SPUTUM MICROSCOPY AFTER UNIVERSAL SAMPLE PROCESSING (USP) TO DIAGNOSE SMEAR NEGATIVE PULMONARY TUBERCULOSIS UNDER RNTCP

BY

DR P SAVITHA *MBBS*

DISSERTATION SUBMITTED TO

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UNDER THE GUIDANCE OF

DR S.R.PRASAD *MD*

PROFESSOR

DEPARTMENT OF MICROBIOLOGY
SRI DEVARAJ URS MEDICAL COLLEGE, KOLAR
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DATE: SIGNATURE OF THE GUIDE

PLACE: KOLAR **DR S.R.PRASAD**

MD

PROFESSOR

DEPARTMENT

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DR S.R.PRASAD *MD*

PROFESSOR,

DEPARTMENT OF MICROBIOLOGY.

SEAL & SIGNATURE OF THE HOD

DR BEENA P.M

DATE:
PLACE: KOLAR

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TO BE SUBMITTED TO THE

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Date: *Signature of the Candidate*

Place:Kolar *DR P.SAVITHA*

LIST OF ABBREVATIONS

ATT - Anti tubercular Therapy

AFB - Acid Fast Bacilli

DOTS - Directly Observed Short Course Chemotherapy

IUALTD – International Union against Tuberculosis and Lung Disease

LJ - Lowenstein-Jensen

MDR - Multi Drug Resistant

MTB - Mycobacterium tuberculosis

RNTCP – Revised National Tuberculosis Control Programme

RSC - Rapid Slide Culture

WHO - World Health Organization

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Abstract

Background

Tuberculosis (TB) is a major health problem in developing countries like India. Even today Ziehl-Neelsen (ZN) staining is the main stay to diagnose tuberculosis at the RNTCP centers. Though ZN staining is a simple procedure, easy to train the staff to perform the test and also to identify the Acid Fast Bacilli (AFB) it has a low sensitivity. There have been many attempts made to increase the sensitivity of the tests for the early diagnosis and treatment of pulmonary tuberculosis.

A study conducted by Chakravorty et al. in 2005 claimed that a new technique – Universal Sample Processing (USP) method was more sensitive when compared to conventional direct smear microscopy and also could detect AFB in samples which were negative by direct smear microscopy. Hence we conducted this study to evaluate the USP technique to detect AFB when compared to the direct smear microscopy under RNTCP setup.

Materials and methods

Sputum samples (spot and early morning) were collected from 1000 patients with suspected tuberculosis visiting the two RNTCP centers situated at two different hospitals at Kolar.

The sputum samples collected were subjected to direct and USP smear microscopy. The USP method consists of processing the sample with chemicals like guanidinium hydrochloride (a chaotropic agent which is commonly used in molecular Microbiology), Tris chloride, EDTA, Sarkosyl and Beta mercaptoethanol. The

chemically processed samples were subjected to centrifugation at 6000 rpm (rotations per minute) for 20 minutes. The smear was made from 10% of the final sediment formed which was stained later by ZN method. The samples which were positive for AFB were put up for culture on Lowenstein-Jensen (LJ) medium and the mycobacterial growth was confirmed by niacin test. To detect whether there was any difference in the time taken to identify the AFB in the direct and USP smear microscopy, the positive smears were subjected to screening by two RNTCP technicians and the time taken was noted.

Results

Among the sputum samples processed, 704 (70.4%) were from SNR hospital and 296 (29.6%) from R.L.Jalappa hospital, Kolar. Of the samples collected which were processed by Direct smear microscopy 641 (64.1%) were from male patients with 66 (10.2%) positive for AFB and 359 (35.9%) were from female patients with 28 (7.7%) positive for AFB.

All the sputum samples were subjected to direct smear microscopy and also USP technique. The samples which were positive for AFB by direct smear microscopy 94 (9.4%) were also positive by USP smear microscopy 94 (9.4%). None of the samples which were negative by direct smear microscopy turned out to be positive by USP smear microscopy. The USP smear microscopy showed AFB clumped together against a clear background in contrast to the direct smear microscopy where AFB was seen distributed in the field on a backdrop of blue counter stain. The USP smear microscopy could not be graded as the AFB were seen clumped together.

Of the samples processed by USP method 94 were positive for AFB. Only 40 (42.55) smear positive samples grew on LJ media which were later confirmed by niacin test.

Contamination was seen in 23 (24.46%) of the samples inoculated onto LJ medium. Fungal contamination [14 (60.86%)] was predominant, most commonly seen in 2nd week followed by bacterial contamination [9 (39.1%)], seen in first week.

There was no difference in the time taken to detect the AFB by direct and USP smear microscopy as screened by the two Revised National Tuberculosis Control Programme (RNTCP) technicians.

Conclusion

Our study showed that USP technique did not offer any additional advantage over direct smear microscopy for the detection of AFB in the diagnosis of pulmonary tuberculosis. The USP method did not detect any positives from the sample which were negative by direct smear microscopy. Also the USP method showed low positive culture rate and high contamination rate. Thus USP smear microscopy is not an efficient test to be followed under RNTCP set up.

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1. Introduction

Tuberculosis is an ancient disease causing immense damage to the human race from time immemorial. It has been and continues to be a major public health problem in India & other developing countries[1]. With urbanization, industrialization, overcrowding facilitating its spread, the emergence of HIV infection and also the spread of multi drug resistant (MDR) TB, the situation of tuberculosis has worsened to such an extent that it has been declared a global emergency[2].

Tuberculosis caused by the acid fast bacilli (AFB), Mycobacterium tuberculosis (MTB) infects one third of the world's population. Pulmonary tuberculosis is the most common condition caused by MTB however it can affect any organ system in the body. It usually gets transmitted by air-borne droplet nuclei, produced by patients who are open cases of pulmonary tuberculosis. Therefore an important step to prevent this infection is to diagnose early and treat the patients appropriately.

Clinical diagnosis of TB has always played an important role in the control of TB even before the discovery of its causative agent. Hence, the diagnosis of TB was for a very long time, only 'clinical' and such a diagnosis was made mainly to identify and isolate, and to a small extent to attempt a cure of such patients. Robert Koch opened a new avenue in TB diagnosis, which along with the discovery of the anti -TB drugs, has given new hopes to put an end to the reign of this dreaded disease.

'Early diagnosis & treatment' was found to be an effective way to reduce the spread of TB. It is one of the mainstays in the strategy of TB control throughout the world even today, as evidenced by the World Health Organization (WHO) & Revised National Tuberculosis Control Programme (RNTCP) recommendations. Today the techniques of diagnosis have reached such an advanced stage that, even as research in

TB diagnosis continues at a rapid pace, advancing the techniques to new heights, the practical applicability of most such advanced techniques is a challenge for the TB control programmes in developing countries. In India, RNTCP runs the TB diagnostic tests to lakhs of patients annually. Hence, the programmes need a test which has good sensitivity and specificity, as well as being cost effective.

