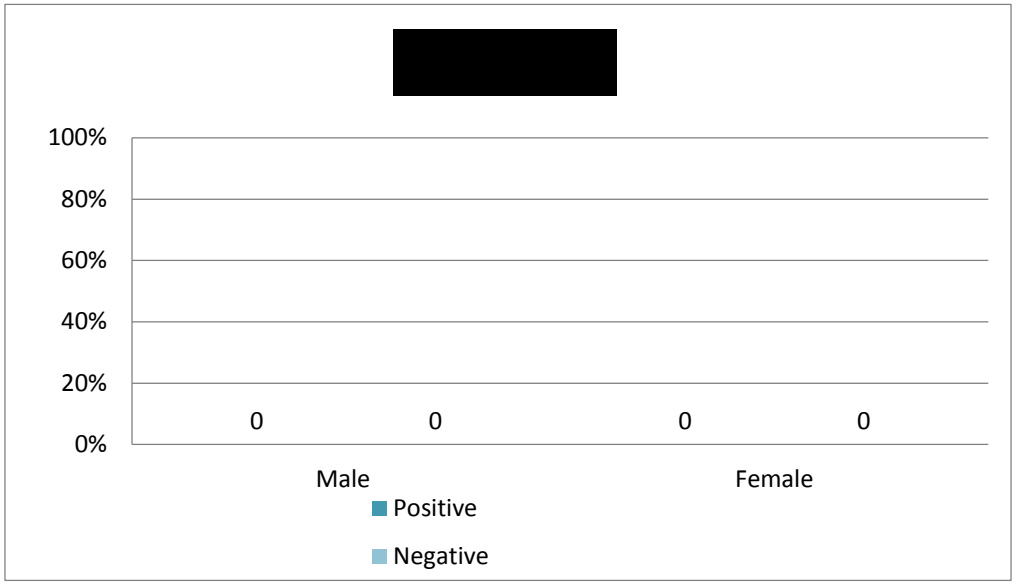
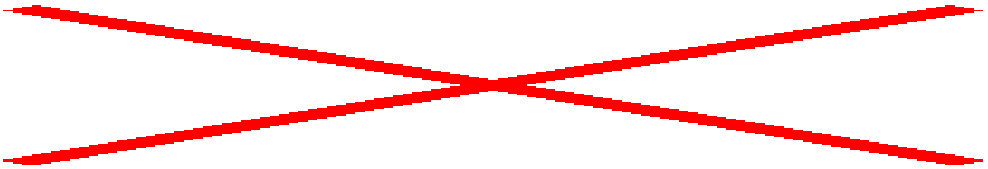


table -6comparison of smear

with culture:

table 7-sensitivity and specicity of smear -fluoresence microscopy



when compared with the gold standard “culture in solid media” the smear microscopy using LED fluorescence showed sensitivity of 62% and specificity of 48%

positive predictive value was 46%

negative predictive value was 64%

p value in McNemar test is 0.21

kappa agreement is 9.72%

p value for kappa agreement is 0.474

table-8comparison between PCR and culture:

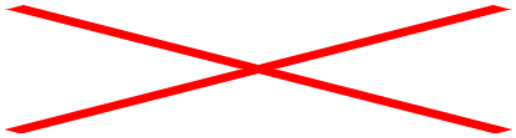
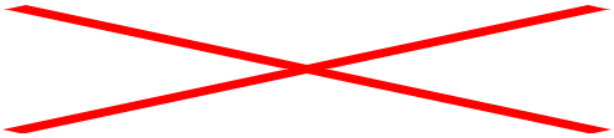


table 9-sensitivity and specificity of PCR:

Statistic	Formula	Value	95% CI
Sensitivity	$\frac{a}{a+b}$	95.24%	76.18% to 99.88%
Specificity	$\frac{d}{c+d}$	79.31 %	60.28% to 92.01%
Positive Likelihood Ratio	$\frac{Sensitivity}{100-Specificity}$	4.60	2.24 to 9.45
Negative Likelihood Ratio	$\frac{100-Sensitivity}{Specificity}$	0.06	0.01 to 0.41
Disease prevalence	$\frac{a+b}{a+b+c+d}$	42.00% (*)	28.19% to 56.79%
Positive Predictive Value	$\frac{a}{a+c}$	76.92% (*)	56.35% to 91.03%

Negative Predictive Value	$\frac{d}{b+d}$	95.83 % (*)	78.88% to 99.89%



the sensitivity of the LAMP PCR was 95.24% , specificity being 79.31% with a confidence interval of 95%

positive predictive value was 76.92%

negative predictive value being 95.83%

McNemar test P value :0.125

cohen's kappa :72%

kappa agreement P value:<0.0001

true positives: 20

true negatives:23

false positive:6

false negative:1

Discussion:

as tuberculosis is one of major causes of death and is preventable if taken appropriate measures.the foremost is detecting the disease in an early stage and initiating treatment thereby preventing its spread.the earlier methods of “sanatorium” ,isolation have been proved inadequate when compared with antibacterial treatment in preventing the spread.

In this study we compare LAMP PCR & fluoresce microscopy with culture using Lowenstein Jenson media in 50 clinical specimens(sputum) collected from cases suspected of tuberculosis for the diagnosis of *Mycobacterium tuberculosis.*

The specimens were collected from the district tuberculosis center, tirunelveli by informed consent to the patients.

the ethical committee clearance from the ethical committee board, tirunelveli medical college was obtained.

The suspicion criteria was based on the symptoms of cough with expectoration of more than 2 weeks and decided by the thoracic physicians in the department.

The laboratory diagnosis of tuberculosis chiefly relies on smear microscopy. The Ziehl Neelson method of acid fast staining is routinely carried out and the RNTCP has phased the fluorescence microscopy as an alternative method whereby the time to examine more fields can

be reductionis is because the low magnification used to examine the fields, thereby examining more fields in a single sitting. The time consumed by fluorescence microscopy to examine 100 fields is 2 minutes by experienced personnel.

However the technique has limitations in that its sensitivity and specificity are less and identification of species is restricted which makes the need for a better technique with same robustness, speed, cost effective with no additional expenses for manpower training and bio safety levels which can penetrate the rural and inaccessible areas of the TB endemic areas to find the TB bacilli and thereby initiating treatment.

In a study by Mathew P et al in journal of clinical microbiology and expert review of molecular diagnostics 2010 states that "while cheap and relatively easy to perform, the more than 125 year old smear microscopy method has only modest sensitivity (35-80%) and cannot differentiate between drug sensitive and drug resistant *Mycobacterium tuberculosis*"[47]

in a study by Steingarta KR et al "a meta analytical study of 45 studies show that the sensitivity advantage of fluorescence microscopy over conventional microscopy is greater by 10%".[46]

The culture is a gold standard, useful in identification of species as well as high sensitivity and specificity, it has shortcomings in that it needs 8 weeks time.

In this study we compare LAMP PCR & fluoresce microscopy with culture using Lowenstein Jenson media in 50 clinical specimens(sputum) collected from cases suspected of tuberculosis for the diagnosis of *Mycobacterium tuberculosis.*

The specimens were collected from the district tuberculosis center, tirunelveli by informed consent to the patients.

the ethical committee clearance from the ethical committee board, tirunelveli medical college

was obtained.

The suspicion criteria was based on the symptoms of cough with expectoration of more than 2 weeks and decided by the thoracic physicians in the department.

The specimens once collected were processed and stored at -20ºC for culture and PCR.

Bio safety precautions were observed during handling of the specimens and processing.

The test results were read during every week for 8 weeks before declaring negative if there was no growth at the end of 8 weeks.

Specimens were used for culture & PCR.

The PCR test was performed with kit from helini biomolecules.

DNA purification and PCR was done as per the manufacturers instructions.

The sensitivity of the smear microscopy in this study is 62%.this is in accordance with earlier study by mathew P et al in which "the sensitivity of the smear microscopy is 30-80%"[46]

it should also be noted that fluoresce microscopy improves the detection rate by another 10%.

The 62% sensitivity rate of the smear microscopy in this study indicates better lab practices, experienced technicians and the high prevalence of TB in this area.

In the study geojith et al says "Smear microscopy detects the morphology of the bacilli while culture methods differentiate based on the physiology of the organism". [45]

LAMP detects the presence of DNA in the specimen whether live or dead the organism may be.these two techniques are complementary to each other in the sense LAMP detects the genetic material more when the smear and culture results are positive.

In this study LAMP shows an excellent sensitivity of 95% which is in higher than earlier study by geojith et al in which the sensitivity was 79.5% .earlier studies showed better specificity.

This is in accordance with our study.

in a study by Pal N sharma et al in the indian journal of pathology “78.65% specimens were culture positive when processed within 48 h by the NaOH method. The culture positivity in the same specimen that were stored with cetylpyridinium chloride and processed after 7-8 days was 70.22%, whereas those stored without CPC and processed by the NaOH method was 46.62%”[85].

In a study by catherine Boehme et al “The sensitivity of LAMP in smear- and culture-positive sputum specimens was 97.7% and the sensitivity in smear-negative, culture-positive specimens was 48.8%”.[82]

the sensitivity of the above study is higher than the present study.

In a meta analytical study by daphne I, madhukar pai et al “commercial NAAT techniques are highly variable , the sensitivity is inconsistent with the specificity. The study further holds that based on the observation its not recommended for NAAT to be replaced for the conventional tests in the present situation”.[86]

It is to be mentioned that a study should have a higher sensitivity and specificity. It it has lower specificity then more persons who dont have disease are identified to have disease and further investigations are ordered or treatment started which is detrimental to patients health and unnecessary expenditure.

According to WHO report on the use of nucleic acid amplification tests for the diagnosis it states that in one large study the sensitivity was only 53%.

in a study by olga l et al on the Assessment by Meta-Analysis of PCR for Diagnosis of Smear-Negative Pulmonary Tuberculosis [87] Sensitivity ranged from 9-100% while the specificity ranged from 25 to 100%.

in a study by lydia kivihya et al on the Comparison of PCR with the Routine Procedure for Diagnosis of Tuberculosis in a Population with High Prevalences of Tuberculosis and Human Immunodeficiency Virus[88]

“The sensitivity and specificity of PCR were 93 and 84%, respectively. HIV status did not affect the sensitivity of PCR. A total of 99.7% of the true smear-positive and 82.1% of the true smear-negative TB patients were correctly identified by PCR. PCR detected M. tuberculosis in 11.7% of the culture-negative suspects, 60% of which had one or two PCR-positive sputum specimens”.

This indicates that the PCR technique can be applied to identify smear negative pulmonary tuberculosis

in a study on application of loop mediated isothermal amplification (lamp) assay as an alternative diagnostic test for rapid tuberculosis diagnosis in limited resource setting

Sensitivity of LAMP was 97.47% , the specificity being 60.31. the higher sensitivity means this test could be used as an alternative for smear microscopy in TB endemic countries like india for rapid and cost effective diagnosis.

In a study by mitarai ,okumura et al on the evaluation of a simple loop-mediated isothermal amplification test kit for the diagnosis of tuberculosis [89]

: The sensitivity of LAMP using raw sputum in smear- and culture-positive specimens was 98.2% while the sensitivity in smear-negative, culture-positive specimens was 55.6% (95%CI 43.4–68.0).

the test says that the sensitivity of lamp was not lower than the commercial amplicor assays for tuberculosis.

In a study by basu dev pandey et al "Using this system, a total of 200 sputum samples from Nepalese patients were investigated. The sensitivity of MTB-LAMP in culture-positive samples was 100% (96/96), and the specificity in culture-negative samples was 94.2% (98/104, 95% confidence interval 90.5–97.9%). [90]

in the study by geojith G et al on the efficacy of LAMP in lab detection of MTB the sensitivity was 91.7% and specificity was 90.9% in 2 hour format.[45]

In the present study the incubation time was 1 hour.increase in incubation time increases the sensitivity of the test as per the above mentioned study.The need for modification of protocols to give a better effficacy needs to researched in further studies.

In a study by ehsan aryana et al " the accuracy of the LAMP method was tested using restriction endonucleases enzymes to digest the byproducts of LAMP and then visualised using agarose gel electrophoresis"[91]

this study confirms the accuracy of the LAMP reaction.

Recently another study by lee et al 2009,

One technique called reverse transciption loop mediated isothermal amplification combined with enzyme linked immunosorbent hybridisation (RT-LAMP-ELISA) was used to target the 16S ribosomal RNA.the test detected one copy of 16S RNA per reaction .this is comparable with the IS6110 based MTB LAMP assay. This technique however is costly compared with

LAMP MTB .[92]

The cost of single LAMP reaction in our study is Rs.500-/-.whereas the technique mentioned above could cost upto 660/reaction.

if both smear and PCR are positive in clinical specimens then the diagnosis of tuberculosis is assumed.

However in studies by ru-yi zhua et al on the LAMP test correctly identified the MTB DNA(M.tuberculosis, M.bovis and BCG strains) & did not detect the non tuberculos mycobacteria and other bacteria. This shows the specificity of this test.[92]

In the study by ehsan aryana et al shows identical sensitivities were obtained for LAMP and nested PCR, but the LAMP assay was more rapid and cost-effective than the latter.

The p value of our study is <0.0001 which is highly significant.

Summary:

the present study aimed at detection of *Mycobacterium tuberculosis* in clinical specimens from patients suspected of tuberculosis with history of fever, cough with expectoration for 2 weeks ,loss of weight, apettite and were referred for lab diagnosis of *Mycobacterium tuberculosis* by expert thoracic physicians from the department of thoracic medicine, tirunelveli medical college.all the sample were collected within a time of 2 months, decontaminated and homogenised by modified petroff's method using TBC guidelines.fluorescence microscopy was done on unprocessed samples and their results collected, however the test is a blinded study and the results were compared at the end of the study.

The processed specimens were then inoculated in LJ media , freshly prepared using a standardized method and the rest of pellet was used to detect MTB by loop mediated isothermal amplification.the results were compared at the end of the study.the results of the study were analysed and summarised below

the age wise percentage of patients included in the study:

18% of the study population was below the age of 30,

18% between 31-40,

18% between 41-50,

24% of the cases were between 51-60 and

22% of the cases were more than 61 years of age.

50 samples were randomly selected from the regional tuberculosis center of which

22% of the study population were females and the rest 78% were males.

culture was positive in 42% of the subjects and negative in 58%.

graph 4 shows PCR was positive in 52% of the study population and negative in 48% cases.

Graph 5 shows fluorescence microscopy was positive in 56% of the subjects were as negative in the rest 44%.

false negative was seen in one sample. which may be due to contamination.

False positive was seen in 6 samples

true positive in 20 samples&

true negatives in 23 samples.

fluoresence microscopy using auromine staining when compared with the gold standard "culture in solid media" shows

sensitivity of 62%

specificity of 48%

positive predictive value was 46%,

negative predictive value was 64%,

P value in McNemar test is 0.21,

kappa agreement is 9.72%,

P value for kappa agreement is 0.474.

comparison of LAMP PCR with gold standard technique which is culture in solid LJ media shows

sensitivity of the LAMP PCR was 95.24%,

specificity being 79.31%,

positive predictive value was 77%,

negative predictive value being 96%

P value for Mcnemar test 0.125

P value for kappa agreement was <0.0001,

kappa agreement was 72%

HIV infected samples were too low and hence were neglected for consideration

also there was no relevance in the positivity rate for male and female patients

age wise distribution of the samples did not bear any specific outcome for the study.

The P value is highly significant with 95% sensitivity rate.

Conclusion:

LAMP PCR can be used for detection of mycobacterium tuberculosis comples in sputum samples with a high degree of sensitivity and moderate levels of specificity.

In culture and smear analagous samples the sensitivity is higher.

the important advantage of LAMP is its robustness, speed in detecting the organism.the detection time is one hour and needs little training to be done

However whether its an active or latent infection needs clinical findings and other assays like Interferon gamma release assays.

When used in conjunction with smear microscopy the results are good.

a large study and subsequent research is required for its implementation as an accepted methodology of diagnosis.

Bibliography

1) Carl Zimmer, “tuberculosis is newer than thought, study says”, new york times 21.08.2014

2)"Robert Koch."World of Microbiology and Immunology.Ed. Brenda Wilmoth Lerner and K. Lee Lerner. Detroit: Gale, 2006. Biography In Context. Web. 14 Apr. 2013.

3)Corbett El, Watt CJ, Walker N et al. the growing burden of Tb: global trends and interactions with the HIV epidemic. Arch of internal medicine 163(9), 1009-1021(2003).

4) WHO.global TB control, surveillance, planning and financing. WHO report 2005.1-247(2005)

5)http://www.tbcindia.nic.in/key.html

6) Nicholas (September 15, 1998),"Scientist at Work/Kary Mullis; After the 'Eureka', a Nobelist Drops Out",The New York Times.

7) http://www.tbfacts.org/tb-india/

8)The Significance of the Tuberculin Skin Test in Elderly Persons by William W Stead in annals of internal medicine 1987;107(6);837-842.

9)Exposure of emergency department personnel to tuberculosis: PPD testing during an epidemic in the community by Peter E Sokolovo published in annals of emergency medicine 1994

Sep;24(3):418-21.

10) Kolappan C, Gopi PG. Tobacco smoking and pulmonary tuberculosis. *Thorax* 2002; 57: 964-6.

11) www.who.int/tb/publications/global_report/gtbr14_main_text.pdf

12) Wirth T et al "origin, spread and demography of *Mycobacterium tuberculosis* complex" PLoS Pathog. 2008 Sep; 4(9).

13) Pease, Arthur Stanley (April 1940). "Some Remarks on the Diagnosis and Treatment of Tuberculosis in Antiquity".*Isis*31(2): 380–393.

14) Transmission of Drug-Susceptible and Drug-Resistant Tuberculosis and the Critical Importance of Airborne Infection Control in the Era of HIV Infection and Highly Active Antiretroviral Therapy Rollouts Clin Infect Dis. 2010 May 15; 50(Suppl 3): S231–S237.

15) Riley EC, Murphy G, Riley RL. Airborne spread of measles in a suburban elementary school. Am J Epidemiol. 1978;107: 421–432

16) Parsons et al -lab diagnostic aspects of drug resistant tuberculosis front.biosci 9:2086-2105

17) Kiwanuka et al -effect of HIV type 1 on disease progression in persons from rakai, uganda with incident HIV-1 infection ,journal of infectious diseases 197:707-713; 2008

18) Lama et al 2007-host factors influencing susceptibility to HIV infection and AIDS progression. journal of retrovirology 4:57

19) SK Sharma and Mohan et al on extra pulmonary TB in Indian J Med Res.2004 Oct;120(4):316-53.

20) Diagnosis and management of miliary tuberculosis: current state and future perspectives the journal of Clin Risk Manag. 2013; 9: 9–26.

21) Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis

worldwide: a meta-analysis and assessment of cost-effectiveness by Bourdin Trunz published in Volume 367, No. 9517, p1173–1180, 8 April 2006

22) NR Gandhi et al on Multidrug-resistant and extensively drug-resistant tuberculosis: a threat to global control of tuberculosis published in lancet 2010 May 22;375(9728) .

23) Diperri Giovanni nosocomial epidemic of active tuberculosis among hiv-infected patients Volume 334, No. 8678-8679, p1502–1504, 30 December 1989

24) Karp et al in oxford journal of clincial infectious diseases study on Coinfection with HIV and Tropical Infectious Diseases. II. Helminthic, Fungal, Bacterial, and Viral Pathogens volume 45 issue 9, Pp.1214-1220

25) AIDS epidemic update 2009 and WHO global TB control update 2009

26) Parsons et al on the lab diagnosis of TB in resource poor countries -challenges and opportunities published in clinical microbiology reviews April 2011, p 314-350

27) Mukadi Y., Perriens J. H., Louis M. E. St., Brown C., Prignot J., Willame J. C., Pouthier F. et al. Spectrum of Immunodeficiency in HIV-1-Infected Patients with Pulmonary Tuberculosis in Zaire. Lancet.1993;342:143–46

28) Ackah et al on response to treatment, mortality and cd4 lymphocyte counts in HIV , lancet 1995 Mar 11;345(8950):607-10.

29) Farba Karam et al on a study on Sensitivity of IFN-γ Release Assay to Detect Latent Tuberculosis Infection Is Retained in HIV-Infected Patients but Dependent on HIV/AIDS Progression in PLoS ONE. 2008; 3(1): e1441.

30) Harries A. D., Hargreaves N. J., Gausi F., Kwanjana J. H., Salaniponi F. M. High Early Death Rate in Tuberculosis Patients in Malawi. International Journal of Tuberculosis and Lung Disease.2001

31) Pai et al on Novel and improved technologies for tuberculosis diagnosis: progress and challenges.Clinics in Chest Medicine 30: 701-716,2009

32) Lange C, Mori T. Advances in the diagnosis of tuberculosis. Respirology.2010;15:220–240.

33) Madebo T, Lindtjorn B. Delay in treatment of pulmonary tuberculosis: an analysis of symptom duration among Ethiopian patients.MedGenMed 1999:E6.