Conventional TB diagnosis mainly depends upon the bacteriological examination. Even today Ziehl-Neelsen staining remains the main stay to detect the AFB and to diagnose tuberculosis, which is recommended by RNTCP. Direct smear microscopy has a poor sensitivity of 60%– 70%, and detects AFB only at concentrations of 10,000 bacilli per mL of the sample[3]. Methods to improve the smear microscopy by using fluorescent microscopy, bleach method, LED microscopy have been explored. However, smear microscopy after USP technique is claimed to detect AFB as low as 250 -300 bacilli per mL[4]. Here we conducted a study to evaluate the utility of ZN staining after Universal Sample Processing (USP) technique on the sputum samples collected from two RNTCP centers, one situated at Sri Narasimha Raja Wadeyar (SNR) hospital and the other at R.L.Jalappa hospital, Kolar for the detection of AFB.

2. Objectives

1. To compare the detection rate of microscopy done after USP with that of direct microscopy.

2. To perform microscopic examination after subjecting the sputum to Universal Sample Processing (USP) methodology on sputum samples of patients found to be negative by direct smear microscopy under RNTCP.

3. The positives detected by the above method will be confirmed by culturing Mycobacterium on LJ medium.

3. Review of literature

3.1 History

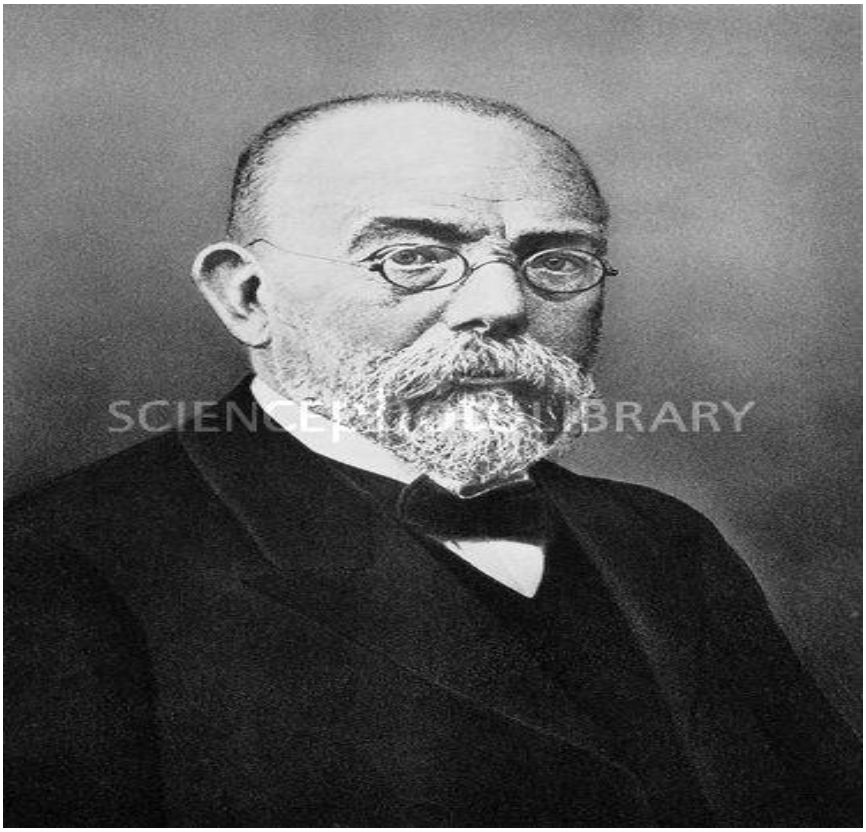
In India tuberculosis is a common disease caused by Mycobacterium tuberculosis, an acid fast bacillus. The disease tuberculosis has been present in the human population since antiquity. The evidence for the existence of this disease has been found since 4000BC, by the skeletal examination of the mummies with spinal tuberculosis and from Egyptian tomb paintings [5]. The first reference to tuberculosis in Asian civilization is found in the Vedas. The oldest of them (Rig-Veda, 1500 BCE) calls the disease *yaksma*[6]. One of the prayers says 'God give me a life without tuberculosis'[7]. The Atharvaveda calls it by another name: *balasa*. The first description of scrofula is given in Atharvaveda.

Tuberculosis may have emerged as a human disease during Neolithic times when human populations increased aggregated and cattle were domesticated. Examination of a skeletons dating back to fourth millennium BC, which were excavated from Arene Candida Cave in Liguria, Italy, demonstrated the evidence of tuberculosis [8].

Around 460 B.C Hippocrates and Aristotle identified tuberculosis described as 'phthisis'(consumption) as the most widespread disease of the times, and noted that it was almost always fatal. In 17th century, the tuberculosis epidemic occurred in Europe, which lasted for two hundred years, was known as the Great White Plague [9]. John Bunyan the 17th century writer referred to tuberculosis as the 'captain of all the men of death'.

Sylvius (1614-1672) was the first to identify the characteristic nodules at autopsy as 'tubercles' in the lungs and other organs [10]. He also described their progression to

abscesses and cavities. Villemin (1868), a French military surgeon, succeeded in transmitting the disease to animals [11].



ROBERT KOCH (1843-1910)

In 1882, Robert Koch a Prussian physician identified a new staining method and applied it to the sputum of tuberculosis patients, revealing the causal agent of the disease for the first time which came to be known as Mycobacterium tuberculosis, or Koch's bacillus. He also cultivated the tubercle bacilli on inspissated serum. Robert Koch reported the isolation of tubercle bacilli to the Berlin Phthisiological society on 24 March 1882 where he quoted, *"I have no business to live this life if I cannot eradicate this horrible scourge from the mankind"*. Since then, 24th March has been

known as World Tuberculosis Day. Robert Koch received the Nobel Prize in physiology or medicine for this discovery in 1905 [12].

The improvement of the staining technique by Paul Ehrilch in 1885 and the subsequent modification by Ziehl-Neelsen were important landmarks in the diagnosis of the disease. Paul Ehrlich (1854-1915), an assistant Professor at the Charite Hospital, Berlin, who was present at Koch's lecture on 24 March 1882 recalled his observation of tubercle bacilli in various clinical materials. Ehrlich who had already devised staining technique for mast cells, using aniline water, fuchsin and gentian-violet, experimented the same technique to demonstrate tubercle bacilli but used a shorter staining time (15 to 30 minutes, instead of Koch's 24 hours) and he also applied 30% nitric acid and alcohol for a few seconds to decolorize the surrounding tissues, while the tubercle bacilli retained the stain[12]. On counterstaining with a yellow or blue dye, the red tubercle bacilli showed up more clearly. In 1887, Ehrlich tested his own sputum, in which he found tubercle bacilli, was diagnosed to have pulmonary tuberculosis[12].

Later Ziehl introduced carbolic instead of aniline, while Neelsen advocated the use of sulphuric instead of nitric acid which was later on called as "Ziehl-Neelsen" (ZN) stain [12]. The Ziehl-Neelsen staining is an important technique followed even today in diagnosing tuberculosis. In 1890 Koch developed tuberculin, a purified protein derivative (PPD) of the bacteria for immunization but it was declared ineffective. In 1908, Charles Mantoux found it was an effective intradermal test for diagnosing tuberculosis. The invention of X-rays by Roentgen in 1895 further helped in the confirmation of clinical diagnosis of tuberculosis. Fluorescent microscopy was introduced by Hageman in 1937 for the diagnosis of tuberculosis.

In 1907 A.S.Griffith and F.Griffith working for Royal commission of tuberculosis, reported dorset egg as the most satisfactory medium for cultivation of Mycobacterium tuberculosis (MTB). In 1930 Lowenstein modified the dorset egg medium. In 1946 Copper and Cohn introduced the use of malachite green for the suppression of contaminants. Jensen in 1955 further modified it by using glycerol as carbon source instead of starch. To date culture on Lowenstein- Jensen medium is an important technique followed for the isolation of Mycobacterium tuberculosis. Canetti et al (1963) described the criteria and techniques for the reliable testing of Mycobacterial resistance to tuberculosis[13]. Deland and Wager (1969) developed techniques for automated detection of bacterial metabolism by measuring radioactive carbon dioxide liberated during decarboxylation of C^{14} labelled substrates in the medium[14]. Middlebrook et al (1977) later modified this technique by using 7H12 medium. Drug susceptibility testing with the above principle was introduced by Snider and colleagues (1981).

The idea of sanatoriums aroused as a result of scientific understanding and the contagious nature of the disease. George Bodington (1840) made the first proposal for a tuberculosis facility in his paper titled '*An essay on the treatment and cure of pulmonary consumption, on principles natural, rational and successful* '. In this paper, he proposed a dietary, rest, and medical care program for the patients[15].

MAMMOTH CAVE (USA)

In United States Dr. John Croghan (1842), the owner of Mammoth Cave, brought 15 tuberculosis sufferers into the cave in the hope of curing the disease with the constant temperature and purity of the cave air[15]. Patients were lodged in stone huts, and each patient was attended by servant to bring meals and other needs. John Croghan himself died of tuberculosis in 1849.

The first anti-tuberculosis sanatorium was established in 1854, 650 meters above sea level, at Görbersdorf. Brehmer and one of his patients, Peter Dettweiler, started the sanatorium movement, and by 1877, sanatoriums began to spread beyond Germany and throughout Europe. Specialized tuberculosis clinics began to develop in other major metropolitan areas. In India, the first sanatorium for the treatment and isolation of patients suffering from tuberculosis was founded in 1906 in Tiluania, near Ajmer. In 1912 the United Mission Tuberculosis Sanatorium (UMTS) was built at Madanapalle, south India[16]. Dr Frimodt Moller, the first Medical superintendent during that period played an important role in India's fight against tuberculosis

through training the TB workers, conducting TB surveys and by introducing BCG vaccination [16].

POSTAGE STAMP - Albert Calmette and Camille Guérin

Albert Calmette and Camille Guérin (1908) developed the vaccine against tuberculosis from attenuated bovine-strain. It was called "BCG" (*Bacille Calmette-Guérin*). Albert Calmette, a French bacteriologist, and his assistant Camille Guérin, a veterinarian, working at the Pasteur institute, France (1908), noted that tubercle bacilli grown in glycerin-bile-potato mixture seemed less virulent. This changed the course of their research, to see if repeated sub culturing would produce a strain that was attenuated enough to be considered for use as a vaccine. The BCG vaccine was first used in humans in 1921 in France. In Lübeck (1930), 240 infants were vaccinated in the first 10 days of life; almost all developed tuberculosis and 72 infants died. It was then discovered that the BCG vaccine that had been administered was contaminated with a virulent strain that was being stored in the same incubator, and led to legal action being taken against the manufacturers of BCG. In 1928, BCG was adopted by the Health Committee of the League of Nations (predecessor to the WHO)[17,18]. Because of opposition, however, it did not become widely used until after World War II.

Rene Jules Dubos (1901-1982) a French born American Microbiologist studied the physiology and immunology of the tubercle bacillus and tuberculosis infection—an investigation which was stimulated by the illness and the death of his wife due to tuberculosis. He introduced wetting agents into the culture medium to enable the diffuse growth of tubercle bacilli which brought about a revolution in tuberculosis research. The wetting agents used were Tween and other factors like albumin, fatty acids, which led to diffuse growth of tubercle bacilli. This culture method helped the researchers to make accurate, quantitative studies of various strains of tubercle bacilli and also to study the virulence and pathogenic properties of tubercle bacilli. He also pioneered international standards for the BCG vaccination against tuberculosis and described the social aspects of the disease in "The White Plague" (1952)[9]. Later he investigated how the environmental effects of crowding, malnutrition, pesticides, toxins, and stress increased the susceptibility to tuberculosis.

Streptomycin, the first antibiotic and first bacterial agent effective against M. tuberculosis was discovered by Albert Schatz, Elizabeth Bugie, and Selman Waksman in 1944. In 1952 Isoniazid, the first oral mycobactericidal drug was discovered. In 1950 para-aminosalisylic acid (PAS) was added to the therapy. In 1956 it was shown that pyrazinamide was active within the macrophage against the tubercle bacilli and by mid I970s this agent was also included in the anti-tuberculosis regimen. The advent of Rifampicin in the 1970s significantly reduced the number of tuberculosis cases until the 1980s[19]. With the discovery of anti-tuberculosis drugs, sanatorium based treatment became unpopular.

In India, the National Tuberculosis Programme (NTP) was started in 1962 in view of reducing the burden of tuberculosis, which was based on the research by Tuberculosis research centre (TRC), Chennai and National TB Institute (NTI), Bangalore[20]. At first the National programme was designed for domiciliary treatment using self-administered standard drug regimens. Though a large network of District TB centers were created with trained staff and infrastructure, the impact on the TB burden till 1992 was very minimal. A review of this programme in 1992 revealed that the NTP did not achieve the objectives because of low priority, managerial weaknesses, over dependence on X-rays for diagnosis, inadequate funding and incomplete treatment due to low rates of treatment adherence and lack of supervision.