34) Liam CK, Tang BG. Delay in the diagnosis and treatment of pulmonary tuberculosis in patients attending a university teaching hospital.Int J Tuberc Lung Dis 1997; 1:326–32.

35) Perkins MD on facing the crisis: improving the diagnosis of tuberculosis in the HIV era in journal of infectious diseases 2007,196:S15-27

36) Reducing the global burden of tuberculosis: the contribution of improved diagnostics.
By Keeler et al in Nature.2006 Nov 23;444 Suppl 1:49-57.

37) Detection of *Mycobacterium tuberculosis* in clinical specimens by polymerase chain reaction and Gen-Probe Amplified *Mycobacterium tuberculosis* Direct Test by Abe c et al *J Clin Microbiol. 1993 Dec; 31(12):3270-4.*

38) Evaluation of Amplicor PCR for direct detection of *Mycobacterium tuberculosis* from sputum specimens by Beaves et al in *J Clin Microbiol. 1995 Oct; 33(10):2582-6.*

39) Detection of *Mycobacterium tuberculosis* in respiratory specimens by strand displacement amplification of DNA. By Down et al in *J Clin Microbiol. 1996 Apr; 34(4):860-5*

40) Fisher M et al in Expert Rev Mol Diagn.2002 Mar;2(2):151-9.on Diagnosis of MDR-TB: a developing world problem on a developed world budget.

41) Moore DF, Guzman JA, Mikhail LT. Reduction in turnaround time for laboratory diagnosis of pulmonary tuberculosis by routine use of a nucleic acid amplification test. Diagnostic microbiology and infectious disease.2005;52:247–254

42) Campos M, Quartin A, Mendes E, Abreu A, Gurevich S, et al. Feasibility of shortening respiratory isolation with a single sputum nucleic acid amplification test.American journal of respiratory and critical care medicine.2008;178:300

43) Lemaire J, Casenghi M. New diagnostics for tuberculosis: fulfilling patient needs first.Journal of the International AIDS Society.2010;13:40

44) catherine boehme in a study on Operational Feasibility of Using Loop-Mediated Isothermal Amplification for Diagnosis of Pulmonary Tuberculosis in Microscopy Centers of Developing Countries ▿ J. Clin. Microbiol. June 2007vol. 45no. 61936-1940

45) Geogith George et al on Comparison of the Efficacies of Loop-Mediated Isothermal Amplification, Fluorescence Smear Microscopy and Culture for the Diagnosis of Tuberculosis in PLoS One. 2011; 6(6): e21007

46) Steingkart KR in Lancet Infect Dis.2006 Sep;6(9):570-81. A study on Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review.

47) Mathew P et al in journal of clinical microbiology and expert review of molecular diagnostics 2010

48) Dye et al on evolution of TB control and prospects of reducing TB incidence JAMA. 2005 Jun 8;293(22):2767-75

49) New tools for the diagnosis of tuberculosis: the perspective of developing countries by Foulds et al in The International Journal of Tuberculosis and Lung Disease, Volume 2,Number 10, October 1998, pp.778-783(6)

50) Cambanis A, Yassin MA, Ramsay A, Squire SB, Arbide I, et al. (2006) A one-day method for the diagnosis of pulmonary tuberculosis in rural Ethiopia. Int J Tuberc Lung Dis10: 230–232

51) Getahun H., Harrington M., O'Brien R., Nunn P. 2007.Diagnosis of smear-negative pulmonary tuberculosis in people with HIV infection or AIDS in resource-constrained settings: informing urgent policy changes. Lancet 369:2042–2049

52) D. Maher and A. Harries, "Tuberculosis case fatality rates in high HIV prevalence populations in sub-Saharan Africa,"AIDS, vol. 15, no. 2, pp. 143–152, 2001

53) Monkongdee P., et al. 2009.Yield of acid-fast smear and mycobacterial culture for tuberculosis diagnosis in people with human immunodeficiency virus. Am. J. Respir. Crit. Care Med.180:903–908

54) Mase S. R., et al. 2007.Yield of serial sputum specimen examinations in the diagnosis of pulmonary tuberculosis: a systematic review. Int. J. Tuberc. Lung Dis.11:485–495

55) Bonnet M, Ramsay A, Gagnidze L, Githui W, Guerin PJ, Varaine F. Reducing the number of sputa examined, and thresholds for positivity: An opportunity to optimize smear microscopy. Accepted for publication, Int J Tuberc Lung Dis.

56) Mase S, Ramsay A, Ng N, Henry M, Hopewell PC, Cunningham J, Urbanczik R, Perkins M, Aziz MA, Pai M. Yield of serial sputum specimen examinations in the diagnosis of pulmonary tuberculosis: a systematic review. Int J Tuberc Lung Dis 2007;11(5):485-95.

57) Williams-Bouyer N, Yorke R, Lee H, Woods GL. Comparison of the BACTEC MGIT 960 and ESP culture system II for growth and detection of mycobacteria. J Clin Microbiol 2000;38:4167-70.

58) Van Kampen SC et al; The realistic performance achievable with mycobacterial automated culture systems in high and low prevalence settings. *BMC Infect. Dis.* 10, 93 (2010).

59) KocagÖz T Program and abstracts of the American Society for Microbiology General Meeting. Washington, DC: American Society for Microbiology;2000.

60) Baylan O et al on Evaluation of a new automated, rapid, colorimetric culture system using solid medium for laboratory diagnosis of tuberculosis and determination of anti-tuberculosis drug susceptibility. Int J Tuberc Lung Dis2004;8:772-7

61) Farnia P et al. Application of oxidation-reduction assay for monitoring treatment of patients with pulmonary tuberculosis.J Clin Microbiol2004;42:3324-5.

62) Moore DA, Mendoza D, Gilman RH, et al. Microscopic observation drug susceptibility assay, a rapid, reliable diagnostic test for multi drug resistant tuberculosis suitable for use in resource-poor settings. J Clin Microbiol 2004; 42:4432–7.

63) ICMR bulletin dated 2002

64) Isenberg et al on a study of a rapid biphasic system for detection of TB journal of clinical microbiology 29:1719 ,1991

65) Venkatraman et al comparison of bactec radiometric method with conventional DST indian journal of medicine 108:120;1998

66).Tortoli E, Russo C, Piersimoni C, et al. Clinical validation of Xpert MTB/RIF for the diagnosis of extrapulmonary tuberculosis.Eur. Respir. J.2012

67) Rohner et al in evaluation of MB/BACT system with solid media for isolation of tubercle bacilli j of clinical microbiology 35:3127;1997

68) Clinical Evaluation Of Difco Esp Culture System Ii For Growth And Detection Of Mycobacteria By Woods Et Al In Journal Of Clinical Microbiology, Jan. 1997, P. 121–124

69) Albay et al in a study on a fast plaque test for the rapid diagnosis of tuberculosis, journal of diagnostic micr infectious diseases 2003;46(3)

70) Rapid diagnosis of *Mycobacterium tuberculosis* by mycobacteriophage. international journal of tuberculosis and lung diseases 2004:8(7)

71) Alcaide et al in a study in usefulness of a new mycobacteriophage for the diagnosis of tuberculosis J of clin microbiology 2003;41(7)

70) Gennaro M. L., Doherty T. M., editors. (ed.). 2010.Immunodiagnosis of Tuberculosis: New questions, New Tools conference, 2008.

71) Daniel T. M., Oxtoby M. J., Pinto E., Moreno E. 1981.The immune spectrum in patients with pulmonary tuberculosis.Am. Rev. Respir. Dis.123:556–559

72) Bothamley G. H., Schreuder G. M., de Vries R. R., Ivanyi J. 1993.Association of antibody responses to the 19-kDa antigen of *Mycobacterium tuberculosis* and the HLA-DQ locus.J. Infect. Dis.167:992–993

73) Laal S., et al. 1997.Surrogate marker of preclinical tuberculosis in human immunodeficiency virus infection: antibodies to an 88-kDa secreted antigen of *Mycobacterium tuberculosis*.J. Infect. Dis.176:133

74) New tools and emerging technologies for the diagnosis of tuberculosis: Part II. Active tuberculosis and drug resistance Expert Review of Molecular Diagnostics(Impact Factor: 3.52).06/2006; 6(3):423-32 by Madhukar Pai

75) Int J Tuberc Lung Dis.1998 Jul;2(7):541-6.MPB64 mycobacterial antigen: a new skin-test reagent through patch method for rapid diagnosis of active tuberculosis. By Nakamura et al

76) Tortoli, E., Russo, C., Piersimoni, C. et al, Clinical validation of Xpert MTB/RIF for the diagnosis of extrapulmonary tuberculosis.*Eur Resp J*.2012;40:442–447.

77) Green, C., Huggett, J.F., Talbot, E. et al, Rapid diagnosis of tuberculosis through the detection of mycobacterial DNA in urine by nucleic acid amplification methods. Lancet *Infect Dis*.2009;9:505–511

78) Brodie et al on the diagnosis of tuberculosis in the journal of clinical chest medicine 26(2)

79) Flores LL, Pai M, Colford JM, Riley LW. In-house nucleic acid amplification tests for the detection of *Mycobacterium tuberculosis* in sputum specimens: meta-analysis and meta-regression Microbiol. 2005;5:55. doi: 10.1186/1471-2180-5-55

80) Suffys, P., J. C. Palomino, S. Cardaso Leão, C. Espitia, A. Cataldi, A. Alito, M. Velasco, J. Robledo, J. Fernandez, P. S, Rosa, and M. I. Romano.2000. Evaluation of the polymerase chain reaction for the detection of *Mycobacterium tuberculosis*.Int. J. Tuberc. Lung Dis.4(2):179-183.

81) Iwamoto, T., T. Sonobe, and K. Hayashi.2003. Loop-mediated isothermal amplification of direct detection of *Mycobacterium tuberculosis* complex, M. avium, and M. intracellulare in sputum samples.J. Clin. Microbiol.41:2616-2622

82) Operational Feasibility of Using Loop-Mediated Isothermal Amplification for Diagnosis of Pulmonary Tuberculosis in Microscopy Centers of Developing Countries by Catherine Boehme et al in J Clin Microbiol. 2007 Jun; 45(6): 1936–1940.

83) http://loopamp.eiken.co.jp/e/lamp/

84) A commercial line probe assay for the rapid detection of rifampicin resistance in *Mycobacterium tuberculosis*: a systematic review and meta-analysis by Morgan et al in BMC Infectious Diseases 2005; 5:62

85)Transport and storage of sputum specimen by using cetylpyridinium chloride for isolation of mycobacteria Year: 2009 Volume: 52 Issue: 1Page: 59-61 by Pal N Sharma et al

86) Commercial Nucleic-Acid Amplification Tests for Diagnosis of Pulmonary Tuberculosis in Respiratory Specimens: Meta-Analysis and Meta-Regression

Daphne I. Ling, DOI: 10.1371/journal.pone.0001536

87) Assessment by Meta-Analysis of PCR for Diagnosis of Smear-Negative Pulmonary Tuberculosis Olga L. Sarmiento,1 Kristen A. Weigle,1,2 Janet Alexander,1 JOURNAL OF CLINICAL MICROBIOLOGY, July 2003, p. 3233–3240

88) J Clin Microbiol.2004 Mar;42(3):1012-5. Comparison of PCR with the routine procedure for diagnosis of tuberculosis in a population with high prevalences of tuberculosis and human

immunodeficiency virus by Kivihya et al

89) int J Tuberc Lung Dis.2011 Sep;15(9):1211-7, i. doi: 10.5588/ijtld.10.0629 Evaluation of a simple loop-mediated isothermal amplification test kit for the diagnosis of tuberculosis by Okumara et al

90) Development of an in-house loop-mediated isothermal amplification (LAMP) assay for detection of *Mycobacterium tuberculosis* and evaluation in sputum samples of Nepalese patients by Basudev Pandey et al in . Med. Microbiol., April 2008 57: 439-443

91) A novel and more sensitive loop-mediated isothermal amplification assay targeting IS6110 for detection of *Mycobacterium tuberculosis* complex by Ehsan Aryan et al in Microbiological Research Volume 165, Issue 3, 31 March 2010, Pages 211–220

92)Evaluation of reverse transcription loop-mediated isothermal amplification in conjunction with ELISA-hybridization assay for molecular detection of *Mycobacterium tuberculosis* J Microbiol Methods, 76 (2) (2009), pp. 174–180

93) Use of visual loop-mediated isotheral amplification of rimM sequence for rapid detection of *Mycobacterium tuberculosis* and *Mycobacterium bovis* by ru-yi zhu Journal of microbiological methods(Impact Factor: 2.03).08/2009; 78(3):339-43.

Annexure -1

Preparation of culture media:

Ingredients:

Mineral salt solution with malachite green:

- potassium dihydrogen phosphate-2.4 g
- magnesium sulphate -0.24 g
- magnesium citrate-0.6 g
- asparagine-3.6 g
- glycerol-12 ml
- malachite green 2% solution-20 ml

the above components are dissolved in 600 ml of distilled water and autoclaved at 121º C for 15 minutes and then cooled at room temperature.

Homogenised whole eggs:

- the eggs are scrubbed with soap and water
- soaked in soap water for 30 minutes
- rinsed
- soaked in 70% ethanol for 15 minutes
- eggs cracked into a sterile glass beaker

- using an electrical blender , blended for 1 minute

for 600 ml of mineral salt solution 1 litre of homogenised whole eggs are added and 8 ml are injected into sterile autoclaved Mcartney bottles .the bottles are then screw capped.

the inspissator is heated to 85ºC ,the bottles are put in a slanting position and the media allowed to coagulate for 50 minutes.

Later the bottles were kept in a refrigerator for inoculation .

Proper bio safety level 3 precautions were taken .

Proforma:

name:

age:

sex:

laboratory number:

specimen number

date of sputum collection

reason for examination:

a) diagnosis

b) follow up

HIV status

smear results:

a)first specimen

b)second specimen

culture results: type of growth

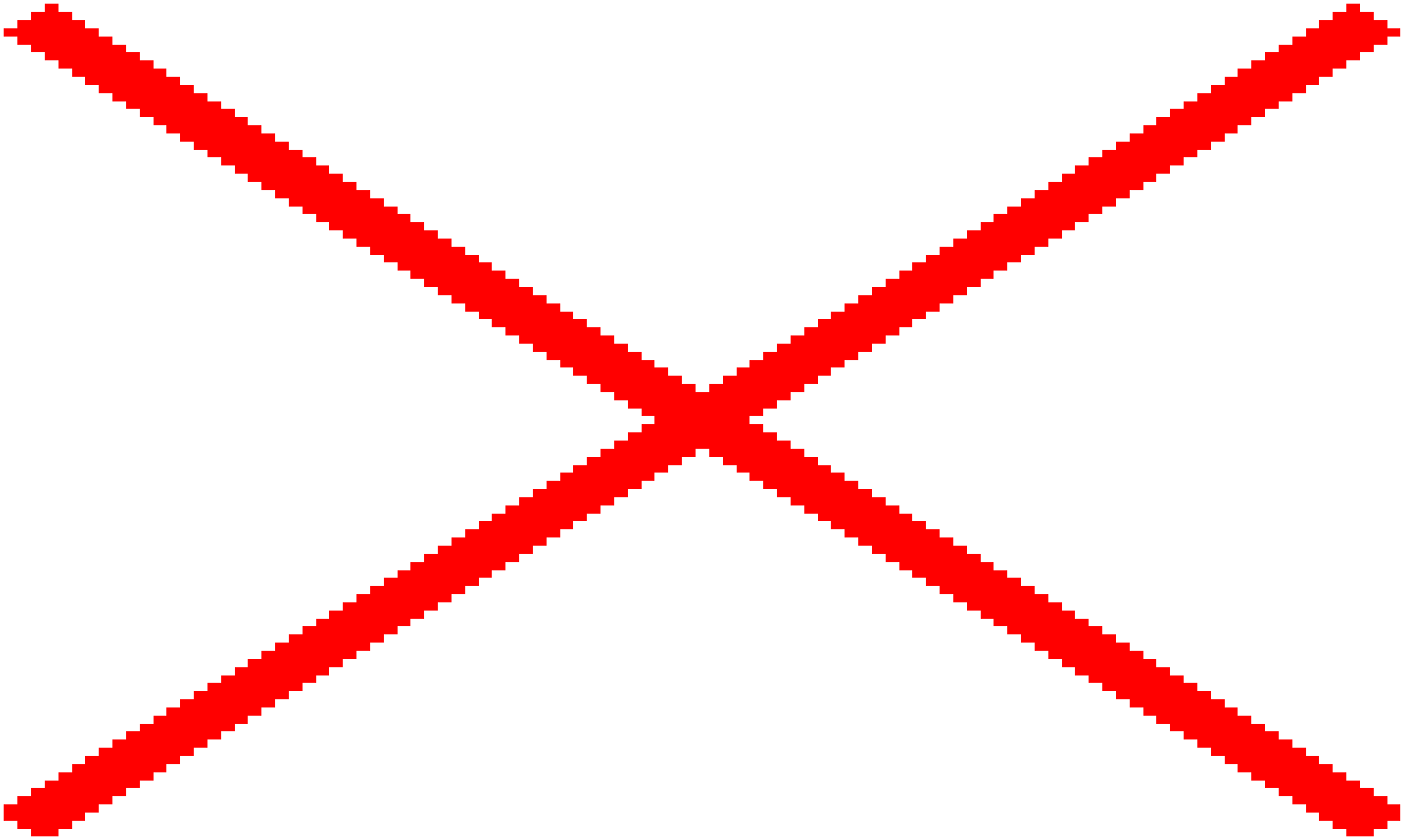
biochemical tests:

a) niacin

b) nitrate:

LAMP PCR result

master data sheet



Introduction:

Tuberculosis is disease known since ancient times. The earliest discovery dates back to 6000 years ago among the human remains in Egypt[1]. It was also isolated from the mummies around 2400-3000 B.C. It's also a disease which has defied the scientific achievements and miraculous cure for so many diseases that modern medicine was able to do in last century. The immunology and understanding of infectious diseases started with the identification of bacteria under a microscope by Anton Van Leuwenhoek in the 16th century. It's to be noted that earlier systems of medicine such as the Greek, Persian medicine though formulated that certain diseases are contagious and are transmitted by "invisible particles", the modern medicine or the western medical system proved a breakthrough in making these "invisible particles" as visible.

Since Koch's discovery of the TB bacilli in 1882, microscopic detection of the bacilli in clinical specimens has remained as the cornerstone of TB diagnosis in low and middle income countries.

Many of us would know that the killer diseases of ancient times such as anthrax, plague, small pox were almost eradicated from the face of the earth by the deeper understanding of science, how our body reacts to foreign agents, and the discovery of penicillin and so on. Only a few infectious diseases still exist which defies the human developments...it would not be an exaggeration to say that though effective treatments, diagnostic methods had been evolved to tackle tuberculosis it is still largely "invisible" or undetected.

The statistics by the world health organization says that one third of every human being in this world is infected with *Mycobacterium tuberculosis* [3, 4]. Not to say it's more prevalent in developing countries or the "third world countries". This disparity in its geographical distribution is due to the poor living conditions, inadequate housing, poor sanitation, malnutrition and to a greater extent on the health care measures taken by the respective governments.

India is one of world's nest in tuberculosis an estimated 2 patients per second die of tuberculosis[5]. And the post independent India started its national tuberculosis program since 1962 which was evolved in to the revised national tuberculosis control program in 1992 with the help of international agencies and adopted the international strategy of DOTS-direct observed short term treatment strategy[5].

The importance of tuberculosis and the need for further research into it is obligated by its "invisible nature". Why it is said as "invisible"? Because the difficulties in the diagnosis whether clinical or non-clinical. In clinical spectrum the diagnosis includes symptoms such as cough with expectoration for more than 2 weeks, evening rise in temperature, night sweats, loss of weight and appetite and radiologically in finding any cavity lesions in the lungs. In microbiology the diagnosis includes the classical acid fast staining method, the gold standard culture on solid media. The developments in molecular biology and the development of polymerase chain reaction has made numerous strides in the field of microbiology in diagnosing infectious diseases. Scientists have gone to the extent of describing the era of molecular biology as "before PCR" and "after PCR".

The PCR techniques enables to diagnose small quantities of the infectious material and has proved to be a good diagnostic method in terms of sensitivity, specificity and rapidness. However they are not without negatives. The negatives include its cost, and cannot differentiate between live or dead organisms.

The PCR technique originally developed by Gary Mullis in 1983 was based on amplifying a single gene or a few gene to many fold times, thereby enabling to identify them. He used a polymerase enzyme taq polymerase isolated from the thermophilic bacterium thermus aquaticus. In fact the process is named after that enzyme. Since then the process has been used in a wide variety of applications such as gene cloning, DNA fingerprinting and to identify infectious organisms. The method relies on the thermal cycling and enzymatic replication of the DNA. Primers which are short DNA fragments which are complementary to the target gene along with the polymerase enzyme make this possible.

It is to be noted that PCR is not without errors, the polymerase enzyme can mutate the PCR fragments made and hence affect their specificity.