Based on the recommendations of an expert committee, a revised strategy to control TB, Revised National Tuberculosis Control Programme (RNTCP), a state-run tuberculosis control programme was initiated by the Government of India in 1992. It incorporates the principles of directly observed treatment-short course (DOTS), the global TB control strategy of the World Health Organization. The program provides, free anti-tubercular drugs across the country through the numerous Primary Health Centers .It was pilot-tested in 1993 in a population of 2.35 million which was later increased in phases. A full-fledged revised programme was started in 1997 which rapidly expanded with good results[20].

By June 2005 the RNTCP had covered more than 1 billion population (more than 90% of the country was covered). Since the introduction of RNTCP, up to June 2005, more than 4.5 million patients were initiated on treatment and about 750,000 additional lives were saved.

Directly observed short course chemotherapy (DOTS) is the internationally recommended strategy for TB control with the objective of improving patient compliance and ensuring complete treatment and also decrease the prevalence of drug resistance in tuberculosis [20].

With the emergence of human immunodeficiency virus (HIV) infection and acquired immunodeficiency syndrome (AIDS), there was a resurgence of tuberculosis in the late 1980s in the developed countries while the poor socioeconomic conditions and increased immigrant population contributed to the situation in the developing countries which were already burdened with tuberculosis[21]. Noncompliance by the patients and incomplete and inadequate treatment further aggravated the problem with the emergence of multi drug resistance strains. Multi drug resistant strains of Mycobacterium tuberculosis made current chemotherapy regimens ineffective. In response to the resurgence of tuberculosis, the World Health Organization issued a declaration of a global health emergency in 1993.

In 1994, WHO an International Union Tuberculosis and Lung Disease (IUALTD) proposed a global tuberculosis surveillance programme to collect data on the global extent of severity of anti TB drug resistance in a standardized manner at country or regional level. In 2006, WHO launched the STOP TB strategy, which is to be implemented from 2006-2015[22].

Table I: Historical milestones in Tuberculosis

Year	Scientist	Discovery
460 BC	Hippocrates and Aristotle	Identified tuberculosis as 'phthisis'(consumption).
1670	Sylvius	Identified 'tubercles'
1854	Sanatorium	First tuberculosis sanatorium was established , at Görbersdorf
1868	Villemin	Succeeded in transmitting the disease to animals.
1882	Robert Koch	new staining method cultivated the tubercle bacilli Koch's bacillus
1885	Paul Ehrlich	Improvement in staining technique
1890	Robert Koch	Tuberculin – purified protein derivative
1895	Roentgen	X- rays for diagnosis
1907	A.S.Griffith and F.Griffith	dorset egg medium for cultivation of MTB
1908	Albert Calmette and Camille Guérin	BCG vaccine
1930	Lowenstein	Modified dorset egg media
1937	Hageman	Fluorescent microscopy
1944	Rene Jules Dubo	Introduced wetting agents into the culture medium to enable the diffuse growth of tubercle bacilli which

		helped in quantification and to study the virulence and pathogenic properties of tubercle bacilli
1946	copper and Cohn	Malachite green to inhibit the contaminants in LJ medium
1955	Canetti et al	glycerol as carbon source instead of starch
1962		National Tuberculosis programme by government of India
1963	Canetti et al	Tests for mycobacterial resistance
1969	Deland and Wager	techniques for automated detection of bacterial metabolism
1992	TRC, Chennai and NTI, Bangalore	DOTS therapy India
2006	Government of India	STOP TB strategy

3.2 Genus definition:

M.tuberculosis belongs to Order – Actinomycetales **Family –** Mycobacteriaceae **Genus –** Mycobacterium

The Greek prefix *myco*= "fungus" is used, as mycobacteria when cultured in liquid medium shows mould-like growth on the surface. The genus includes pathogens known to cause serious diseases in mammals,

including tuberculosis (Mycobacterium tuberculosis) and leprosy(Mycobacterium leprae).

The genus characteristics are,

1. Mycobacteria are non-motile, non-spore forming, weakly Gram positive, aerobic and micro-aerophilic. Straight or slightly curved rod -shaped bacteria, measuring 0.2-0.6x 1-10 micrometer in size[23].

2. Acid fastness (resistance to decolourisation by acid being stained with a basic fuchsin dye)

3. The cell wall consists of the hydrophobic mycolate layer and a peptidoglycan layer held together by a polysaccharide, arabinogalactan.

4. Mycolic acid is made up of 70 carbon atoms

5. These are oxygen loving organisms

6. G+C content of the DNA of 6I-70mol%

Genomics of H37Rv strain of Mycobacterium tuberculosis

The strain H37 was originally isolated in 1905. Unlike some clinical isolates that

often lose virulence after repeated passages in the laboratory, this strain has maintained its full virulence in animals experimented since its isolation. In 1934, H37 was dissociated into "virulent" (Rv) and "avirulent" (Ra) strains). The original 1905 H37 isolate was then discontinued, and the H37Rv and H37Ra isolates have been maintained at the Trudeau Institute. Its size is 4 million base pairs, with 3959 genes; 40% of these genes have had their function characterized. There are also six pseudogenes.

The genome consists of 250 genes which are involved in fatty acid metabolism, with 39 of these involved in the polyketide metabolism generating the waxy coat. About 10% of the coding capacity is taken up by two clustered gene families that encode acidic, glycine-rich proteins. These proteins have a conserved N-terminal motif, deletion of which impairs growth in macrophages and granulomas. Nine noncoding sRNAs have been characterized in M. tuberculosis, with a further 56 predicted in a bioinformatics screen.

3.3 Epidemiology

It is estimated that one third of the world's population is infected with M.TB, and that each year, about 9 million people develop TB, one in ten of whom is HIV positive, equivalent to about 1.1 million (13%) in 2010[24].

In countries worldwide, the reported percentage of all TB cases occurring in children varies from 3% to more than 25%. About 5 per cent of those infected are likely to develop disease in the first year after infection and the remaining 5 per cent during their lifetime. These rates increase about six-fold in HIV infected individuals.

According to the Global TB report 2011, there were 8.8 million (range, 8.5–9.2 million) cases of TB, 1.1 million (range, 0.9–1.2 million) deaths from TB among HIV-negative people and an additional 0.35 million (range, 0.32–0.39 million) deaths from HIV-associated TB in 2010. Most of these occurred in Asia (59%) and Africa (26%)[24].

The five countries with the largest number of incident cases in 2010 were, developing countries like India (2.0 million–2.5 million), China (0.9 million–1.2 million), South Africa (0.40 million–0.59 million), Indonesia (0.37 million–0.54 million) and Pakistan (0.33 million–0.48 million). India ranks number 1 among all countries in terms of the total number of incident cases of TB & harbors one fifth (20%) of global TB burden. Of the 9 million annual TB cases, about 1 million (11%) occur in children (under 15 years of age). Of these childhood cases, 75% occur annually in 22 high-burden countries that together account for 80% of the world's estimated incident cases[25].

India alone accounted for an estimated one quarter (26%) of all TB cases worldwide, and China and India combined accounted for 38%.

The proportion of TB cases co-infected with HIV is highest in countries in the African Region; overall, the African Region accounted for 82% of TB cases among people living with HIV. Globally, incidence rates fell slowly from 1990 to around 1997, and then increased up around 2001 as the number of TB cases in Africa was driven upwards by the HIV epidemic.

The tuberculosis mortality is decreasing globally and the Stop TB Partnership target of a 50% reduction by 2015 compared with 1990 can be met if the current trend is

sustained. The target could also be achieved in all WHO regions with the exception of African Region.

India has more new TB cases annually than any other country. In 2009, out of the estimated global annual incidence of 9.4 million TB cases, 2 million were estimated to have occurred in India, thus contributing to a fifth of the global burden of TB. It is estimated that about 40% of Indian population is infected with tuberculosis. The high burden of tuberculosis in India is illustrated by the estimation that TB accounts for 17.6% of deaths of all communicable diseases and 3.5% of all causes of mortality.

The prevalence of MDR-TB is less than 3% amongst new cases and 12-17% in re-treatment cases. These surveys also indicate that the prevalence of MDR-TB is stable in the country as similar prevalence results were obtained in studies conducted by TRC, Chennai and NTI, Bangalore.

The risk factors for tuberculosis infection include poverty, overcrowding, poor ventilation, immune suppressed status and extreme age group.

3.4 Pathogenesis [23]

The mode of infection in primary pulmonary tuberculosis is by direct inhalation of the aerosolized bacilli present in droplet nuclei of expectorated sputum. An open case whose sputum sample contains more than 10,000 bacilli per ml has more chances of spreading the disease. Most of the inhaled bacilli are arrested by the natural defenses of upper respiratory tract. On reaching the lungs the bacilli are ingested by the alveolar macrophages. The virulence of tubercle bacilli depends on its ability to survive within the macrophages and also to inhibit the fusion of phagosomes with lysosomes. The bacteria are disseminated by the lymphatic circulation to regional

lymph nodes in the lung, forming the primary or Ghon complex. There is hematogenous circulation of the bacteria to many organs including Central nervous system (CNS) and other parts of the lung, where the disease may manifest as fatal tubercular meningitis or miliary (disseminated) tuberculosis respectively.

The outcome of infection is influenced by many other factors like genetic susceptibility of an individual, age, immune status, stress, nutrition, and co existing diseases.

The Mycobacterial antigens are presented by the antigen presenting cells to the antigen specific T-lymphocytes which undergo clonal proliferation. The activated T cells release cytokines, notably interferon gamma, which, together with calcitriol, activate macrophages and cause them to form a compact cluster, or granuloma, around the foci of infection. The center of the granuloma contains a mixture of necrotic tissue and dead macrophages and is called as caseation due to its cheese like appearance. Though most the tubercle bacilli are killed some remain in dormant form, which gets reactivated and cause post-primary tuberculosis[26].

3.5 Immunology

Cell mediated immunity plays an important role in the host defense against the MTB, and the first step in this process is recognition of MTB by cells of the innate immune system. Since the main route of entry is the respiratory route, the alveolar macrophages are the important cell type that fights the pathogen. They form the main component of the innate immune response. MTB initially infects macrophages and dendritic cells. The macrophage-Mycobacterium interaction include the binding of M.tuberculosis to macrophages via the surface receptors, mycobacterial growth

inhibition or killing through free radical based mechanisms (such as oxygen and nitrogen intermediates) and cytokine- mediated mechanisms. They are also involved in mycobacterium escaping these mechanisms and its persistence. Macrophages are involved in recruitment of accessory immune cells for local inflammatory response and presentation of antigens to T cells for development of acquired immunity. Other components of the innate immune response include neutrophils, natural killer cells and natural resistance associated macrophage protein (NRAMP)[27]. MTB is surrounded by lipid-rich outer molecules as a capsule which protects it from the toxic radicals and hydrolytic enzymes produced as a defense by macrophages and inflammatory cells[28].

The specific acquired Th1 response is characterized by the production of TNF-α, IL-2 and INF-γ. These cytokines stimulate macrophages and there by cell-mediated immunity which is important against the intracellular pathogens. Cytokines like interleukins 4, 5 and 10 induce delayed type of hypersensitivity, tissue destruction and progressive disease. Studies have shown that defects in the generation of the Th1 cell effector INF-γ results in susceptibility to TB infection[29]. Robert Koch (1891) noticed a necrotic lesion at the site of inoculation of MTB bacillus in a previously infected guinea pig which came to be known as Koch's phenomenon. It is a delayed type of hypersensitivity reaction which demonstrates the cell mediated immunity and also forms the basis for Tuberculin test.

Tuberculin reaction

The development of DTH reaction is an attempt at preventing reinfection by same the organism. Sub cutaneous inoculation of tubercle bacilli in an normal individual

produces no immediate response, but a nodule develops at the site of inoculation in 10 to 14 days which ulcerates. Regional lymph adenitis is seen. In contrast, inoculation into the previously infected individual results in indurated area at the site of injection within 1 to 2 day with no lymphadenitis. The bacterial cell wall component lipopolysaccharide-protein complex is the responsible antigen. This reaction is permanently / transiently negative in individuals in whom CMI are transiently or permanently impaired which is due to anergy.

Anergy is diminished or absent DTH or type IV hypersensitivity reaction which occurs as a result of lack of responsiveness to commonly used skin test antigens like PPD, histoplasmin. Here there is decreased capapcity of T.lymphocytes to secrete lymphokines when their T cell receptors interact with specific antigens. Anergy is often associated with lack of co stimulation[30].

3.6 Pathology [28, 31]

MTB infection produces characteristic lesions called 'Tubercle' in the infected tissue. These are avascular granulomas composed of a central zone containing giant cells, with or without caseation, and a peripheral zone of lymphocytes and fibroblasts. Depending on the time of infection and the type of response, tuberculosis may be classified as 'primary' and 'post primary'.

Primary tuberculosis is the form of disease that develops in a previously unexposed and therefore unsentisized individuals. The bacilli, which are engulfed by alveolar macrophages multiply and give rise to a sub pleural focus of tuberculous pneumonia, commonly located in the upper part of the lower lobe or lower part of the upper lobe.