The technique has been evolved into many variations and one of the variations is loop mediated isothermal amplification. This LAMP technique employs a Bst polymerase extracted from bacillus stearothermophilus. In contrast to the conventional PCR which uses thermocycling, this technique could be done in a constant temperature. This is made possible by using a polymerase enzyme with a high strand displacement activity in addition to the replication activity. Typically 4 different sets of primers are used to amplify six different areas of the target gene which adds to their specificity. A large number of copies are made and

their diagnosis is done with naked eye due to the increased precipitation of magnesium pyrophosphate which is a byproduct of the amplification. It can also be detected via photometry .dyes such as a SYBR green can be used to intercalate the amplified DNA and hence by measuring the color change it's possible to quantitate the reaction as well.

By eliminating the use of costly thermocyclers this technique promises a great and low cost alternative in diagnosing infectious diseases. The laboratory diagnostic method include Ziehl-Neelson acid fast staining which is routinely used in all the primary health care centers in India. However the sensitivity of the test is only 30%, thereby missing a huge chunk of the positive patients. Culture on the other hand is the "gold standard" but it needs sophisticated centers with biosafety levels and the result cannot be achieved unless 8 weeks which is huge time for which the treatment cannot be delayed. The skin test can determine the immune status of the individual and cannot determine if he's suffering from active diseases or had previous immunization, nevertheless it's useful in screening purposes. The RNTCP has achieved great lengths in combating tuberculosis in India, nevertheless the gaps in diagnosing the infection still remains a hurdle.

In India 40% of population are infected with tuberculosis with a lifetime risk of 100% even in the absence of HIV. The emergence of HIV has made the TB/HIV a dreadful combination as HIV itself is not a killer disease while it lowers the immunity, thereby making tuberculosis an opportunistic infection and thus the rates of Tuberculosis in patients infected with HIV has soared. One more thing which is of great concern is the emergence of the "super bugs", which is the

multidrug resistance Tuberculosis which has higher mortality and morbidity. The RNTCP has launched PCR based techniques in detecting the above two clauses which is CBNAAT which is cartridge based nucleic acid amplification techniques. This has been accepted worldwide to detect *Mycobacterium tuberculosis*. *But* their role in screening *Mycobacterium tuberculosis* and detecting it in patients in outpatient setting has seen limitations owing to the higher costs and still needs further evaluation and research regarding the same. The present study aims to find the efficacy of this newer technique the "loop mediated isothermal amplification" in the detection of *Mycobacterium tuberculosis* in clinical specimens. As tuberculosis remains as one of the diseases which needs a definitive control strategy numerous research works are on the way especially in the light of MDR TB and its association with HIV.

Aims and objective:

- To find a low cost, rapid and efficient microbiological diagnostic method for the detection of *Mycobacterium tuberculosis* from clinical specimens that could be implemented without much training and even in a rural setting
- To evaluate the sensitivity, specificity of loop mediated isothermal amplification in sputum samples collected from both smear positive, smear negative specimens in a blinded study and compare it with a gold standard technique -culture in solid Lowenstein Jensen media.

REVIEW OF LITERATURE:

Epidemiology:

According to ICMR bulletin dated august 2002, about one third of the world's population is infected with tuberculosis.

Around 10 million people acquire tuberculosis annually and 3 million people die of the disease each year. Mortality for tuberculosis is 25% of all avoidable deaths. 95% of all TB cases and 98% of death due to TB are in developing countries which includes India, and among them 75% are in economically active age group.

In India around 2 million people acquire the disease annually and of that 1 million people die of it each year. This means one patient dies of TB every minute. To compound this problem, is the emergence of HIV.

India has 3.5 million HIV patients of which 1.8 million patients have TB. Around 60% of HIV patients acquire active TB during their life time[7] .

Its worth to mention the emergence of the super bugs MDR and XDR and their coincidence with HIV is nevertheless associated with higher fatality rates.

Terminology:

TB incidence: number of new cases of active TB /year

TB prevalence: number of people living with active TB

The revised national tuberculosis control programme was launched in india in 1997.

Definition:

TB relapse: if the patient becomes ill after finishing the first course of treatments Treatment failure: some improvement or total failure to respond to treatment.

Default: patients who havent finished the first course of treatments all the above three categories can develop drug resistance and need drug susceptibility testing.

Exposure to TB depends upon the following factors:

1) Number of active cases in a community

2) Duration of their infection

3) Number and nature of interaction between the active case and contacts in a given time.

The above facts were derived from Tbfacts.org in a study by Dr.William W Stead in the annals of internal medicine say that "person who react after exposure of tuberculosis fall into three types.the reaction here is tuberculin sensitivity test.

1) Patients who have positive TST and have a high risk of tuberculosis, they need prophylaxis

2) Patients where there is no screening done before exposure and are positive to TST should be treated as like above said

3) People who are positive to TST but the risk is too low..they dont need prophylaxis"

Corresponding to the topic I am quoting another study which is worth when considering the screening in epidemics and health care workers[8].

In a study by Peter E Sokolovo in the annals of the internal medicine finds

that" systematic monitoring by tuberculin sensitivity testing is necessary to find the adequacy of respiratory isolation procedures carried out in the emergency department of a hospital during epidemics."

Smoking is a known risk factor which predisposes to tuberculosis.[10]

In a study by Dr.C.Kolappan from the tuberculosis research center, ICMR published in british medical journal of thorax says that " the association between tuberculosis and smoking is a strong dose-response relationship".[11] There's some good news.by implemeting effective diagnosis and treatment strategies a few countries have been able to bring down TB.eg: china,brazil and cambodia.[11]

Causative organism:

Scientific work suggests that *Mycobacterium tuberculosis* evolved from a common human specific pathogen about 40000 years ago. .the evolution of the TB bacilli is closed linked with migration of human beings out of africa .the bovis species evolution has been linked with the domestication of animals.[12]

The tubercle bacilli is an aerobic, acid fast bacilli.the acid fastness is due to the peptidogylcan layer linked with the mycolic acid in the cell wall. This arrangement is one of the virulent characters of the organism as its difficult for the antibiotics to penetrate the cell wall and to the action of the antibodies..cell wall has another component , lipoarabinomannan which eases the intracellular survival of the organism.

Regarding the evolution of TB bacilli, In a study by cristina gutierrraz ,the author says that "scientists show that the TB bacilli is able to exchange parts of their genome with other bacteria , which makes them adaptable to specific hosts".

Transmission:

TB is a disease thats spread by aerosols.factors which influence the transmission are

1) Ventilation in the room

2) Number of bacilli in the droplet

3)Exposure to UV rays

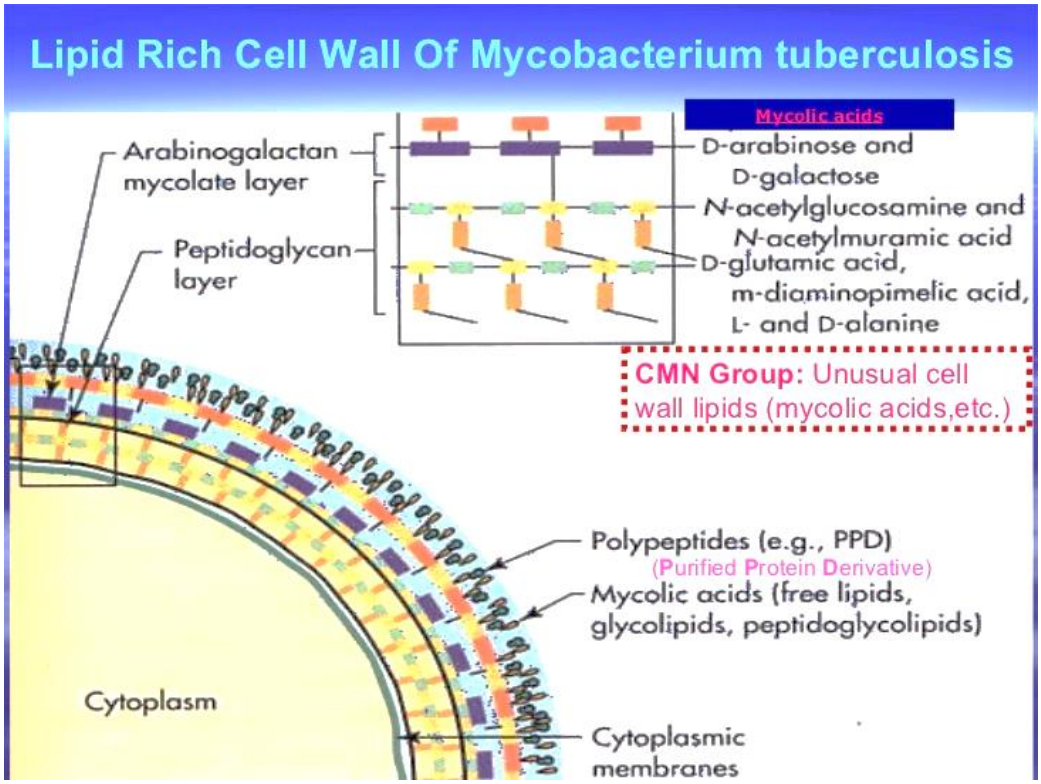
Primarily its a disease affecting the lungs.

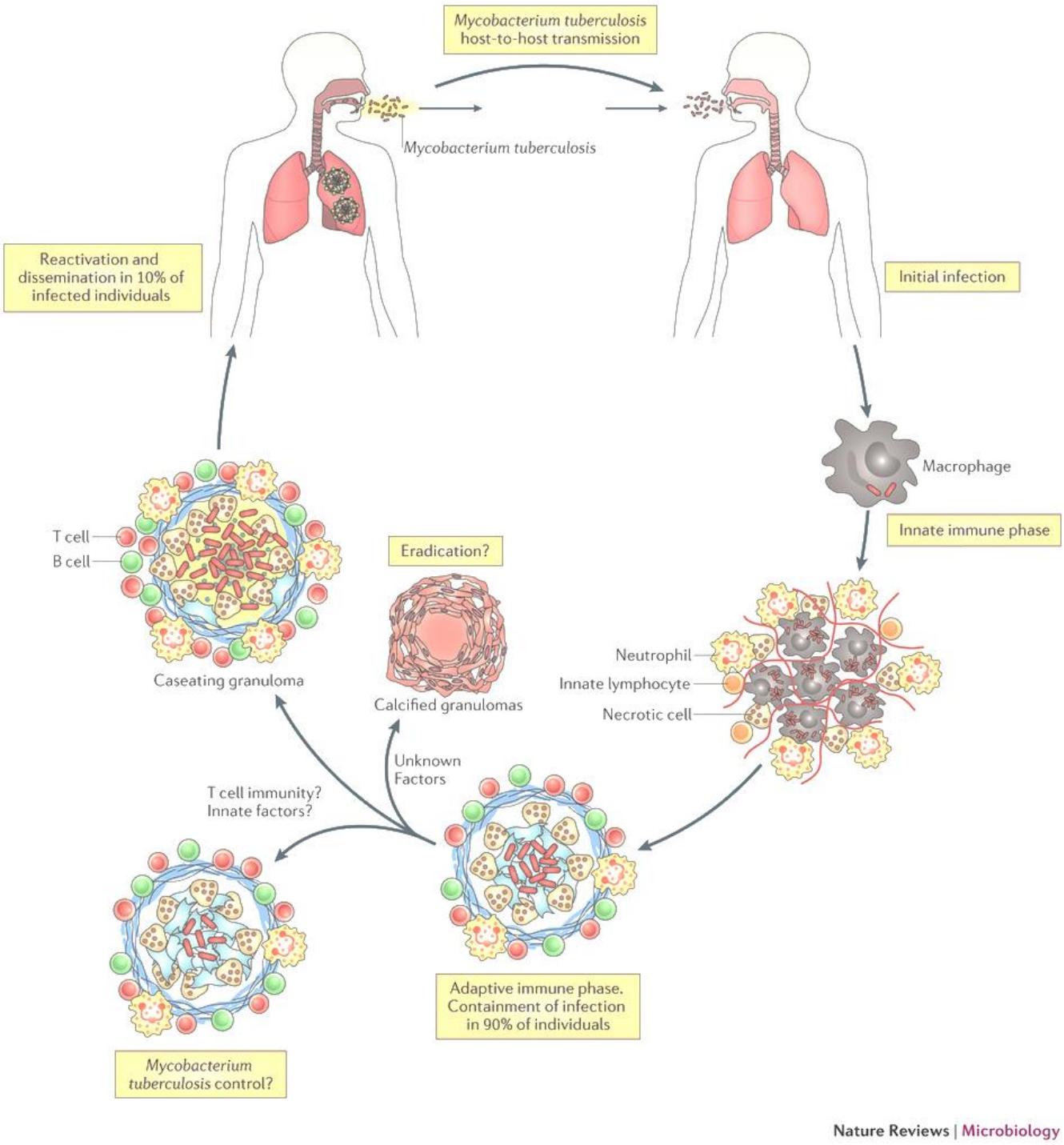
Greek physician and the father of medicine Hippocrates said phthisis, the other name for tuberculosis as “ulceration of lungs, thorax accompanies by cough, fever, and consumption of the body by pus”.[13]

No wonder the disease was also called a consumption disease.

In a study by Sheela V.Shenoi et al in the journal of current HIV reports in 2013 says that “prevention of tuberculosis requires airborne infection control methods and such methods have been ignored and with grave consequences”.[14]

In yet another study by Riley et al published in the American journal of epidemiology confirmed the airborne transmission and its association with quantified exposure risk.[15]


Lipid Rich Cell Wall Of Mycobacterium tuberculosis
Mycolic acids
Arabinogalactan mycolate layer
Peptidoglycan layer
D-arabinose and D-galactose
N-acetylglucosamine and N-acetylmuramic acid
D-glutamic acid, m-diaminopimelic acid, L- and D-alanine
CMN Group: Unusual cell wall lipids (mycolic acids,etc.)
Polypeptides (e.g., PPD)
(Purified Protein Derivative)
Mycolic acids (free lipids, glycolipids, peptidoglycolipids)
Cytoplasm
Cytoplasmic membranes



Pathophysiology:

In a study by parsons et al on the laboratory diagnostic aspects of drug resistant TB states "the main reasons for the requirement of multiple drugs to effectively cure Tb is because of the hydrophobic cell envelope surrounding members of the *Mycobacterium tuberculosis* complex that serves as a permeability

barrier to many compounds; the sequestered, non replicating sub population of TB that is affected by drugs only when the cells reemerge from dormancy; and the drug target or drug activating enzymes in TB that are altered by mutation and result in a population of drug resistant cells".[16]

The primary defense system is the airway itself. The infectious droplets when inhaled are caught in the mucus secreted by the goblet cells of the respiratory tract. The cilia present in columnar epithelium push the trapped bacilli upwards by their movement.

Those bacilli which escape this primary defense mechanism are engulfed by the alveolar macrophages which are present in the lungs. This is the second life of defense. Macrophages are cells which need no previous exposure to get sensitized. The action of macrophages starts a chain of events which result in either containing the infection or the bacilli able to withstand this defense too. At last to be an active disease all depends on this delicate balance, the immunity of the body and the invading bacteria.

Inside the macrophages the bacteria continue to multiply, the macrophages began to release a few immune markers like cytokines and proteolytic enzymes. This attracts the T lymphocytes and marks the cell mediated immunity. This forms the third step of defense.

The end result of this cell mediated immunity is destruction of the lesion (granuloma) into a necrosis, in some cases, the lesion is replaced with fibrosis, while in some cases where the immune system is weak the lesion progresses further to primary progressive tuberculosis.

The TB bacilli is contained within the lesion, which may be activated later when the host immune defenses are weak..this dormant lesion is called latent tuberculosis.

In some cases when the lesion erodes into a blood vessel, the bacteria is carried to distance sites producing extra pulmonary tuberculosis. If it enters the lymphatic system, it reaches the adjacent lymph nodes producing soft cheese like necrosis called caseating necrosis.

Clinical features:

Depends on the stage of tuberculosis

1) **Latent Tuberculosis**:

MTB can be confined, but difficult to eradicate. Patients with latent TB will not have any clinical signs and symptoms. Neither they are infectious. But the bacilli is present in them for long time.in case of immunodeficiency as in case of AIDS,diabetes, steroid therapy etc the disease may get reactivated. Its more common in elderly patients. In a study by kiwanuka et al and lama et al says "coinfection with TB has been associated with an increased replication of HIV-1 and the development of TB disease during HIV infection involves both a reactivation of latent TB and increasingly progressive primary TB"[17,18]

2) **Primary pulmonary tuberculosis:**

This is usually non symptomatic. Only diagnostic tests can detect the disease. However the following findings may be detected in case a patient comes with primary disease: Lymphadenopathy, pleural effusion, dyspnoea, fever.

3) **Primary Progressive Tuberculosis**:

Active disease develops in 5-10% of exposed cases. The clinical features are nonspecific during early stages.The C/F include: malaise, fatigue, loss of weight, evening rise in temperature associated with night sweating. Wasting is due to altered metabolism, loss of appetite. Clubbing due to insufficient oxygenation. The cough is first dry later becomes productive. If any blood vessel becomes ruptured, hemoptysis may occur. Blood tests show anemia and increased leukocyte count.

4) **Extra Pulmonary Tuberculosis**:

It occurs in around 20% of immunodeficient cases. The commonest forms include, TB meningitis, TB spine, miliary TB and lymphatic TB.

In a study by S.K .Sharma and Mohan et al "diagnosing extra pulmonary tuberculosis is very difficult. The recent advances in imaging studies can locate the site of infection .with the help of biopsy its possible to isolate the bacilli from the lesion".[19]

In a study by Sayantan Rat et al

"The main event in causing miliary TB is the massive lympho-hematogenous spread of the bacilli from a focus to multiple organs. The main organs involve liver, spleen and bone marrow. The predilection to these organs is because of many phagocytes in their sinusoidal wall. Miliary TB can be due to

1) Simultaneous reactivation of multiple loci either from primary disease or latent disease

2) Reinfection

The pathogenecity is because of the insufficient T cell response. in this disease, the recruitment of Teff cells fails to provide an adequate level of immunity because of homing of regulatory Treg cells which inhibit teff cells"[20]

In a study by bourdin trunz et al in the lancet edition of April 2006 studied the effect of BCG vaccination in the prevention of TB meningitis and miliary tuberculosis and find that "BCG vaccination is a good cost effective intervention against severe forms of TB such as TB meningitis and miliary TB and it should be retained in high incidence countries as a strategy to supplement the chemotherapy of active tuberculosis".[21]

Multi Drug Resistant Tuberculosis:

Standard anti tuberculosis has been used for many years since the advent of those medicines, however the resistance to them have emerged and is now global..there has been resistance at least one drug in all the countries with tuberculosis.

In a study by dorman et al and gandhi NR et al published in lancet states "recently multi drug resistant TB and extensive drug resistant TB have had devastating effects on populations of HIV infected individuals in developing countries, who ironically had good access to HIV care but dies from some untreatable form of TB".[22]

MDR TB, there is resistance to isoniazid and rifampicin, the first line drugs. The most common cause of MDR TB is faulty treatment. This type of TB can be cured by second line drugs. But these drugs are not easily available. Moreover the

medicines in the second line had to be taken at least for two years consecutively which can reduce patient compliance as well severe side effects. Its to be noted that when starting the primary therapy of first line of drugs patient education is important creating awareness regarding faulting treatment which the can complicate this disease further.

More worse than MDR TB is the XDR TB or extreme drug resistant TB which has high mortality rates.

In the study about half a million people worldwide developed MDR TB in 2013.

HIV and TB:

Statistics of association between HIV and TB have been described above in the statistics. Its noteworthy to mention that the people living with HIV can develop active tuberculosis 26 to 31 times more than normal population. Also Africa "the dark continent" which has has been subject to numerous disease since the origins of mankind is no exception to this association. In 2013 there were 1.1 million cases of TB among HIV patients, 78% of which were from Africa. This may be linked with poor healthcare, AIDS denial ism among many politicians, poverty and malnutrition. The origins of AIDS, HIV have been subject to numerous conspiracy theories and well disputed by reputed scientists all over the world and the disappearance of over a 100 microbiologists in the last decade alone, the treatment for AIDS has been disputed among many main stream scientists.

In studies by klautau GB et al and Murray JF et al in 2005 "HIV mediated immunosuppression impairs granuloma formation, resulting in both ineffective

containment of *Mycobacterium tuberculosis* bacilli and diminished formation of pulmonary cavities" in a study by poprawski D in 2000 "the above mentioned effects cause extra pulmonary disease in HIV-TB subjects".

In further studies by pitchenik AE et al, lee MP et al and colebunders R et al "the HIV-TB manifests with atypical chest findings, greater involvement of lower lobes of the lung and lower concentration of bacteria in sputum".

"The world health organization recommends a 12 component approach of collaborative TB-HIV activities".