The involved area is called the Ghon focus. The Ghon focus together with the enlarged hilar lymph node and intervening lymphangitis constitutes 'the primary complex'. This occurs 3-8 weeks from the time of infection and is associated with the development of tuberculin hypersensitivity. In most cases, the lesion heals spontaneously in 2-6 months leaving behind a calcified node. The implications of primary tuberculosis are hypersensitivity reaction which causes tissue damage, scar formation that may harbor viable bacilli for years which later on may get reactivated and progressive primary tuberculosis which is most commonly seen in immunocompromised individuals. A few bacilli may survive in the healed lesion and remain latent.

The post-primary tuberculosis, also called secondary or adult type of tuberculosis arises in a previously sensitized host. It is either due to the reactivation of latent infection, (in which case it is called post-primary progression or endogenous reactivation) or due to exogenous re-infection. It affects mainly the upper lobes of the lungs and the lesions undergo necrosis and tissue destruction, leading to cavitations. Lymph node involvement is unusual. The necrotic materials break down into the airways, leading to expectoration of bacteria-laden sputum, which is the main source of infection to others. With the dissemination of infection tubercle bacilli can cause meningitis, genital tuberculosis leading to infertility, intestinal tuberculosis leading to fibrosis and perforation. In immunodeficient individuals, instead of cavity formation there is widespread dissemination of lesions in the lungs and other organs.

3.7 Clinical features [32]

Tuberculosis can manifest as pulmonary or extra pulmonary tuberculosis. Pulmonary tuberculosis manifest usually as asymptomatic in early stage of the disease. The disease tuberculosis, according to the status of the patient's immune system may develop differently in each patient. The disease can manifest as latent infection, primary disease and extra pulmonary disease. Each stage has different clinical manifestations

Persons with latent tuberculosis though infected, do not show any signs or symptoms of the disease. But, the viable bacilli can persist in the necrotic material for years or even *lifelong*, and if the immune system of an individual *becomes* compromised later the disease gets reactivated. Co-infection with human immunodeficiency virus is the most common cause for progression to active disease; other factors include uncontrolled diabetes mellitus, chemotherapy, organ transplantation, long-term corticosteroid usage.

Primary pulmonary tuberculosis is often asymptomatic. Lymphadenopathy occurs as the bacilli spreads from the lungs through the lymphatic system. Patient develops pleural effusion because the tubercle bacilli infiltrate the pleural space. The effusion may remain small and resolve spontaneously, or it may become large to induce symptoms such as fever, pleuritic chest pain, and dyspnea. Dyspnea occurs as a result of poor gas exchange in the areas of affected lung tissue[33].

Active tuberculosis develops in only 5% to 10% of persons exposed to M tuberculosis. The early signs and symptoms are often nonspecific as the patient progresses to active tuberculosis. Manifestations often include progressive fatigue, malaise, weight loss, and a low-grade fever usually associated with evening rise of

temperature. Wasting is a classic feature of tuberculosis. It is due to the lack of appetite and the altered metabolism associated with the inflammatory and immune responses. Wasting involves the loss of both fat and lean tissue; the decreased muscle mass contributes to the fatigue. The patient may develop cough which is non-productive initially, but later develops productive cough expectorating thick sometimes blood streaked sputum. The patient also suffers from anemia which causes fatigue and weakness. Leukocytosis occurs in response to the infection and inflammatory process.

Though pulmonary tuberculosis is the most common form, extrapulmonary tuberculosis occurs in more than 20% of immunocompetent individuals , and the risk for extrapulmonary disease increases further in immune suppressed individuals. Lymphatic tuberculosis is the most common extrapulmonary tuberculosis, and cervical adenopathy is involved most commonly. The most serious extra pulmonary tuberculosis is the involvement of central nervous system, where infection may result in meningitis or space occupying tuberculomas which is often fatal. Another fatal form of extra-pulmonary tuberculosis is disseminated or miliary tuberculosis. The bacilli can then spread throughout the body, leading to multi organ involvement. Miliary tuberculosis progresses rapidly and can be difficult to diagnose because of its systemic and nonspecific signs and symptoms. Other possible locations include bones, joints, pleura, and genitourinary system[33].

3.8. HIV infection and Tuberculosis

Tuberculosis is the most common opportunistic infection amongst HIV-infected individuals. The rapid surge of HIV infection in developing countries like Africa, India has resulted in the increased incidence of tuberculosis. The co-existence of HIV infection with tuberculosis is called as 'The cursed duet'[34]. HIV infection is the most important factor responsible for developing tuberculosis and tuberculosis is a leading cause of HIV - related mortality and morbidity.

People once infected by tubercle bacilli have about, 10% chance of developing tuberculosis during the remainder of their lives, whereas an HIV positive person, already infected by tubercle bacillus has a 5-15% chance of developing overt disease annually or up to 50% related morbidity and mortality. According to the sentinel surveillance, India accounts for one eighth of all HIV infections in the world. TB occurs in 60% to 70% of HIV positive patients in India, majority of who are in the economically active age groups. It is being predicted that if India does not cap the HIV/AIDS epidemic, it would affect the economy of the nation in the coming years.

The CD4+ T cell depletion due to HIV infection results in dissemination of M.TB. There is reduced immune response in HIV infected patients, which contributes to their susceptibility to tuberculosis. The susceptibility to get infected as well as to develop tuberculosis disease is higher among HIV infected patients. Also these patients are increasingly susceptible to re-infection even after treatment. At the site of active TB infection, macrophages infected with M. tuberculosis expresses tumour necrosis factor-α (TNF-α), which along with Monocyte Chemotactic Protein 1(MCP 1), transcriptionally activates HIV-1 replication. The tubercle bacillus also enhances the HIV replication by inducing nuclear factor kappa-B, the cellular factor that binds to promoter regions of HIV. Thereby, the immune response to TB bacilli and the

bacilli themselves increase HIV replication (viral load increases by 6 to 7 fold), as a result there is rapid progression of HIV infection [35].

The clinical presentation of tuberculosis in most patients with HIV is indistinguishable from those who do not have HIV infection. The clinical manifestation of TB in HIV infected patients depends on the degree of immunosuppression. Post primary TB (classical reactivation-type disease) is the most common among People living with HIV/AIDS (PLHAs). In the presence of severe immune deficiency, the disease presents atypically. Cavity formation is less common in patients with pulmonary disease. Most commonly the lower lobes are involved when compared to the apical lobe, this leads to smear negativity but culture could be positive. Induced sputum or bronchoscopy specimens may be useful in diagnosis. Pleural effusions also occur more frequently with HIV infection

More than 50% of cases with PTB occur in patients with CD4 counts more than 200cells/microL. Patients with low CD4 T-cell counts(<200/μL) are associated with an increased frequency extra pulmonary TB (EPTB) , disseminated TB(DTB), positive mycobacterial blood cultures, with atypical chest radiographic findings. The overall prevalence of EPTB is more among people living with HIV/AIDS (being more frequent in severely immunocompromised) when compared to HIV- uninfected.