In a study by NR Gandhi et al in Lancet says that "MDR tuberculosis is more prevalent than previously realized in this setting. XDR tuberculosis has been transmitted to HIV co-infected patients and is associated with high mortality. These observations warrant urgent intervention and threaten the success of treatment programmes for tuberculosis and HIV".[22]

In another study by Diperri giovani et al in lancet says that "In an investigation of a nosocomial outbreak of tuberculosis, 18 HIV-infected inpatients were found to have been exposed to *Mycobacterium tuberculosis*; active tuberculosis developed in 8, 7 within 60 days of diagnosis of the index case. The patients with lower total lymphocyte and CD4 lymphocyte counts were more likely to get the disease than were those with higher counts. A low score on multiple antigen skin testing was also associated with the development of active tuberculosis. 4 of the 18 patients had a positive tuberculin skin test before exposure to *M tuberculosis*; none of them subsequently got the disease"[23]

In a study by karp et al on co infection with HIV and tropical infectious

diseases states “much of the deadliness of tuberculosis epidemics, especially in sub-Saharan Africa has to do with the virulent synergy between HIV and tuberculosis”.[24]

According to AIDS epidemic update 2009 and WHO global TB control update 2009 “out of the 9.27 million incident cases of TB in 2007,an estimated 1.37 million were HIV positive ;79% of these HIV positive cases were in the African region and 11% were in south east Asia region”.[25]

In a study by Parsons et al in clinical microbiology reviews “an estimated 1.3 million deaths occurred among incident TB cases who were HIV positive. Infection with HIV 1 increases the risk of reactivating latent TB infection by 80 to 100 fold and HIV patients acquire new TB infections have higher rates of TB progression. Tuberculosis can occur at all points in the immunosuppressive spectrum of HIV disease with variable presentations and particularly in high burden countries TB may be the first presentation of HIV disease”.[26]

According to a study by mukadi et al on tuberculosis fatality rates in high hiv prevalence populations in sub Saharan Africa “fatality rates are higher for HIV-TB co infected patients who are on anti-TB treatment but not on ART than for treated TB patients who are HIV negative”.[2\7]

In a study by ackah et al on response to treatment, mortality and cd4 lymphocyte counts in HIV infected patients with tb in abidjan states that “highest death rates occur in co infected patients with lowest CD4 counts”.[28]

According to world health organization

“Latent tuberculosis infection (LTBI) is a state of persistent immune response to stimulation by *Mycobacterium tuberculosis* antigens without evidence of clinically manifested active TB. A direct measurement tool for M. tuberculosis infection in humans is currently unavailable. One-third of the world’s population is estimated to have LTBI: they do not have active TB disease but may develop it in the near or remote future, a process called TB reactivation. The lifetime risk of reactivation for a person with documented LTBI is estimated to be 5–10%, with the majority developing TB disease within the first five years after initial infection. However, the risk is considerably higher in the presence of predisposing factors”

In a study by farba karam et al,

“The identification and treatment of latent TB infection in HIV positive individuals is one of the main recommendations developed by the World Health Organization in order to reduce morbidity and mortality in HIV patients living in high TB prevalence areas. Tuberculin skin testing has been shown to be highly inadequate for identifying LTBI in HIV infected individuals, as the anergy to skin testing may lead to false negative results. More sensitive and specific diagnostic tools are therefore urgently required. The study showed that, EC-ELISPOT (enzyme linked immuno spot) assay appears more sensitive than the TST in HIV infected individuals, but this sensitivity appears impaired in those with severe immuno-suppression.”[29]

Lab Diagnosis :

Research in new diagnostics, treatment and prophylaxis for tuberculosis is

happening in a fast pace. The global plan to stop TB formulated a plan to make these new modalities available to resource less countries and high TB prevalent areas. This organization involved in the adoption, introduction and implemention of these new techniques.

In study by harries AD in 2001 “Up to 20% of all the TB patients in sub Saharan Africa, whom the treatment is initiated die within a year and 2/3 rd of these deaths occur in first 2 months”.[30]

Isn’t this pathetic? This study reveals that the delay in detection of TB due to inadequate diagnostic techniques resulting in patients presenting in advanced stage of the disease.

In a study by Pai M on novel and improved technologies for tuberculosis diagnosis in the journal of clinical chest medicine states that “the WHO stop TB partnership's new diagnostics working group and foundation for innovative new diagnostics (FIND) have classified tools for the diagnosis of TB as the following:

1) WHO endorsed Tools

2) Tools in late stage development or evaluation

3) Tools in early stage development.”[31]

In a study by lange C et al and pai M et al states that “considerable effort and resources have been invested in developing novel diagnostics and improving existing ones”.[32,31]

In studies by madebo T et al &liam CK et al “patients in many countries

with high TB burden do not receive diagnosis for 3-6 months".[33,34]

The world health organization said "although the treatment success rates are improving every year, case detection rates are increasing more slowly".

In the journal of clinical microbiology review linda M parsons et al states "the global awakening to TB's devastating impact on vulnerable populations has been brought about in many countries by its deadly synergy with HIV. In response WHO has provided several key guidance documents for national TB control and laboratory programs for the use of LED microscopes to improve the sensitivity of a turnaround time of front line assay, sputum smear microscopy, and for improving the accuracy of TB detection and drug susceptibility testing through the use of liquid culture and molecular line probe assays"[26]

In a study by madhukar pai in the new tools and emerging technologies for diagnosis of tuberculosis states that "despite this enormous burden (high prevalence of TB) case detection rates are low posing major hurdles for TB control".[31]

In the African continent alone, less than 50% of cases are being detected by the currently adopted DOTS programmes. There are new challenges to this like drug resistance and HIV co infection.

In studies by perkins MD et al & WHO global tuberculosis control report 2010 states that "the dual specters of TB and AIDS have drawn recent attention to the lack of suitable diagnosis for TB"[35]

In study by study by Keeler E et al on reducing the global burden of

tuberculosis states that “TB case detection is the first hurdle towards tackling the TB epidemic”.[36]

In studies by abe C et al, beavis et al, down et al states that “to address the need for rapid and sensitive identification of *Mycobacterium tuberculosis* and other mycobacteria, various genotyping methods for routine diagnosis have been introduced during the past decade”.[37,38,39]

In a study by fisher M on diagnosis of MDR TB states that “Theres a long standing need for new, rapid, accurate and convenient tests for TB diagnosis and drug resistance is currently being addressed by several agencies and group”[40]

In a study by perkins et al in the topic admitting defeat in a journal states that “conventional tests for detecting drug resistance are slow, complicated, expensive and difficult to perform in field conditions”[35]

In a study by Moore DF et al & campos M et al states that “a highly sensitive rule in test can significantly improve the case detection whereas a highly specific rule out test can reduce the turnaround time and the duration of respiratory isolation as well as unnecessary administration of potentially toxic drugs”.[41,42]

In a study by lemaire J et al on advances in the diagnosis of tuberculosis states “despite the recent advances in TB diagnosis, cost and accessibility continue to be the major limiting factors in the effort to eradicated tuberculosis”.[43]

In a study by catharina C.Boehme et al states that “improved technologies that can abbreviate the diagnostic process and facilitate early diagnosis can save patients time and money, decrease morbidity, improve treatment outcomes, and interrupt

the transmission of the disease".[44]

In a study by perkins in the international journal of tuberculosis and lung diseases states that "such technologies need to be more sensitive, faster in yielding results and simpler to use than microscopy"[35] in a study by Pai M et al in the national medical journal of india states "better efforts to control TB require faster and more accurate diagnostic tests".[31]

According to WHO any new technique for diagnosing TB should have the following things to be considered:

- Should be evidence based. Not only should the test be effective in controlled trials but also should perform well in actual field conditions
- The program readiness: the implementation of any technique requires change in national policy and treatment guidelines.
- Turnaround time: results should be faster than existing ones
- cost effective
- quality control measures strictly adhered
- biosafety precautions adopted
- adequate staff training

The currently available diagnostic techniques at a glance

1) Sputum microscopy: its 125 years old technique and remains the most common method used in the diagnosis of TB.

In a study by geojith george et al states that "notwithstanding the advent of

novel diagnostic techniques, smear microscopy remains the most practical test available in resource limited settings, where majority of the TB is present".[45]

Its rapid and requires little training to the technicians and no bio safety levels are required.

In a study by steingart KR on fluorescence vs conventional sputum smear microscopy for TB states that "microscopy is rapid, specific and inexpensive but has low sensitivity"[46] it depends on the bacterial load in the specimens and detects around 50% of all active cases. Sensitivity is very low (20%) in children and HIV patients. The cons include inability to detect resistance to drugs.

In a study by mathew P et al in journal of clinical microbiology and expert review of molecular diagnostics 2010 states that "while cheap and relatively easy to perform, the more than 125 year old smear microscopy method has only modest sensitivity (35-80%) and cannot differentiate between drug sensitive and drug resistant *Mycobacterium tuberculosis*"[47]

In a study by dye et al on evolution of TB control and prospects of reducing TB incidence report "the human immunodeficiency virus epidemic has further diminished the utility of routine microscopy and smear negative TB has arisen as a particular problem in sub Saharan Africa".[48]

The patient has to come twice to the clinic. Once to give the specimen and second to collect the results. This means the transportation costs and the loss of pay.

In studies by foulds J et al in the new tools for diagnosis of tuberculosis and

perkins MD et al in international journal of tuberculosis and lung diseases the limitations of conventional methods of diagnosis including smear microscopy have been explained.[49&35]

In parsons et al on clinical microbiology reviews states "in high incidence countries, TB control relies on passive case finding among individuals self-presenting to healthcare facilities, followed by either diagnosis based on clinical symptoms or lab diagnosis using sputum smear microscopy. Serial sputum specimens are required which means that the people are asked to make repeated visits to the health care center for specimen delivery and collection results. For many patients, the costs of repeated visits to health care facilities are prohibitive and patient dropout is a significant problem"[26]

In a study by squire sb et al in 2005 "in places where HIV is prevalent, the proportion of cases detected by smear microscopy is 20-30%.to address this issue multiple sputum examinations was done due to which a relatively large number of patients do not complete the testing".[50]

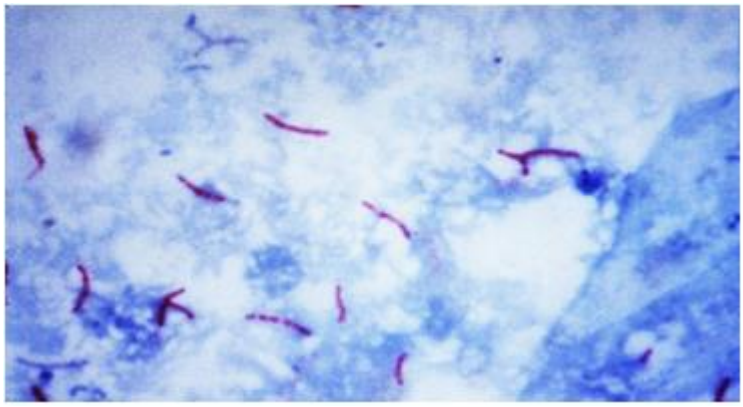
In a study by steingart et al published in lancet "sensitivity of sputum smear microscopy has been reported to vary from 20-80% often depending on the diligence with which specimens are collected ,smears are made and stained smears are examined".[46]

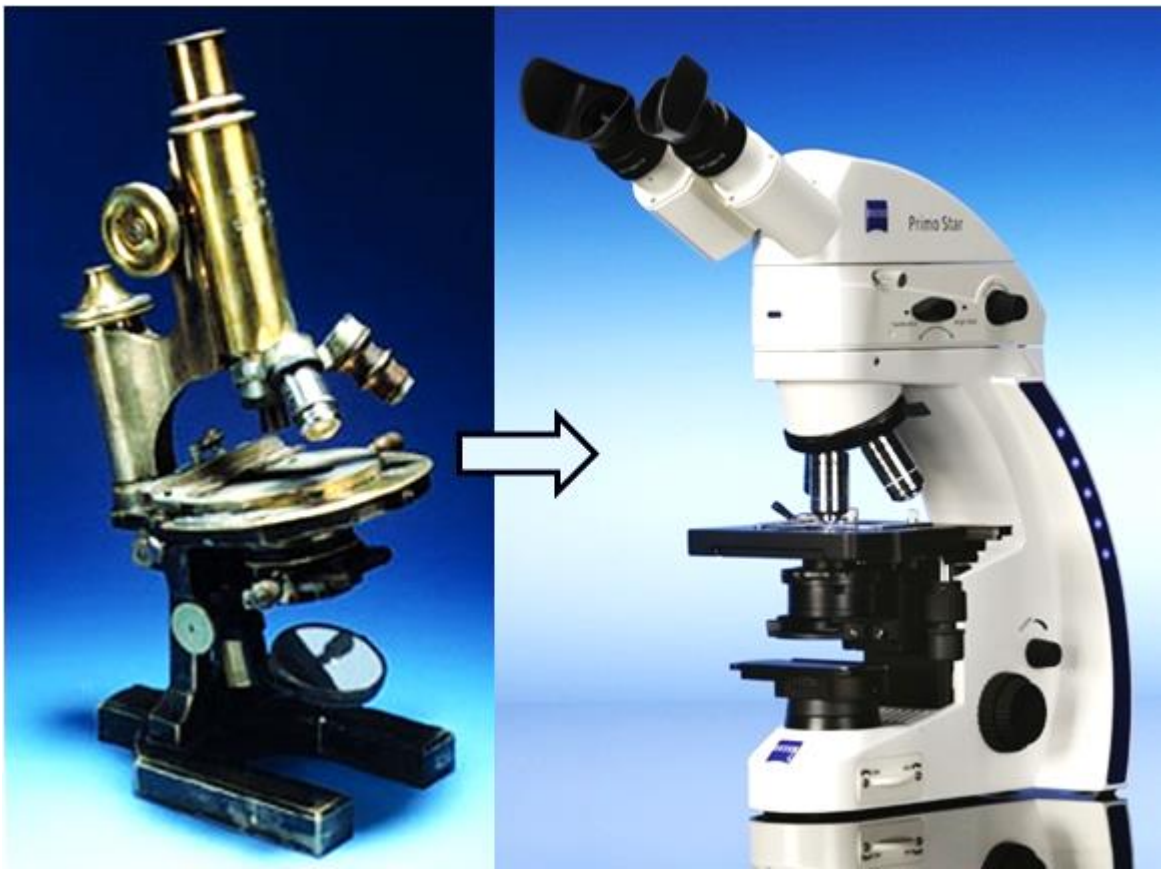
In a study by getahun et al published in lancet states "Tb smear microscopy is highly insensitive for HIV co infected individuals and for children due to the reduced pulmonary bacillary loads in these patients".[51]

In study by harries ad et al in 2003 “more than a third of patients with smear negative TB in Malawi needed more than 6 visits to a health care center for treatment to be initiated”[52]

This is due to the difficulties in confirming smear negative TB, underlines the need the new and simple, yet rapid and effective diagnostic methods to address such cases.

In parsons et al clinical microbiology reviews states “in resource poor countries many smear microscopy labs are single room and insufficiently staffed with poor maintenance of microscopes. These labs don’t have consistent clean water supply and electricity. There are few opportunities for the training of staff and little staff capacity to handle high volume workloads. Quality assurance programs including quality control and external quality assessments are often lacking. This a critical need for new, sensitive, easy and paid point to care diagnostics and also for investments in laboratory infrastructure, quality assurance programs and well trained staff”.[26]





In a study by monkongdee et al states “the who recommendation is two rather than three sputum examinations be used was generated largely for non -HIV patients. However this approach was evaluated in HIV infected patients in Southeast Asia and the incremental yield of microscopy was found to rapidly diminish to 2% after the second smear.[53]

According to who laboratory policy in 2007 “for TB detection two sputum smear are to be collected and examined”.

The above policy was drafted based on a meta-analysis of 37 studies which quanitified the incremental yields of serial sputum specimens.

In a study by mase at al in 2007 states “85.8% of Tb cases were detected with the first sputum specimen. With second sputum specimen, the average

incremental yield was 11.9%, while the incremental yield of third specimen, when the first two were negative was 3.1%"[54]

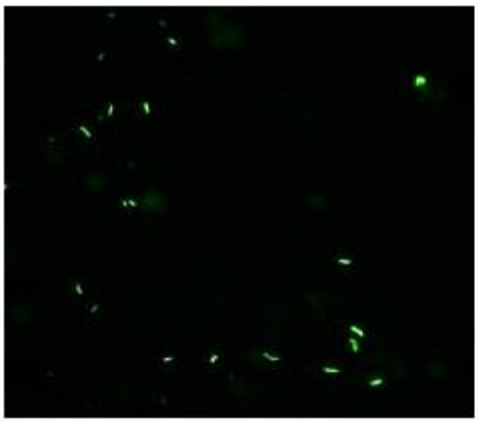
In a study by bonnet et al in 2007 conducted in Kenya shows "decreasing the number of smears examined for detection of TB leads to reduction of patient visits to clinic and lab workload".[55]

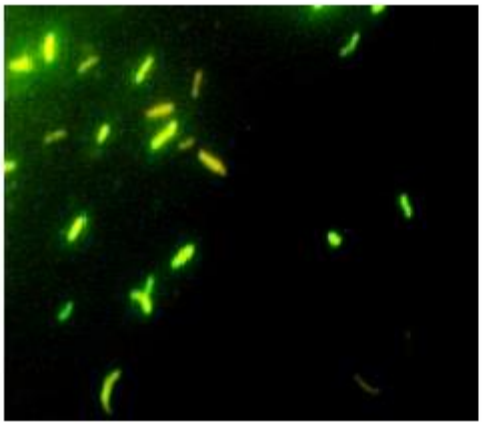
It is to be mentioned that the above reasons are major drawbacks of multiple smear examinations and prevent patient from taking from collecting the results thereby diagnosing TB.

In a study by aziz et al tells "screening of TB patients from three to two specimens should be recommended only in settings with a well-established lab network and a fully functional EQA program for smear microscopy including on site evaluation and follow up training for problem laboratories".[56]

It's a well-known phenomenon that most of the labs in our country don't have quality control assessments.

In a report by world health organization-strategic and technical advisory group for Tuberculosis (STAG-TB) dated 2009 recommends "conventional fluorescence microscopy be replaced by LED microscopy in a phased manner."





In a study by steingarta kr et al "a meta analytical study of 45 studies show that the sensitivity advantage of fluorescence microscopy over conventional microscopy is greater by 10%".[46] Also sputum processing increases the chance of finding TB bacilli my microscopy by 13-33%.

Tb Culture:

It is the gold standard test. Most sensitive method and accommodates DST and speciation. Takes about 2-8 weeks since the TB bacilli is a slow growing organism having a slow generation time of 14-15 hours. Standard method is using

solid LJ media.

WHO STOP TB report 2008 “some national TB programmes in developing countries have no functioning TB culture facility at all. In others its done only in national reference labs and high grade hospitals. Few countries have the capacity for DST”.

Because of the shortcomings, automated liquid systems have been developed.

In a study by william -bouyer n et al “these systems detect the O2 production, CO2 consumption by radiometry, calorimtery, pressure sensors and decrease the time to detection by half”.[57]

Disadvantage if this system includes contamination especially when inexperienced and difficult to examine the colony morphology.

In a study by van kampen sc “the liquid culture systems suffer from important shortcomings as they are prone to contamination, human resource intensive, costly”.[58]

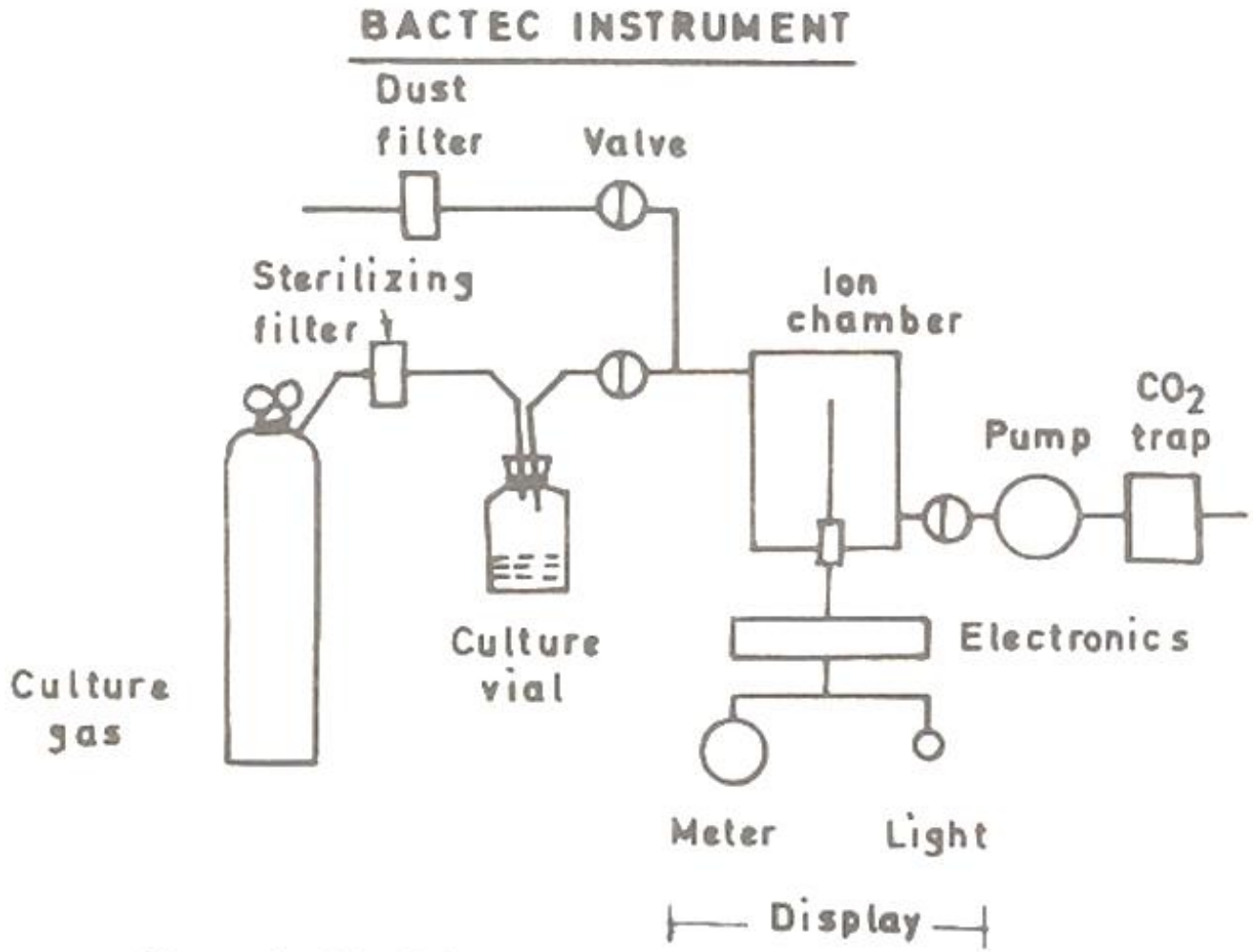


Figure 1. Block Diagram of the principle of operation of Bactec unit.

In studies by kocagoz t etal &baylan o et al “solid media called TK uses a colorimetric method to find the bacterial growth. Its possible to discriminate and differentiate non tuberculous bacteria in this method. IT’S cheap and rapid.

Detection time is 10 days less than conventional LJ media".[59&60]

In study by farnia p et al "a number of redox reagents have been developed to find the bacterial growth in early cultures". This needs further evaluation.[61]

MODS-microscopic observation drug susceptibility:

Manual technique in which the colonies of the culture are examined under a microscope, in this case an inverted microscope is used.

Advantages: DST could be done, inexpensive and could be done in one week time.

Inverted microscope

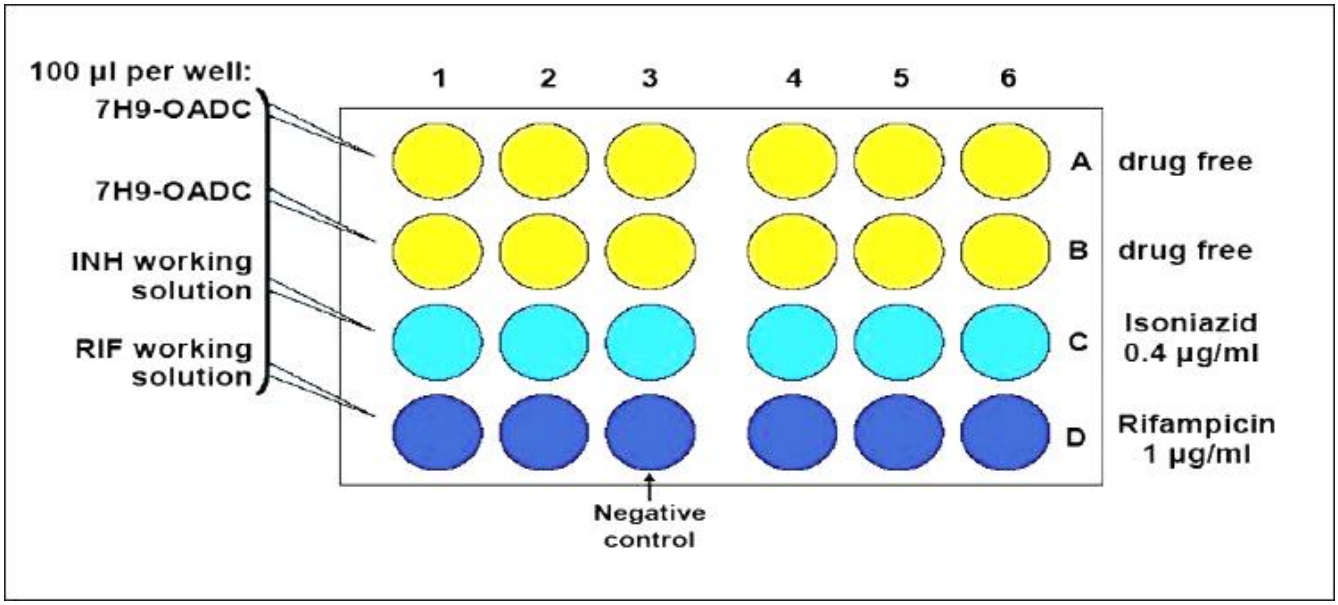
Cons: the technique need training to technicians, bio safety level 2 precautions and difficulty in differentiating between NTM and MTB.

In a study by Moore DA et al "by adding Anti TB antibiotics to adjacent

wells and examining for comparative growth, MODS can be applied for early detection of DST as well".[62]

In a recent study on a large scale carried out in Peru by Moore DA et al "MODS detected 94% of 1908 culture positive cases whereas LJ medium detected only 87%".[62] The average detection time in this method was only 8 days.

This seems to be a promising too.



Septi check AFB method:

In the ICMR bulletin dated 2002 "it is non radiometric method has the unique advantage of simultaneous detection of MTB, NTM and contaminants".[63]

In a study done in USA by isenberg HD et al shows "the system gives a better culture result compared with other methods including BACTEC 460 TB".[64]

Radiometric BACTEC 460 TB method:

14C labeled palmitic acid in 7H12 medium is used. When MTB grows 14C is generated which is measure by the system. The system also identifies MTB as well as can be used for DST.

In a study done in TRC, chennai by Venkatraman Et al shows “rate of isolation of positive cultures was significantly faster with the BACTEC method with 87% of the positives obtained by 7days and 96% by 14 days.DST could be done in 8 days”.[65]

MGIT 960 mycobacteria detection method:

In this method, O2 utilization by MTB is determined by a fluorescent dye in the medium. Its an automated system.

In a study carried out in usa by tortoli et al "this system exhibits a potential for rapid, accurate and cost effective detection of MTB especially in high volume settings"[66]

MB/Bact system:

It's a non-radiometric methods based on the colorimetric detection of Co2.

In a study by rohner et al concludes "this systems is a good alternative to BACTEC 460 despite some disadvantages like contamination".[67]

ESP culture system 2:

Automated system based on the detection of pressure changes in the media in a study by woods et al “it’s a reliable, less labour alternative to BACTEC, however it should not be used as a standalone system”.[68]

Phage based test:

Underlying principle: the ability of phages to infect bacteria has been used. A lytic mycobacteriophage D29 infects the TB bacilli and killed with a virucide.this procedure only kills the virus and not the bacteria inside, later the cells are plated on a lawn of *Mycobacterium smegmatis* producing plaques indicating the disease (TB). The test could be done in days.

In studies by albay a et al, butt t et al "this test detects 29%-87% of smear positive cases and 13-78% of smear negative cases in 2 days".[69,70]

In a study by alcaide F et al "the capacity of D29 phage to replicate in

nontuberculous mycobacteria has not impaired clinical specificity which remained high (99.1%) even in a study in which 30% of all culture isolates were non tuberculous mycobacteria.[71]

This technique requires necessary infrastructure.

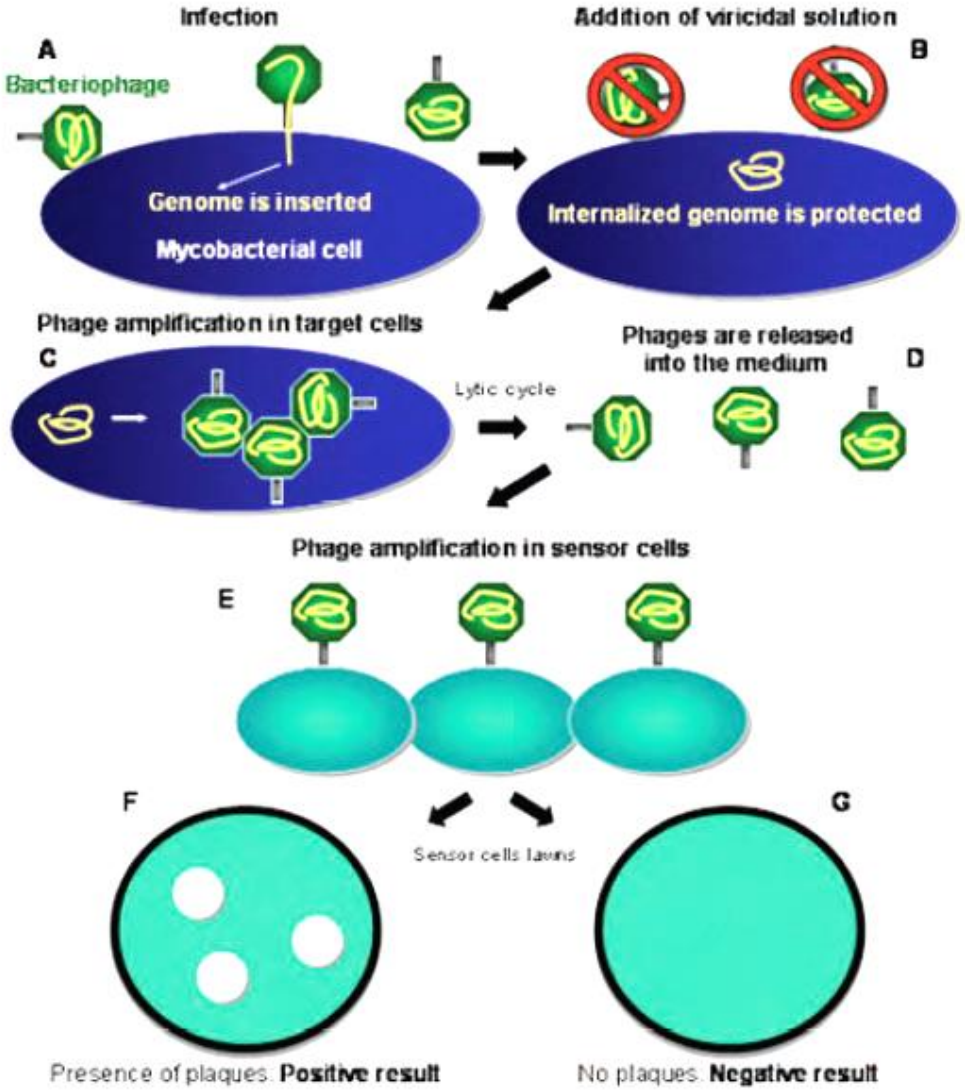


Figure 5. Detection of *M. tuberculosis* by PhaB assay. A. Phages are added to the decontaminated clinical sample. Phages will infect viable mycobacterial cells present in the solution. B. Ferrous ammonium sulphate is added (viricidal solution) to destroy all non-infecting phages. C. Phages begin to replicate leading to cell lysis and release of the new phage particles into the medium (D). E. This solution is added onto a lawn of sensor cells (*M. smegmatis*) to produce plaques (F). In case the sample was negative, no plaques will be present (G).

Immunodiagnosis

In studies by gennaro ML et al, daniel TM et al ,bothamley GH et al, "immune based tests for detection of antigen, antibody and immune complexed

have been reviewed".[70,71,72]

There's no such test available yet to replace smear microscopy and culture. The problem is these test aim at detecting the humoral response rather than the cell mediated immunity which plays a prominent role in TB.

The above mentioned studies have analyzed the reason for failure of such tests.

In studies by Laal S et al "the Tb is characterized by a wide spectrum from latent to active and in each stage different set of antigens are expressed, and even in a single stage the antigens are not the same, for eg: its different from cavitary lesions and the disease with non cavitary lesion"[73]

Hence any serological test should encompass to detect all these antigenic responses. Previously crude antigens were used, which led to poor specificity.

In review by Madhukar Pai et al "the development of recombinant proteins specific to MTB has partially overcome this problem".[74]

Many serological tests employ a single antigen and hence the detection of disease during all stages is less. In the above mentioned studies "the development of a cocktail of antigens specific to each stage of the disease has partially addressed this problem".

Lipoarabinomannan assay:

It's a heat stable glycolipid present in the cell wall of mycobacteria.

Instead of searching for an antibody response specific to an antigen, this

test aims at finding the specific antigen using a capture ELISA method

This method has demonstrated some success. Further evaluation needed.

Interferon gamma release assays (IGRAs):

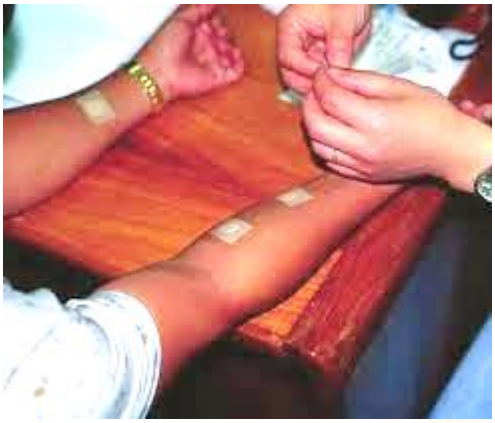
It's a diagnostic immune response Tb test. The standard Mantoux or tuberculin sensitivity test has many limitations. This new tests measures the cell mediated immunity in response to MTB specific antigens which are not present in BCG and NTM there by specific to MTB. Two tests are currently available. The quantiferon TB gold test and the T spot TB test. The best thing is prior BCG vaccination does not give a positive test, thereby useful in screening.

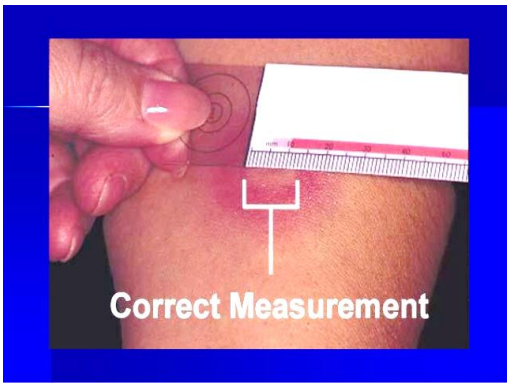
This test is not without limitations. There's no evidence to find which case with progress to active disease based on this test.

MPB64 skin patch test:

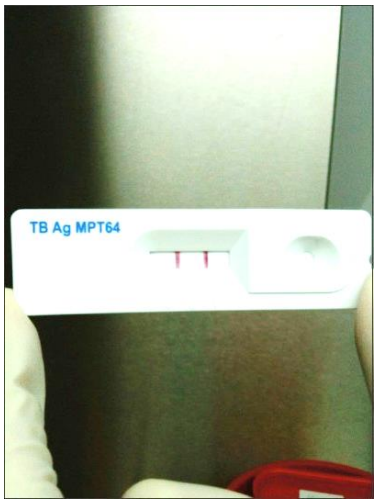
MPB 64 is a Mtb specific protein. In research by Nakamura et al in japan in 1998 "the test was capable of eliciting a distinct response from individuals with active but not latent TB, the sensitivity was 98% and specificity was 100%".[75]

An aqueous solution containing MPB64 is applied to the gauze and over the forearm and measurement made.





Also it could be used in lateral flow assays in differentiating Mtb from NTM. The exact mechanism is unclear.



Molecular methods:

Nucleic acid amplification tests (NAT) are designed to amplify regions specific to MTB complex. They are available as commercial kits. In USA amplicor and MTD are FDA approved. In studies by Piersimoni C et al & Hugget Jf et al "the 3 widely used methods are PCR, transcription mediated amplification (TMA) and strand displacement amplification. These methods show excellent speed and specificity, sensitivity approaching but not equaling culture".[76,77]

In house NAT are predominantly used in research settings. In studies by Brodie et al, Pai M et al "the literature of NAT has been extensively reviewed"

In meta analytical studies by Flores L et al, Pai M et al "the results demonstrate that AT have high specificity for both pulmonary and extra pulmonary TB".[78&31]

In review by Madhukar Pai et al in the journal of expert review on molecular diagnosis "high specificity and positive predictive value confer clear advantages in terms of the tests ability to rule in TB. A positive NAT in a patient with a high pretest probability is fairly confirmatory of TB, particularly in smear positive cases".[79]

It also says "the sensitivity of NAT is lower and highly variable across studies. In many studies, sensitivity estimates have been lower in paucibacillary forms of TB. Therefore a negative test does not rule out TB".

A negative NAAT with a positive smear can be non-tuberculous mycobacteria.

Disadvantages: Cannot differentiate between live and dead bacilli as the DNA can be amplified in both cases.

In a meta analytical study by Flores et al on the accuracy of in house PCR tests "the use of IS6110 as an amplification target and the use of nested PCR were associated with higher accuracy"[79] In the ICMR bulletin dated august 2002 "IS6110 is specific for MTB and is present upto 20 times in its genome, thus offering multiple targets for amplification".[63] The author Madhukar Pai et al in this review concludes that "in general, the concerns about accuracy, reliability and

requirement for necessary infrastructure reduce their applicability in resource limited settings".[74]

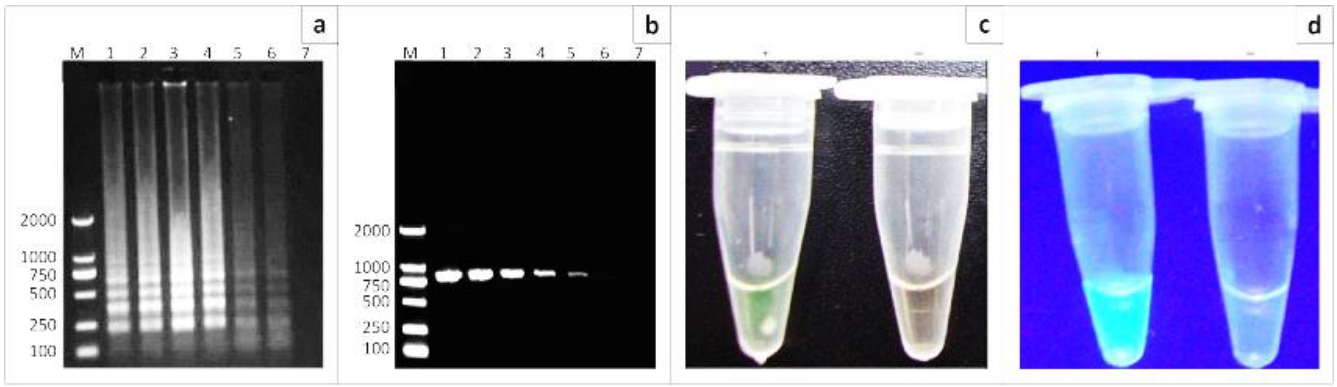
In a study by Suffys P "even in established molecular labs, the performance of NAT is highly variable, implying the need for more robustness".[80]

One such promising technology is the LAMP, loop mediated isothermal amplification. It was invented by Eiken chemicals, japan.