3.9 Laboratory diagnosis of mycobacterium tuberculosis

The diagnosis of pulmonary tuberculosis involves demonstration of mycobacterium tuberculosis in the sputum sample of a patient with compatible clinical picture by smear examination, culture or by molecular methods.

India has four National Reference Laboratories (NRLs), one Intermediate Reference Laboratory (IRL) for mycobacterial culture and drug susceptibility testing in each state and almost 13,000 designated microscopy centers (DMCs) which serves a population of 1,00,000 or 50,000 in tribal or difficult/mountainous areas.

At the level of Primary Health centre (PHC) - Sputum testing for mycobacterium is done as per the guidelines of RNTCP.

Community health centre (CHC) provides diagnostic services through the microscopy centres and treatment services as per the Technical and Operational Guidelines for Tuberculosis Control.

Sample collection: Presently two sputum samples, one early morning sample and another spot sample, are collected from each patient with history of cough for 2 weeks. The patient is asked to collect well coughed-up sputum sample into a clean wide mouthed container[20].

Laboratory diagnosis includes,

I. Smear examination

II. Culture and identification

III. Molecular techniques

I. Smear examination

Smear examination is the most common method followed to diagnose pulmonary and extra pulmonary tuberculosis. This method is rapid and inexpensive. To detect acid fast bacilli by microscopy the clinical specimen should contain more than 10,000 organisms per ml[36].

Acid fast staining

In 1882 Ehrilch demonstrated that Mycobacteria resist decolourisation with mineral acids. This property of Mycobacteria is due to the presence of mycolic acid in its cell wall[35].

Though Ehrilch used Aniline oil as a mordant to demonstrate acid fastness, Frank Ziehl a German Bacteriologist changed the mordant to carbolic acid. In 1883 Neelsen a German Pathologist modified the concentration of the mordant Carbolic acid and incorporated it with the dye to form carbolfuchsin which is now used as Ziehl-Neelsen stain.

a. Ziehl-Neelsen staining [37]

Is the most common technique followed to demonstrate the acid fastness of Mycobacteria. The primary stain used is concentrated carbolfuchsin. Heating with the flame along with phenol present in carbolfuchsin helps in penetration

of the dye, 25% sulphuric acid acts as a decolorizing agent. This is counterstained with 1% Loeffler's methylene blue.

The acid fast bacilli are stained pink and the background including pus cells and other organisms appear blue. Ziehl-Neelsen staining method has 33.79% of sensitivity and 100% specificity.

Grading is a technique followed as per the RNTCP guidelines based on the number of AFB. It is an important indicator which gives an idea about bacterial laod, whether the patient is on regular treatment or not and also about the development of resistance to the anti-tubercular drugs.

The RNTCP grading for AFB as follows [20]

	Result	Grading	Number of fields examined
More than 10 AFB per oil immersion field	Positive	3+	20
1-10 AFB per oil immersion field	Positive	2+	50
10-99 AFB per oil immersion field	Positive	1+	100
1-9 AFB per oil immersion field	Scanty	Actual number	200
No AFB per oil immersion field	Negative	0	100

Factors influencing the results of smear examination[37] are:

a. Thickness of the smear

b. Extent of decolorization

c. Type of counterstain used

d. The person examining the smear

b. Fluorescent Microscopy [36,37]

In this method flurochrome stains are used which have the property to absorb the shorter wavelength light rays and emit light rays of longer wavelength. This results in fluorescence. Auramine or Rhodamine is used as the flurochrome where in the organism appears as bright yellow-orange rods.

It is a popular screening method and is also less laborious for the technician. For positive results the specimen should contain 10,000 bacilli per ml. However as fluorescent microscope are expensive they are not be available in all laboratories.

c. LED microscopy [38]

Recently World health organization (WHO) has implemented the use of Light emitting diode (LED) microscope for the diagnosis of tuberculosis instead of conventional ZN staining. LED microscope is a newly developed technique to diagnose tuberculosis in resource-limited settings. Compared to conventional fluorescent microscopes, LED microscopes are less expensive, require less power and are able to run on batteries with bulbs having long half-life and also do not have the risk of releasing toxic products.

LED microscopy showed a sensitivity of 84% and specificity of 98% against culture as reference standard. LED microscopy showed a statistically significant increase in sensitivity of 6% when compared to direct ZN microscopy, 5% more sensitive and 1% more specific than conventional fluorescent microscopy[38].

II. <u>Culture</u>

Culture is more sensitive than the smear examination and is the gold standard. The clinical specimen should contain at least 100 bacilli per ml for the culture to become positive[36]. The samples collected is digested and decontaminated before culture. The most common method followed is Petroff's method, in which 4% sodium hydroxide (NaoH) is used for digestion and decontamination. Other agents used are N-acetyl-cystiene along with 2% NaoH, 5% Oxalic acid, 13% trisodium phosphate and 1% Cetylpyridium chloride along with 2% NaoH.

There are 3 Types of media used [36]:

Egg based: LJ, Petragnani, Dorset's egg medium.

Agar based: Middlebrook 7H10 or 7H11.

Liquid based: Dubo's medium, Kirschner's, Middlebrook 7H9.

Lowenstien-Jensen (LJ) media is the commonest medium used for culture of mycobacteria. It is an egg based medium, with malachite green as a selective agent. Culture of Mycobacteria on LJ media provides a definitive diagnosis. It is more sensitive and is considered gold standard. The main drawback is the prolonged time for the organism to grow, about 6-8 weeks [37, 39].

Rapid slide culture (RSC) [40]

Robert Koch was the first person to use RSC technique which consists of human blood medium. The major drawback was the high rate of contamination. Recently it has been modified by adding antimicrobial drugs like Amphotericin B, Polymixin B, Carbenicillin and Trimethoprim to overcome contamination by bacteria and fungus. The growth in this medium is obtained in 7 days. Recently there is renewed interest in RSC as it gives faster results than the growth on LJ medium. But it could detect only an additional 1.3% more for the detection of positive over that detected by direct microscopy under the RNTCP.

Newer culture methods

BACTEC system [35]

The BACTEC 460 TB Radiometric system was first developed by Becton Dickinson in 1980. This technique includes Middle brook 7H12 or 7H13 liquid media for the growth of the mycobacteria which contains ^{14}C – labelled palmitic acid. The growth of the Mycobacteria utilizes the acid and releases radioactive CO_2 which is measured by BACTEC instrument and expressed as “growth index”. The time required to detect

the growth averages 9-14 days. To distinguish tuberculosis from other mycobacteria, NAP(Beta nitro alpha acetylamine beta hydroxyl propiophenone) is used. M.tuberculosis is inhibited by NAP but not by other mycobacteria. This system can be adapted for antimicrobial sensitivity testing also. The limitations of this method are that, it cannot be used to study the colony morphology, high cost of the instruments and the disposal of radioactive material used.