In study by Iwamoto T et al "the advantages of this technology is that it uses 6 specifically designed primers and a single polymerase with strand displacement activity, requires no thermocycler, it's a closed system needing no bio safety levels and gives a visual readout by naked eye".[81]

In study by Boehme C et al "preliminary data suggests that this test can be done in a benchtop by technicians with no molecular training detecting all smear positive specimens and half of the smear negative specimens".[82]

The inventor Eiken in its website claims that "the amplification efficiency is high and DNA can be amplified 10^9 -10^{10} times in 15-60 mins".[83]

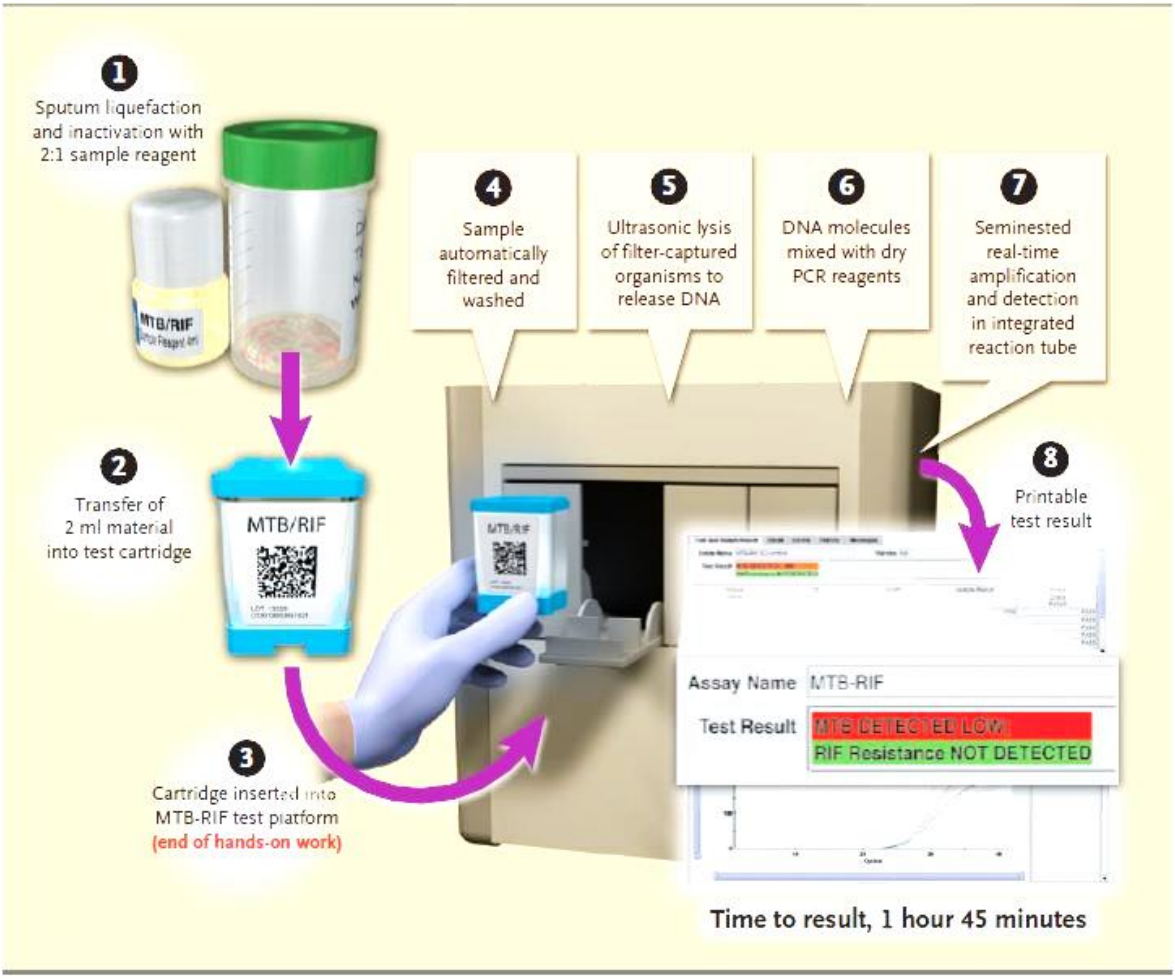


Lane M, DNA marker; Lanes 1–6, 1 × 10^6 CFU/mL, 1 × 10^5 CFU/mL, 1 × 10^4 CFU/mL, 1 × 10^3 CFU/mL, 1 × 10^2 CFU/mL and 1 × 10^1 CFU/mL of *Haemophilus parasuis* used as a reaction template, respectively; Lane 7, negative control; +, positive reaction; –, negative reaction.

FIGURE 1: Detection of (a) *inf*B loop-mediated isothermal amplification (LAMP) and (b) polymerase chain reaction products, as well as visual detection of *inf*B LAMP products under (c) daylight and (d) ultraviolet light.

Gene x pert:

This test detects both MTB and rifampicin resistance. This is a hemi nested PCR

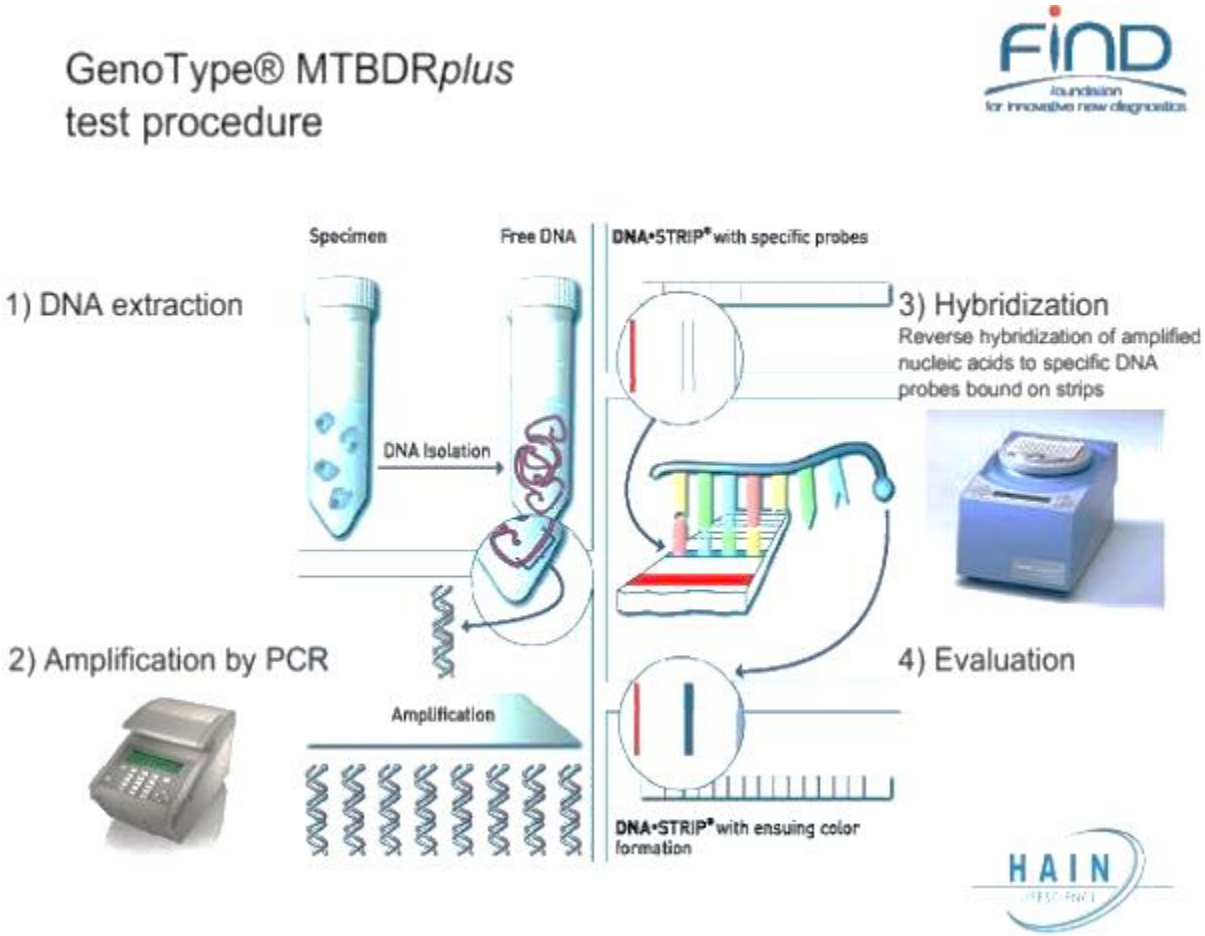


In a study by Helb D, Blakemore R "in this test PCR amplification of five overlapping probes that are complementary to the entire 81 base pair RIF resistance determining region of the MTB rpo B gene and subsequently probes this region for mutations that are associated with Rifampicin resistance".

Disadvantages include high cost. In a review by Mark D Perkins et al "FIND(foundation for innovative and new technologies) is working with cepheid to develop a real time PCR assay for TB on its gene xpert platform that automates sputum processing, DNA extraction, gene amplification and target detection into a single, hands free test".[35]

Line probe assays:

In review by Madhukar Pai et al says “line probe assays are are strip based tests that use PCR and reverse hybridization methods for the rapid detection of mutations associated with drug resistance and detection of MTB complex”.[74]



In a meta analytical study Morgan et al “the line probe assay has high sensitivity and specificity when culture isolates are used, less when directly applied on clinical specimens”[84]

These tests are expensive and need sophisticated labs.

Materials and methodology:

This is a comparative evaluation of three techniques, fluorescence microscopy, culture using solid media and LAMP PCR.

This study is done in Tirunelveli medical college hospital over a period of four months from June 2015 to September 2015

Sample selection: 50 samples randomly collected from the district tuberculosis center, from patients suspected of tuberculosis as referred by expert thoracic physicians in the department of thoracic medicine.

1) Fluorescence microscopy:

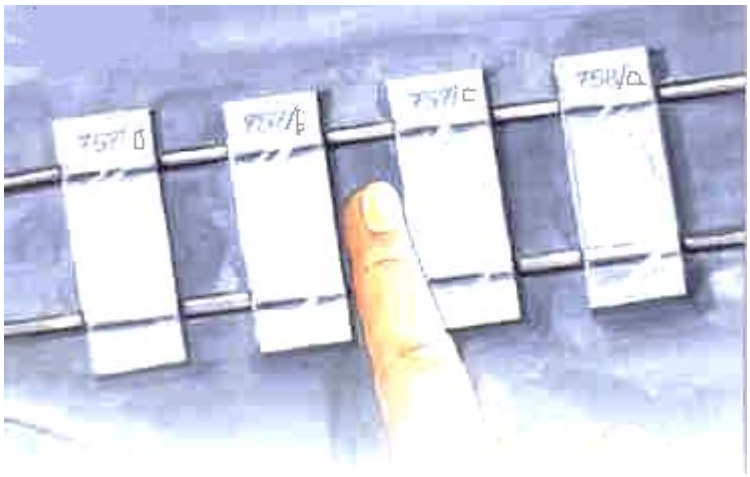
Principle:

Mycobacteria are acid fast.ie. They resist decolorizing even after exposure to acid. In this technique carbol fuschin is replaced with auromine -O. The mycolic acid in the cell wall has an affinity for fluorochromes. The counter stain to differentiate the background is potassium permanganate. The advantage is greater area can be examined under low power microscope thereby enabling a larger area to be examined in a less time.

The mycobacteria appear as yellow luminous rods.

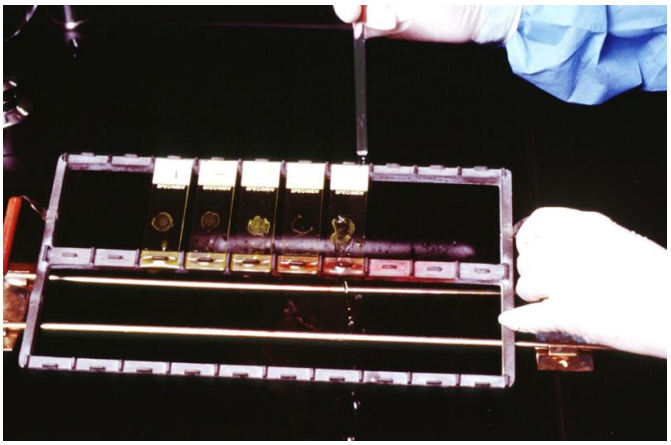
Sputum smear preparation:

Label a new clean slide using a 5mm nichrome sterile loop transfer sputum to the slide smear the slide for an area of 2-3 cm air dry for 15 minutes heat fix by passing over the flame 3-4 times place the smeared slides on the rack

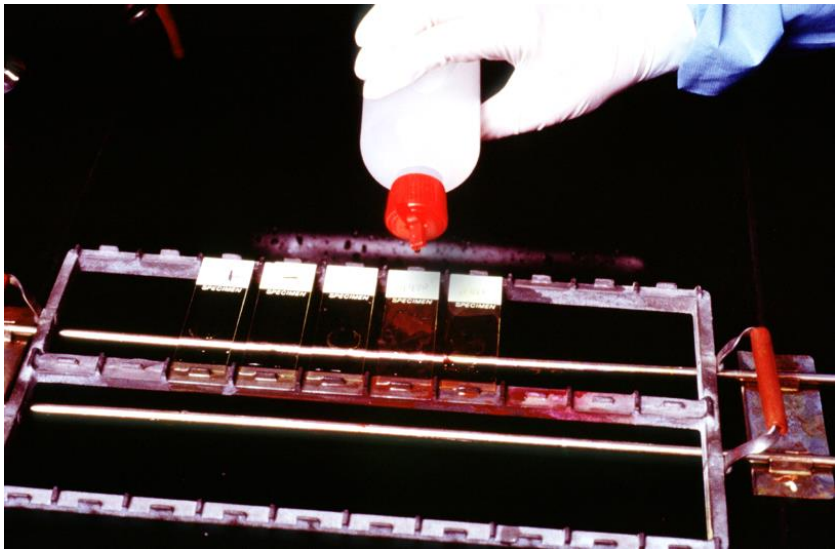


pour auramine phenol over the slides

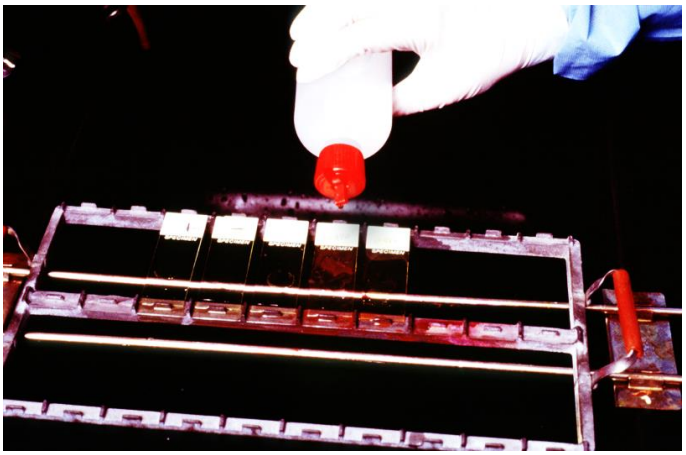
wash with running water



decolorize with acid alcohol for 2 minutes twice



counter stain with KMnO4 for 30 seconds



wash with running water and let them air dry.

The procedure was followed as per TBC India guidelines.

The following reagents were required

1) 3% phenol:

2) Auramine-phenol solution

3) Acid alcohol

Auramine-phenol preparation:

3% phenol 100 ml was warmed to 40ºC

0.3 gm of auramine was added and shaked for 10 minutes

The solution was filtered using whatman No.1 filter paper and stored in a dark glass bottle.

Acid Alcohol preparation

0.5 ml HCL

0.5 gm NaCl

75 ml absolute alcohol

25 ml distilled H20

4)0.1% KMnO4

2) **Culture In Lowen Stein Jenson Media:**

Mucoid or mucopurulent sputum for cases suspected of pulmonary tuberculosis was collected from the district TB center, department of thoracic medicine, tirunelveli.

The specimen was labeled and the laboratory number of each specimen noted.

Strict bio safety precautions were used. N95 mask and gloves were used for

sputum handling.

The specimens were decontaminated by modified petroff's method as per TBC guidelines.

Specimen should be processed as described below:

1.5 ml of sputum in universal container is transferred to centrifuge tube and double the volume of NaOH 4% is added

↓

2. the tubes are inverted and the sputum is mixed well.

↓

3. the tubes are kept in votex shaker for a minute and then in incubator at 37ºC for 15 minutes

↓

4.after 20 minutes, 15 ml of distilled water is added to the tubes

↓

5. centrifuged at 3000 x g for 15 minutes.

↓

6.supernatant fluid discorded in a disinfectant solution(bleach solution)

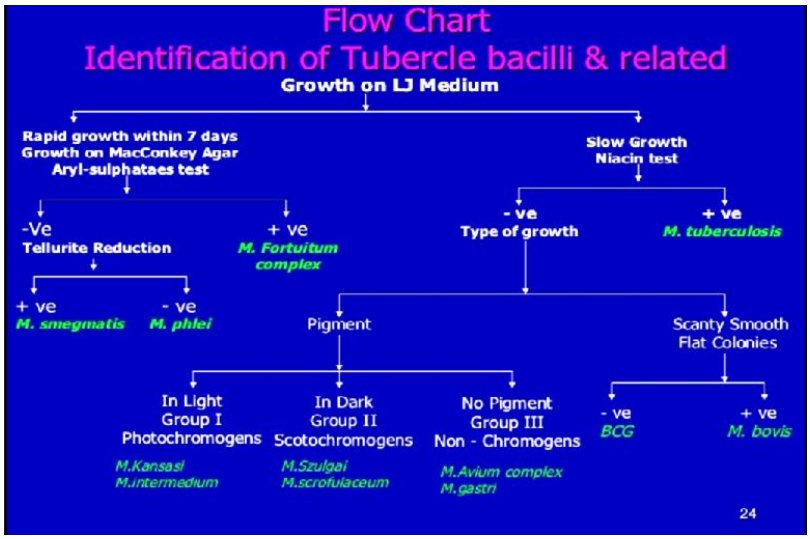
↓

7. another 15 ml distiller water added and centrifuged at 3000 x g for 15 minutes and the supernatant discarded.

↓

8.the pellet is inoculated in 2 slopes of LJ media.

Growth is checked weekly for 8 weeks.



Reading Of Cultures:

The colonies of *Mycobacterium tuberculosis* appear as rough, buff, like bread crumbs and appear 2- weeks after incubation

If the colony has doubtful morphology then Ziehl Neelson staining is done.if no afb is seen then the culture is discarded as contamination.

If smear positive then the following biochemical tests were done

1) Niacin

2) Nitrate

If both were positive, then *Mycobacterium tuberculosis* was confirmed. If niacin is negative then depending on the pigmentation and morphology they were grouped under BCG or *M.bovis* and non-tuberculous mycobacteria.

Flow chart depicting the identification of tubercle bacilli based on growth on LJ medium

LJ media after 3 weeks incubation showing bread crumbs like colonies

centrifuge tube containing pellet

the findings were reported as per below

Reading of culture	report
No growth	Negative
1-100 colonies	positive(number of colonies)
>100 discrete colonies	Positive 2+
Confluent growth	Positive 3+
contaminated	contaminated
<20 colonies of only NTM is one or both slopes	No growth
>20 colonies of only NTM in both slopes	Negative for M.Tb

media was prepared as per annexure 1 attached

freshly prepared lowenstein jenson medium to be inoculated before keeping in

incubation biosafety cabinet for sputum processing and inoculation

3) **LAMP PCR:**

DNA purification procedure:

1) From the pellet prepared by homogenisation and decontamination after inoculation into the LJ slopes the remainingis used.

2) Add 180µl of digestion buffer and 20µl of lysozyme .votex for 10 seconds

3) Incubated at 37ºC for 15 minutes

4) 200µl of binding buffer and 20µl of proteinase K added.mixed well using votex

5) Incubated at 56º C for 15 minutes

6) 300µl of ethanol added and mixed well by inverting several times.

7) Pipette entire sample into the spincolumn. Centrifuged at 10000 rpm for 1 minutes. Discard the flow through and place the column back into the same collection tube.

8) 500µl of wash buffer-1 added to the spin column. Centrifuged at 12000 rpm for I minute and the flow through discarded. Place the column back into the same collection tube.

9) Add 500µl of wash buffer-2 to the spin column .centrifuged at 12000 rpm for 1 minutes and the supernatant discarded. The column is placed back into the same collection tube.

10) Repeat wash buffer-2 wash once

11) Centrifuge the spin column with collection tube at 13000 rpm for 1 minute

12) Discard collection tube and transfer the spin column into a fresh 1.5 ml fresh micro centrifuge tube.

13) Add 100µl of prewarmed elution buffer to the centre of the spin column membrane

14) Incubate for 2 min at room temperature and centrifuge at 13000 rpm for 1 minute.discard the spin column and store the purified DNA at -20ºC.

Materials required:

Pure fast bacteria; DNA mini spin purification, isothermal master mix, SYBR green Dye and primers are from helini biomolecules isothermal master mix contains: reaction buffer, 1.6mM of dNTP mix, 0.5 M of betaine, 6mM of MgSO4,8U of Bst DNA polymerase

Loop isothermal primers:

F3: GGTGAGGTCTGCTACCCA

B3: CGTGAGGGCATCGAGGT

FIP: ATCGCTGATCCGGCCACAGCGTTAGGTGCTGGTGGTCCG

BIP: CGTGGTCCTGCGGGCTTTCAGATGCACCGTCGAACG

***Mycobacterium tuberculosis* IS6110 genes**

TCAGCCGGCGGCTGGTCTCTGGCGTTGAGCGTAGTAGGCAGCCTCGA

GTTCGACCGGCGGGACGTCGCCGCAGTACTGGTAGAGGCGGCGATGG

TTGAACCAGTCGACCCAGCGCGCGGTGGCCAACTCGACATCCTCGATG

GACCGCCAGGGCTTGCCGGGTTTGATCAGCTCGGTCTTGTATAGGCCG

TTGATCGTCTCGGCTAGTGCATTGTCATAGGAGCTTCCGACCGCTCCGA

CCGACGGTTGGATGCCTGCCTCGGCGAGCCGCTCGCTGAACCGGATCG

ATGTGTACTGAGATCCCCTATCCGTATGGTGGATAACGTCTTTCAGGTC

GAGTACGCTTTCTTGTTGGCGGGTCCAGATGGCTTGCTCGATCGCGTC

GAGGACCATGGAGGTGGCCATCGTGGAAGCGACCCGCCAGCCCAGGA

TCCTGCGAGCGTAGGCGTCGGTGACAAAGGCCACGTAGGCGAACCCT

GCCCAGGTCGACACATAGGTGAGGTCTGCTACCCACAGCCGGTTAGGT

GCTGGTGGTCCGAAGCGGCGCTGGACGAGATCGGCGGGACGGGCTGT

GGCCGGATCAGCGATCGTGGTCCTGCGGGCTTTGCCGCGGGTGGTCCC

GGACAGGCCGAGTTTGGTCATCAGCCGTTCGACGGTGCATCTGGCCAC

CTCGATGCCCTCACGGTTCAGGGTTAGCCACACTTTGCGGGCACCGTA

AACACCGTAGTTGGCGGCGTGGACGCGGCTGATGTGCTCCTTGAGTTC

GCCATCGCGCAGCTCGCGGCGGCTGGGCTCCCGGTTGATGTGGTCGTA

GTAGGTCGATGGGGCGATCGGCACACCCAGCTCGGTCAGCTGTGTGC

AGATCGACTCGACACCCCACCGCAA

Reaction Mix

components	Volume
Loop Isothermal Master Mix	10µl
Primer Mix	5µl
Purified DNA	5µl
Final volume	20µl

Negative control and Positive control are included in every run.

Centrifuged and incubated at 65°C for 1hour.

Visualization

1µl of 10X SYBR Green dye added to each PCR tube and gently mixed by vortex and placed over UV transilluminator and illumination are compared with Negative control and interpreted.

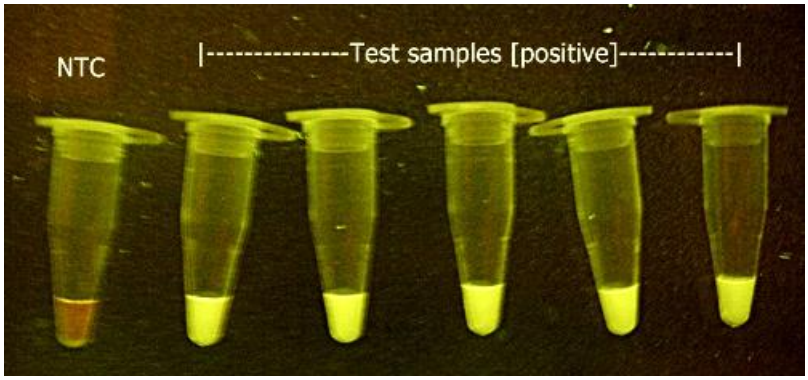
MTB Lamp Primer:

GGTGAGGTCTGCTACCCA

CGTGAGGGCATCGAGGT

ATCGCTGATCCGGCCACAGCGTTAGGTGCTGGTGGTCCG

CGTGGTCCTGCGGGCTTTCAGATGCACCGTCGAACG

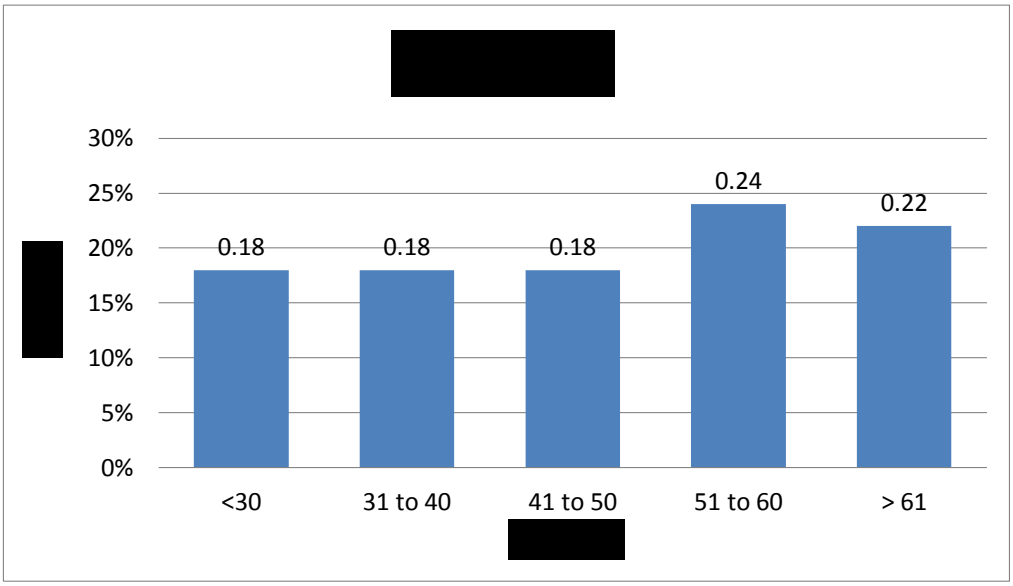


Results:

Table 1- age wise distribution:

Age	No of Subjects	Percentage
<30	9	18%
31 to 40	9	18%
41 to 50	9	18%
51 to 60	12	24%
> 61	11	22%

Graph 1

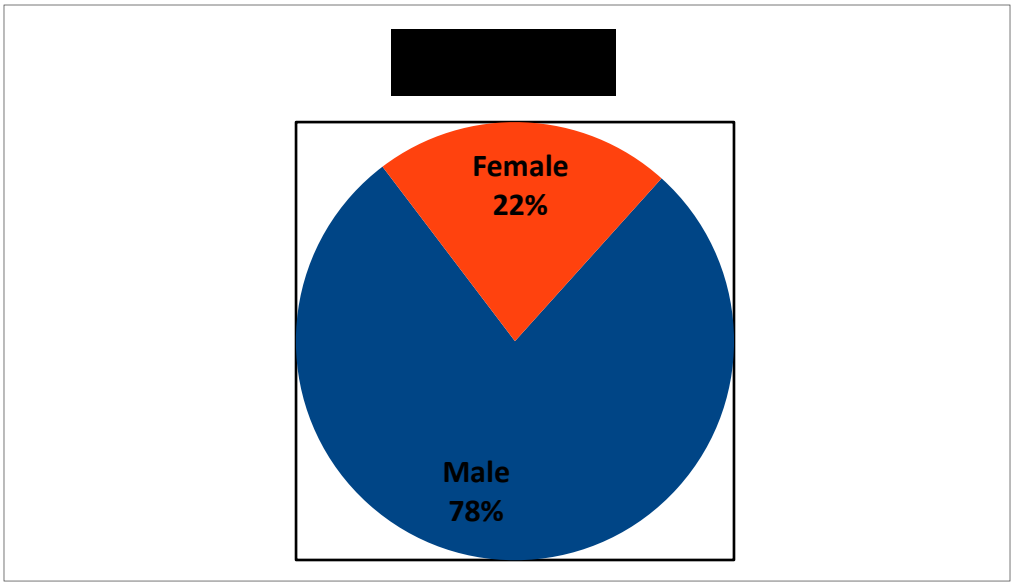


In the present study 18% of the study population was below the age of 30, 18% between 31-40, another 18% between 41-50, 24% of the cases were between 51-60 and 22% of the cases were more than 61 years of age.

Table 2-Gender wise distribution:

Sex	No
Male	39
Female	11

Graph 2:

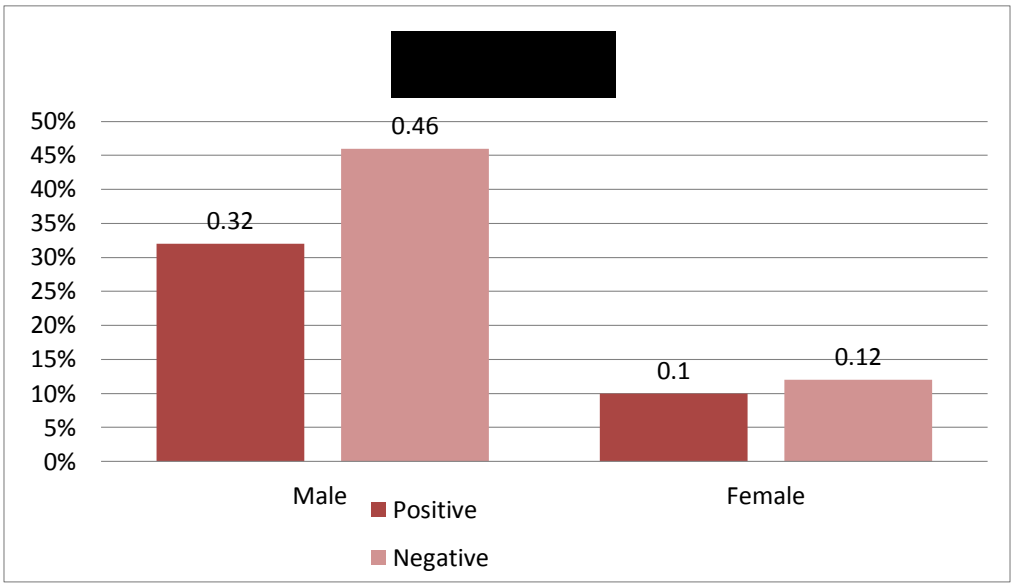


In the present study among 50 samples collected , 39 (78%) were males and 11(22%) were females.

Table 3-Culture results:

Culture		
	Positive	Negative
Male	32%	46%
Female	10%	12%

Graph 3:

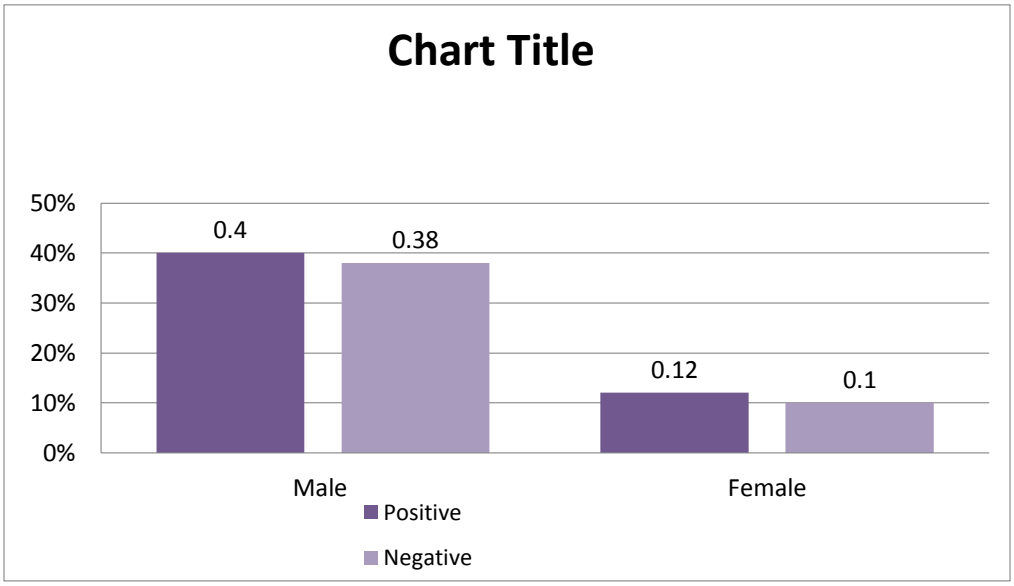


In the present study culture was positive in 42% of the subjects and negative in 58%.

Table 4-PCR results:

Lamp PCR		
	Positive	Negative
Male	40%	38%
Female	12%	10%

Graph 4:

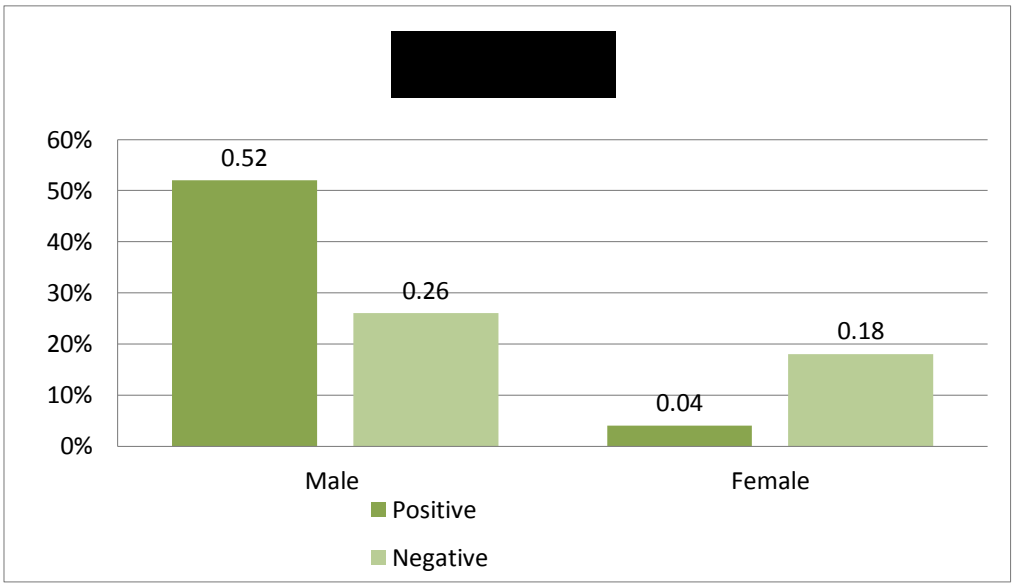


LAMP PCR was positive in 52% of the study population and negative in 48% cases.

Table 5-Smear results:

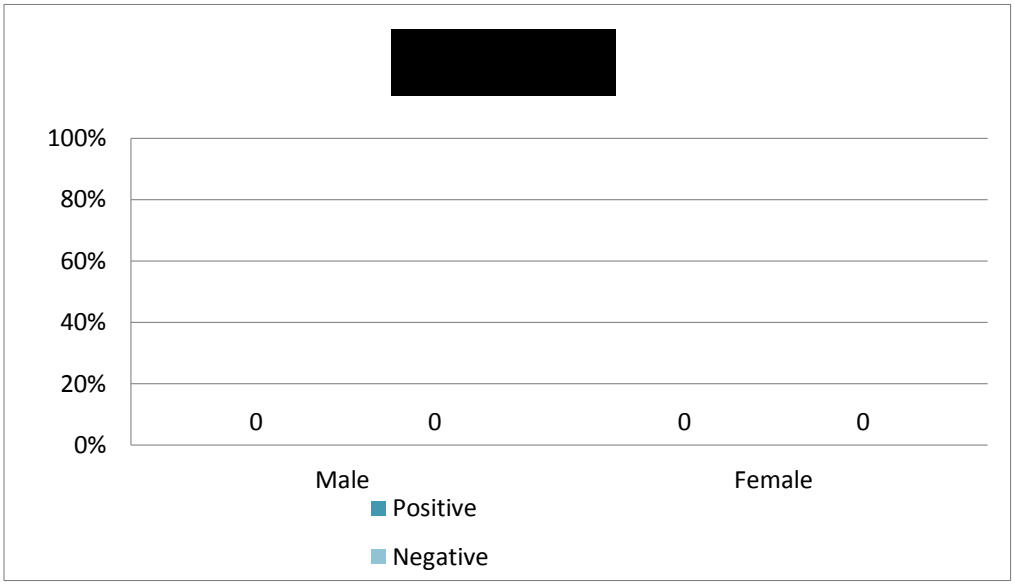
Smear		
	Positive	Negative
Male	52%	26%
Female	4%	18%

Graph 5:



by fluorescence microscopy was positive in 56% of the subjects were as negative in the rest 44%.

This is the sample graph for reference:



Sample table		
	Positive	**Negative**
Male	**0%**	**0%**
Female	**0%**	**0%**

Table -6comparison of smear

with culture:

		Culture	
		Positive	Negative
Smear	Positive	13	15
	Negative	8	14

Table 7-sensitivity and specicity of smear -fluoresence microscopy

Mc Nemar Test	Kappa Agreement	9.7Z%	
P value	P value	0.474	
0.21			

When compared with the gold standard “culture in solid media” the smear microscopy using LED fluorescence showed sensitivity of 62% and specificity of 48%

Positive predictive value was 46%

Negative predictive value was 64%

p value in McNemar test is 0.21

kappa agreement is 9.72%

p value for kappa agreement is 0.474

Table-8 comparison between PCR and culture:

		Culture	
		Positive	Negative
PCR	Positive	20	6
	Negative	1	23

Table 9-sensitivity and specificity of PCR:

Statistic	Formula	Value	95% CI
Sensitivity	$\frac{a}{a+b}$	95.24%	76.18% to 99.88%
Specificity	$\frac{d}{c+d}$	79.31 %	60.28% to 92.01%
Positive Likelihood Ratio	$\frac{Sensitivity}{100-Specificity}$	4.60	2.24 to 9.45
Negative Likelihood Ratio	$\frac{100-Sensitivity}{Specificity}$	0.06	0.01 to 0.41
Disease prevalence	$\frac{a+b}{a+b+c+d}$	42.00% (*)	28.19% to 56.79%
Positive Predictive Value	$\frac{a}{a+c}$	76.92% (*)	56.35% to 91.03%
Negative Predictive Value	$\frac{d}{b+d}$	95.83 % (*)	78.88% to 99.89%

Mc Nemar Test	Kappa Agreement	72%
P value	P value	<0.0001
0.125		

The sensitivity of the LAMP PCR was 95.24% , specificity being 79.31% with a confidence interval of 95%

Positive predictive value was 76.92%

Negative predictive value being 95.83%

McNemar test P value :0.125

Cohen's kappa :72%

Kappa agreement P value:<0.0001

True positives: 20

True negatives:23

False positive:6

False negative:1

DISCUSSION:

As tuberculosis is one of major causes of death and is preventable if taken appropriate measures. The foremost is detecting the disease in an early stage and initiating treatment thereby preventing its spread. The earlier methods of "sanatorium", isolation have been proved inadequate when compared with antibacterial treatment in preventing the spread.

In this study we compare LAMP PCR & fluoresce microscopy with culture using Lowenstein Jenson media in 50 clinical specimens (sputum) collected from cases suspected of tuberculosis for the diagnosis of *Mycobacterium tuberculosis.*

The laboratory diagnosis of tuberculosis chiefly relies on smear microscopy. The Ziehl Neelson method of acid fast staining is routinely carried out and the RNTCP has phased the fluorescence microscopy as an alternative method whereby the time to examine more fields can be reductionis is because the low magnification used to examine the fields, thereby examining more fields in a single sitting. The time consumed by fluorescence microscopy to examine 100 fields is 2 minutes by experienced personnel.

However the technique has limitations in that its sensitivity and specificity are less and identification of species is restricted which makes the need for a better technique with same robustness, speed, cost effective with no additional expenses for manpower training and bio safety levels which can penetrate the rural and inaccessible areas of the TB endemic areas to find the TB bacilli and thereby initiating treatment.

In a study by Mathew P et al in journal of clinical microbiology and expert

review of molecular diagnostics 2010 states that “while cheap and relatively easy to perform, the more than 125 year old smear microscopy method has only modest sensitivity (35-80%) and cannot differentiate between drug sensitive and drug resistan*t Mycobacterium tuberculosis*”[47]

In a study by Steingarta KR et al “a meta analytical study of 45 studies show that the sensitivity advantage of fluorescence microscopy over conventional microscopy is greater by 10%”.[46]

The culture is a gold standard, useful in identification of species as well as high sensitivity and specificity, it has shortcomings in that it needs 8 weeks’ time. In this study we compare LAMP PCR & fluoresce microscopy with culture using Lowenstein Jenson media in 50 clinical specimens (sputum) collected from cases suspected of tuberculosis for the diagnosis of *Mycobacterium tuberculosis.*

The sensitivity of the smear microscopy in this study is 62%.this is in accordance with earlier study by Mathew P et al in which “the sensitivity of the smear microscopy is 30-80%”[46]

It should also be noted that fluoresce microscopy improves the detection rate by another 10%.

The 62% sensitivity rate of the smear microscopy in this study indicates better lab practices, experienced technicians and the high prevalence of TB in this area.

In the study Geojith et al says “Smear microscopy detects the morphology of the bacilli while culture methods differentiate based on the physiology of the organism”. [45]

LAMP detects the presence of DNA in the specimen whether live or dead the organism may be.these two techniques are complementary to each other in the sense LAMP detects the genetic material more when the smear and culture results are positive.

In this study LAMP shows an excellent sensitivity of 95% which is in higher than earlier study by geojith et al in which the sensitivity was 79.5% .earlier studies showed better specificity.

This is in accordance with our study.

In a study by Pal N sharma et al in the indian journal of pathology "78.65% specimens were culture positive when processed within 48 h by the NaOH method. The culture positivity in the same specimen that were stored with cetylpyridinium chloride and processed after 7-8 days was 70.22%, whereas those stored without CPC and processed by the NaOH method was 46.62%"[85].

In a study by catherine Boehme et al "The sensitivity of LAMP in smear- and culture-positive sputum specimens was 97.7% and the sensitivity in smear-negative, culture-positive specimens was 48.8%".[82]

The sensitivity of the above study is higher than the present study.

In a meta analytical study by daphne I, madhukar pai et al "commercial NAAT techniques are highly variable, the sensitivity is inconsistent with the specificity. The study further holds that based on the observation its not recommended for NAAT to be replaced for the conventional tests in the present situation".[86]

It is to be mentioned that a study should have a higher sensitivity and specificity. It it has lower specificity then more persons who dont have disease are

identified to have disease and further investigations are ordered or treatment started which is detrimental to patients health and unnecessary expenditure. According to WHO report on the use of nucleic acid amplification tests for the diagnosis it states that in one large study the sensitivity was only 53%.

In a study by olga l et al on the Assessment by Meta-Analysis of PCR for Diagnosis of Smear-Negative Pulmonary Tuberculosis [87] Sensitivity ranged from 9-100% while the specificity ranged from 25 to 100%.

In a study by lydia kivihya et al on the Comparison of PCR with the Routine Procedure for Diagnosis of Tuberculosis in a Population with High Prevalences of Tuberculosis and Human Immunodeficiency Virus[88]

"The sensitivity and specificity of PCR were 93 and 84%, respectively. HIV status did not affect the sensitivity of PCR. A total of 99.7% of the true smear-positive and 82.1% of the true smear-negative TB patients were correctly identified by PCR. PCR detected M. tuberculosis in 11.7% of the culture-negative suspects, 60% of which had one or two PCR-positive sputum specimens".

This indicates that the PCR technique can be applied to identify smear negative pulmonary tuberculosis

In a study on application of loop mediated isothermal amplification (lamp) assay as an alternative diagnostic test for rapid tuberculosis diagnosis in limited resource setting

Sensitivity of LAMP was 97.47% , the specificity being 60.31. the higher sensitivity means this test could be used as an alternative for smear microscopy in

TB endemic countries like india for rapid and cost effective diagnosis.

In a study by mitarai ,okumura et al on the evaluation of a simple loop-mediated isothermal amplification test kit for the diagnosis of tuberculosis [89]

The sensitivity of LAMP using raw sputum in smear- and culture-positive specimens was 98.2% while the sensitivity in smear-negative, culture-positive specimens was 55.6% (95%CI 43.4–68.0).

The test says that the sensitivity of lamp was not lower than the commercial amplicor assays for tuberculosis.

In a study by basu dev pandey et al "Using this system, a total of 200 sputum samples from Nepalese patients were investigated. The sensitivity of MTB-LAMP in culture-positive samples was 100% (96/96), and the specificity in culture-negative samples was 94.2% (98/104, 95% confidence interval 90.5–97.9%). [90]

In the study by geojith G et al on the efficacy of LAMP in lab detection of MTB the sensitivity was 91.7% and specificity was 90.9% in 2 hour format.[45]

In the present study the incubation time was 1 hour.increase in incubation time increases the sensitivity of the test as per the above mentioned study.The need for modification of protocols to give a better effficacy needs to researched in further studies.

In a study by ehsan aryana et al " the accuracy of the LAMP method was tested using restriction endonucleases enzymes to digest the byproducts of LAMP

and then visualised using agarose gel electrophoresis"[91]

This study confirms the accuracy of the LAMP reaction.

Recently another study by lee et al 2009,

One technique called reverse transciption loop mediated isothermal amplification combined with enzyme linked immunosorbent hybridisation (RT-LAMP-ELISA) was used to target the 16S ribosomal RNA.the test detected one copy of 16S RNA per reaction .this is comparable with the IS6110 based MTB LAMP assay. This technique however is costly compared with LAMP MTB .[92]

The cost of single LAMP reaction in our study is Rs.500-/-.whereas the technique mentioned above could cost upto 660/reaction.

If both smear and PCR are positive in clinical specimens then the diagnosis of tuberculosis is assumed.

However in studies by ru-yi zhua et al on the LAMP test correctly identified the MTB DNA (M.tuberculosis, M.bovis and BCG strains) & did not detect the non tuberculos mycobacteria and other bacteria. This shows the specificity of this test.[92]

In the study by ehsan aryana et al shows identical sensitivities were obtained for LAMP and nested PCR, but the LAMP assay was more rapid and cost-effective than the latter.

The p value of our study is <0.0001 which is highly significant.

Summary:

The present study aimed at detection of *Mycobacterium tuberculosis* in clinical specimens from patients suspected of tuberculosis with history of fever, cough with expectoration for 2 weeks ,loss of weight, apettite and were referred for lab diagnosis of *Mycobacterium tuberculosis* by expert thoracic physicians from the department of thoracic medicine, tirunelveli medical college.all the sample were collected within a time of 2 months, decontaminated and homogenised by modified petroff's method using TBC guidelines.fluorescence microscopy was done on unprocessed samples and their results collected, however the test is a blinded study and the results were compared at the end of the study.

The processed specimens were then inoculated in LJ media , freshly prepared using a standardized method and the rest of pellet was used to detect MTB by loop mediated isothermal amplification.the results were compared at the end of the study.the results of the study were analysed and summarised below

The age wise percentage of patients included in the study:

18% of the study population was below the age of 30,

18% between 31-40,

18% between 41-50,

24% of the cases were between 51-60 and

22% of the cases were more than 61 years of age.

50 samples were randomly selected from the regional tuberculosis center

of which

22% of the study population were females and the rest 78% were males.

Culture was positive in 42% of the subjects and negative in 58%.

Graph 4 shows PCR was positive in 52% of the study population and negative in 48% cases.

Graph 5 shows fluorescence microscopy was positive in 56% of the subjects were as negative in the rest 44%.

False negative was seen in one sample. which may be due to contamination.

False positive was seen in 6 samples

True positive in 20 samples&

True negatives in 23 samples.

Fluoresence microscopy using auromine staining when compared with the gold standard "culture in solid media" shows

Sensitivity of 62%

Specificity of 48%

Positive predictive value was 46%,

Negative predictive value was 64%,

P value in McNemar test is 0.21,

Kappa agreement is 9.72%,

P value for kappa agreement is 0.474.

Comparison of LAMP PCR with gold standard technique which is culture in solid LJ media shows

Sensitivity of the LAMP PCR was 95.24%,

Specificity being 79.31%,

Positive predictive value was 77%,

Negative predictive value being 96%

P value for Mcnemar test 0.125

P value for kappa agreement was <0.0001,

Kappa agreement was 72%

HIV infected samples were too low and hence were neglected for consideration also there was no relevance in the positivity rate for male and female patients age wise distribution of the samples did not bear any specific outcome for the study.

The P value is highly significant with 95% sensitivity rate.

Conclusion:

LAMP PCR can be used for detection of mycobacterium tuberculosis comples in sputum samples with a high degree of sensitivity and moderate levels of specificity.

In culture and smear analagous samples the sensitivity is higher. The important advantage of LAMP is its robustness, speed in detecting the organism.the detection time is one hour and needs little training to be done

However whether its an active or latent infection needs clinical findings and other assays like Interferon gamma release assays.

When used in conjunction with smear microscopy the results are good. A large study and subsequent research is required for its implementation as an accepted methodology of diagnosis.

BIBLIOGRAPHY

1) Carl Zimmer, “tuberculosis is newer than thought, study says”, new york times 21.08.2014

2)"Robert Koch."World of Microbiology and Immunology.Ed. Brenda Wilmoth Lerner and K. Lee Lerner. Detroit: Gale, 2006. Biography In Context. Web. 14 Apr. 2013.

3)Corbett El, Watt CJ, Walker N et al. the growing burden of Tb: global trends and interactions with the HIV epidemic. Arch of internal medicine 163(9), 1009-1021(2003).

4) WHO.global TB control, surveillance, planning and financing. WHO report 2005.1-247(2005)

5)http://www.tbcindia.nic.in/key.html

6) Nicholas (September 15, 1998),"Scientist at Work/Kary Mullis; After the 'Eureka', a Nobelist Drops Out",The New York Times.

7) http://www.tbfacts.org/tb-india/

8)The Significance of the Tuberculin Skin Test in Elderly Persons by William W Stead in annals of internal medicine 1987;107(6);837-842.

9)Exposure of emergency department personnel to tuberculosis: PPD testing during an epidemic in the community by Peter E Sokolovo published in annals of emergency medicine 1994 Sep;24(3):418-21.

10) Kolappan C, Gopi PG. Tobacco smoking and pulmonary tuberculosis. *Thorax* 2002; 57: 964-6.

11) www.who.int/tb/publications/global_report/gtbr14_main_text.pdf

12) Wirth T et al “origin, spread and demography of *Mycobacterium tuberculosis* complex”

PLoS Pathog. 2008 Sep; 4(9).

13) Pease, Arthur Stanley (April 1940). "Some Remarks on the Diagnosis and Treatment of Tuberculosis in Antiquity".*Isis*31(2): 380–393.

14) Transmission of Drug-Susceptible and Drug-Resistant Tuberculosis and the Critical Importance of Airborne Infection Control in the Era of HIV Infection and Highly Active Antiretroviral Therapy Rollouts Clin Infect Dis. 2010 May 15; 50(Suppl 3): S231–S237.

15) Riley EC, Murphy G, Riley RL. Airborne spread of measles in a suburban elementary school. Am J Epidemiol. 1978;107: 421–432

16) Parsons et al -lab diagnostic aspects of drug resistant tuberculosis front.biosci 9:2086-2105

17) Kiwanuka et al -effect of HIV type 1 on disease progression in persons from rakai, uganda with incident HIV-1 infection ,journal of infectious diseases 197:707-713; 2008

18) Lama et al 2007-host factors influencing susceptibility to HIV infection and AIDS progression. journal of retrovirology 4:57

19) SK Sharma and Mohan et al on extra pulmonary TB in Indian J Med Res.2004 Oct;120(4):316-53.

20) Diagnosis and management of miliary tuberculosis: current state and future perspectives

the journal of Clin Risk Manag. 2013; 9: 9–26.

21) Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness by Bourdin Trunz published in Volume 367, No. 9517, p1173–1180, 8 April 2006

22) NR Gandhi et al on Multidrug-resistant and extensively drug-resistant tuberculosis: a threat to global control of tuberculosis published in lancet 2010 May 22;375(9728) .

23) Diperri Giovanni nosocomial epidemic of active tuberculosis among hiv-infected patients Volume 334, No. 8678-8679, p1502–1504, 30 December 1989

24) Karp et al in oxford journal of clincial infectious diseases study on Coinfection with HIV and Tropical Infectious Diseases. II. Helminthic, Fungal, Bacterial, and Viral Pathogens volume 45 issue 9, Pp.1214-1220

25) AIDS epidemic update 2009 and WHO global TB control update 2009

26) Parsons et al on the lab diagnosis of TB in resource poor countries -challenges and opportunities published in clinical microbiology reviews April 2011, p 314-350

27) Mukadi Y., Perriens J. H., Louis M. E. St., Brown C., Prignot J., Willame J. C., Pouthier F. et al. Spectrum of Immunodeficiency in HIV-1-Infected Patients with Pulmonary Tuberculosis in Zaire. Lancet.1993;342:143–46

28) Ackah et al on response to treatment, mortality and cd4 lymphocyte counts in HIV , lancet 1995 Mar 11;345(8950):607-10.

29) Farba Karam et al on a study on Sensitivity of IFN-γ Release Assay to Detect Latent Tuberculosis Infection Is Retained in HIV-Infected Patients but Dependent

on HIV/AIDS Progression in PLoS ONE. 2008; 3(1): e1441.

30) Harries A. D., Hargreaves N. J., Gausi F., Kwanjana J. H., Salaniponi F. M. High Early Death Rate in Tuberculosis Patients in Malawi. International Journal of Tuberculosis and Lung Disease.2001

31) Pai et al on Novel and improved technologies for tuberculosis diagnosis: progress and challenges.Clinics in Chest Medicine 30: 701-716,2009

32) Lange C, Mori T. Advances in the diagnosis of tuberculosis. Respirology.2010;15:220–240.

33) Madebo T, Lindtjorn B. Delay in treatment of pulmonary tuberculosis: an analysis of symptom duration among Ethiopian patients.MedGenMed 1999:E6.

34) Liam CK, Tang BG. Delay in the diagnosis and treatment of pulmonary tuberculosis in patients attending a university teaching hospital.Int J Tuberc Lung Dis 1997; 1:326–32.

35) Perkins MD on facing the crisis: improving the diagnosis of tuberculosis in the HIV era in journal of infectious diseases 2007,196:S15-27

36) Reducing the global burden of tuberculosis: the contribution of improved diagnostics.

By Keeler et al in Nature.2006 Nov 23;444 Suppl 1:49-57.

37) Detection of *Mycobacterium tuberculosis* in clinical specimens by polymerase chain reaction and Gen-Probe Amplified *Mycobacterium tuberculosis* Direct Test by Abe c et al *J Clin Microbiol. 1993 Dec; 31(12):3270-4.*

38) Evaluation of Amplicor PCR for direct detection of *Mycobacterium tuberculosis* from sputum specimens by Beaves et al in *J Clin Microbiol. 1995*

Oct; 33(10):2582-6.

39) Detection of *Mycobacterium tuberculosis* in respiratory specimens by strand displacement amplification of DNA. By Down et al in *J Clin Microbiol. 1996 Apr; 34(4):860-5*

40) Fisher M et al in Expert Rev Mol Diagn.2002 Mar;2(2):151-9.on Diagnosis of MDR-TB: a developing world problem on a developed world budget.

41) Moore DF, Guzman JA, Mikhail LT. Reduction in turnaround time for laboratory diagnosis of pulmonary tuberculosis by routine use of a nucleic acid amplification test. Diagnostic microbiology and infectious disease.2005;52:247–254

42) Campos M, Quartin A, Mendes E, Abreu A, Gurevich S, et al. Feasibility of shortening respiratory isolation with a single sputum nucleic acid amplification test.American journal of respiratory and critical care medicine.2008;178:300

43) Lemaire J, Casenghi M. New diagnostics for tuberculosis: fulfilling patient needs first.Journal of the International AIDS Society.2010;13:40

44) catherine boehme in a study on Operational Feasibility of Using Loop-Mediated Isothermal Amplification for Diagnosis of Pulmonary Tuberculosis in Microscopy Centers of Developing Countries□J. Clin. Microbiol. June 2007vol. 45no. 61936-1940

45) Geogith George et al on Comparison of the Efficacies of Loop-Mediated Isothermal Amplification, Fluorescence Smear Microscopy and Culture for the Diagnosis of Tuberculosis in PLoS One. 2011; 6(6): e21007

46) Steingkart KR in Lancet Infect Dis.2006 Sep;6(9):570-81. A study on

Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review.

47) Mathew P et al in journal of clinical microbiology and expert review of molecular diagnostics 2010

48) Dye et al on evolution of TB control and prospects of reducing TB incidence JAMA. 2005 Jun 8;293(22):2767-75

4

Master Sheet

specimen number	lab number	name	age	sex	diagnosis	follow up	results A	results B	net smear result		HIV status	date of specimen collection	culture results		Lamp PCR results	
1	3304	murugeshwari	40	female	yes	no	1	2	positive	1	not available	29.06.2015	positive	1	positive	1
2	2997	Ganapathy	64	male	no	yes	1+	2+	positive	1	neg	08.06.2015	positive	1	positive	1
3	2960	sudalai	41	male	yes	no	2+	2+	positive	1	non reactive	05.06.2015	negative	2	negative	2
4	2962	ganeshan	38	male	yes	no	3	3	positive	1	non reactive	05.06.2015	positive	1	positive	1
5	2948	kannan	48	male	no	yes	2		positive	1	non reactive	04.06.2015	negative	2	positive	1
6	2929	chelladurai	56	male	no	yes	1		positive	1	not available	03.06.2015	positive	1	positive	1
7	3009	selvam	45	male	yes	no	1	1	positive	1	neg	09.06.2015	negative	2	negative	2
8	2954	thomas	40	male	yes	no	3		positive	1	neg	09.06.2015	positive	1	positive	1
9	2935	kanagaraj	27	male		yes	3	3	positive	1	neg	04.06.2015	negative	2	negative	2
10	3031	chelladurai	35	male	yes	no	sc4	sc6	positive	1	positive	10.08.2015	negative	2	negative	2
11	2944	henry	63	male	yes	no	3	3	positive	1	neg	04.06.2015	negative	2	positive	1
12	2966	baskar	28	male	no	yes	3	not available	positive	1	not available	05.06.2015	positive	1	positive	1
13	3032	kumar	40	male	no	yes	neg	sc5	positive	1	neg	10.06.2015	negative	2	negative	2
14	2980	mahalingam	55	male	yes	no	sc12	not available	positive	1	not available	06.06.2015	positive	1	positive	1
15	2987	senthilvel	65	male	no	yes	3	3	positive	1	not available	06.06.2015	positive	1	positive	1
16	3054	subbiah	63	male	yes	no	1	2	positive	1	neg	11.06.2015	positive	1	positive	1
17	3008	sakthivel	42	male	yes	no	3	3	positive	1	neg	09.06.2015	positive	1	positive	1
18	2986	manirusamy	55	male	yes	no	sc12	not available	positive	1	neg	06.06.2015	negative	2	positive	1
19	2939	chandiran	51	male	yes	no	1	3	positive	1	neg	04.06.2015	positive	1	positive	1
20	3062	pavithra	35	female	yes	no	neg	neg	negative	2	neg	02.06.2015	negative	2	negative	2
21	2996	rathinam	50	female	yes	no	neg	neg	negative	2	neg	08.06.2015	negative	2	positive	1
22	2904	karuppasamy	27	male	yes	no	3	3	positive	1	neg	02.06.2015	negative	2	positive	1
23	2905	sankaran	58	male	no	yes	neg	neg	negative	2	neg	02.06.2015	negative	2	positive	1
24	2906	jeyaraj	8	male	yes	no	neg	neg	negative	2	neg	02.06.2015	positive	1	positive	1
25	2907	muthusamy	70	male	yes	no	neg	neg	negative	2	neg	02.06.2015	positive	1	positive	1
26	2908	paramisvam	67	male	yes	no	neg	neg	negative	2	neg	02.06.2015	positive	1	positive	1
27	2909	meeraselvam	26	male	yes	no	2	2	positive	1	neg	02.06.2015	negative	2	negative	2
28	2910	chellamal	51	female	yes	no	neg	neg	negative	2	neg	02.06.2015	positive	1	positive	1
29	2994	vadivu	46	female	yes	no	neg	neg	negative	2	positive	06.06.2015	positive	1	positive	1
30	2911	samudrayan	71	male	yes	no	neg	neg	negative	2	neg	02.06.2015	positive	1	negative	2
31	2912	rajesh	26	male	yes	no	neg	neg	negative	2	neg	02.06.2015	negative	2	negative	2
32	2913	kalimuthu	55	male	yes	no	neg		negative	2	neg	02.06.2015	positive	1	positive	1
33	2914	guruvammal	70	female	yes	no	1	1	positive	1	not available	03.06.2015	positive	1	positive	1
34	2993	mariappan	30	male	yes	no	1	1	positive	1	positive	06.06.2015	positive	1	positive	1
35	2915	kalyani	40	female	yes	no	neg	neg	negative	2	not available	03.06.2015	positive	1	positive	1
36	3007	lakshmi	55	female	yes	no	neg	neg	negative	2	not available	08.06.2015	negative	2	negative	2
37	3010	nainar	58	male	yes	no	1	2	positive	1	not available	09.06.2015	negative	2	negative	2
38	3011	subramaniam	37	male	yes	no	neg	neg	negative	2	positive	09.06.2015	negative	2	negative	2
39	3012	ponnuvel	43	male	yes	no	neg	neg	negative	2	not available	09.06.2015	negative	2	negative	2
40	3021	bakiyanathan	48	male	yes	no	1	2	positive	1	neg	09.06.2015	negative	2	negative	2
41	3023	selvi	27	female	yes	no	neg	neg	negative	2	positive	09.06.2015	negative	2	negative	2
42	3015	sudalai	65	female	yes	no	neg	neg	negative	2	neg	09.06.2015	negative	2	negative	2
43	3027	sanmugapandi	56	male	no	yes	neg	neg	negative	2	neg	09.06.2015	negative	2	negative	2
44	3028	vellapandi	75	male	yes	no	neg	neg	negative	2	neg	10.06.2015	negative	2	negative	2
45	3024	ganeshan	56	male	yes	no	1	2	positive	1	neg	09.06.2015	negative	2	negative	2
46	3026	mani	48	male	yes	no	3	3	positive	1	neg	09.06.2015	negative	2	negative	2
47	2955	kulandhai	35	male	yes	no	neg	neg	negative	2	neg	05.06.2015	negative	2	negative	2
48	2930	beema	28	female	yes	no	neg	neg	negative	2	neg	03.06.2015	negative	2	negative	2
49	2940	paramisvam	55	male	yes	no	neg	neg	negative	2	not available	04.06.2015	negative	2	negative	2
50	3003	ponnusamy	69	male	yes	no	3	3	positive	1	not available	08.06.2015	negative	2	negative	2