According to a study conducted by Mendoza et al. the BACTEC culture system detected mycobacterial growth earlier than the conventional LJ culture by 1-2 weeks and also the culture positivity rate was also more when compared to the conventional LJ medium [42].

MGIT (Mycobacteria Growth Indicator Tube) [36, 37]

MGIT is a non-radiometric, flourochrome based method for the detection of mycobacterial growth. It consists of a glass tube with modified 7H12 broth base, with basic nutrients and antibiotics. An oxygen sensitive fluorescent compound embedded at the bottom of the tube does not fluoresce in the presence of oxygen. The mycobacterial growth causes depletion of oxygen as a result of which there is fluorescence, which is detected visually under ultraviolet light or in the automated system (MGIT 960 system), by a sensor. According to a study conducted by Rodrigues et al. MGIT 960 TB system showed a positivity of 41% when compared to 24% *by* the conventional LJ medium[43].

MB/BaCT [37]

Is an automated system for colorimetric detection of mycobacterial growth. This system utilizes a colorimetric sensor and reflected light to monitor the concentration of

carbon dioxide in the medium. The mycobacterial growth causes increase in the concentration of carbon dioxide in the media. This is indicated by change in the color from green to yellow. The colorimetric method does not require careful handling and disposal as required in case of BACTEC system. According to a study conducted by Brunello et al. the MB/BacT and BACTEC 460 TB systems detected 96.5 and 99.4% of all isolates, respectively, when compared to 95.9% detected by LJ medium[44].

Septi-check [37]

This is a biphasic medium system which consists of an enriched selective broth and a slide with non-selective Middle brook agar on one side and two sections on the other side, one with NAP and egg containing agar, the other with chocolate agar for detection of contamination. The biphasic nature of the system and the advantage gained by repeated exposure of the agar media from beginning to organisms if any in the broth makes it more sensitive than other methods. According to study conducted by Sewell et al. Septi-Chek detected 95% of the positive specimens, BACTEC detected all the isolates and LJ detected 75% of the 4 positive isolates[45].

MB redox

It is a system based on the reduction of tetrazolium salt indicator in liquid medium which forms red to violet particles when reduced. The growth of the mycobacteria allows an easy macroscopic visualization of the bacterial growth. This system does not require specialized machine (can be read by eye or simple spectrophotometer) but cannot be used to measure DST[37].

Thin layer agar culture (TLA)

It is a solid media which includes plates with a thin layer of 7H11 agar medium incubated in Co2 incubator and examined microscopically on alternate days for the first two weeks to look for formation of micro colonies of M. tuberculosis. The results are obtained within 5-10 days but for DST it requires 10-15 days. A study conducted by Silva et al showed that the time taken for the positive result by thin layer 7H11 method was significantly less when compared to the conventional methods [46].

Reporter genes assay [35, 36]

Mycobacterial growth can be detected by using specific reporter genes like luciferase genes. The mycobacterial growth is indicated by the emission of light due to the replication of luciferase gene. The results can be obtained in 48 hours. These genes can also be used for anti-tubercular drug susceptibility tests.

Phage assay [35,36]

It is a phage amplification-based assay where in presence of M. tuberculosis in the clinical samples is detected by the replication of MTB-specific phages. Replication of the phages is identified by the production of plaques which can be visualized. Advantage of this assay is that it can be directly done on the sputum specimens. Drug susceptibility testing can also be performed by incorporating rifampicin into the assay – viable (rifampicin resistant) organisms support phage replication whilst dead (rifampicin susceptible) organisms do not. The assay is available as a commercial kit or as an in-house method.

Identification of MTB [37]

1. Colony morphology

   Mycobacterium tuberculosis appears as dry, rough, raised, wrinkled, buff coloured colonies on solid media, hence known as "rough, buff & tough" colonies compared to the bread crumbs [47]. In liquid media the growth begins at the bottom, creeps up the sides, and forms a pellicle on the top. Virulent strains form long serpentine cords whereas the avirulent forms grow in a dispersed manner; this is due to the presence of cord factor.

2. Culture smear

   Positive cultures are confirmed by acid fast staining

3. Rate of growth

   MTB is slow growing & requires 3 to 8 weeks to form visible colonies on solid media. The generation time of MTB is 15-20 hours.

4. Pigment production

   MTB does not produce any pigment. Produces ruff, buff, tuff colonies

5. Nitrate test

   MTB reduces nitrates to nitrites.

6. Niacin test

   Niacin is accumulates when MTB is grown on ‘egg containing’ medium.

7. Growth on paranitrobenzoic acid (PNB containing medium)

   MTB being sensitive to PNB and does not grow in PNB containing media, whereas non tuberculous mycobacteria grow in the presence of PNB.

Rapid identification techniques

a. Chemical methods based on lipid profile
b. Hybridization with specific gene probes
c. Gene amplification methods

1. Analysis of the Lipid Profile :

Mycobacteria have characteristic lipid profile, which is analyzed by High performance liquid chromatography (HPLC). HPLC detects the mycolic acid 6,7 dimethyl oxycoumarin esters of mycobacterium tuberculosis[36].

2. Identification based on cell wall associated proteins

The cell free extracts prepared from mycobacterial growth are subjected to polyacrylamide gel electrophoresis and the resulting electrophoretic patterns of mycobacterial proteins are studied.

3. Nucleic acid probes[36]

These are well defined oligonucleotide probes which are developed based on the information about specific gene sequences. M.tuberculosis sequences can be detected directly from clinical specimens. This test is based on IS6110 and also on genes encoding 38kDa protein antigen and 65kDa antigen. This test can detect 100 organisms/ml. Ribosomal RNA based probes are also available. These are more sensitive than DNA probes ref. Restriction fragment length polymorphism (RFLP) methods such gene for hsp 65kDa protein, KatG and r RNA genes and sequencing of 16sRNA have been described. The RFLP technique using IS6110 repetitive sequence as a probe is considered as the gold standard for typing Mycobacterium tuberculosis complex.

4. Biomarkers

M.tuberculosis specific biomarkers are Urine tuberculosis DNA, Anti alanine dehydrogenase, Sputum Ag 85 B-RNA and volatile organic compounds. These biomarkers are measurable, which indicates the pathogenic process or pharmacological response to therapeutic interventions[36].

Non Culture methods [35, 36, 37]

1. antigen detection

2. antibody detection

3. Nucleic Acid Amplification (NAA)

1. Antigen detection

Tuberculous meningitis can be diagnosed by detecting the mycobacterial antigen in cerebrospinal fluid (CSF). Latex agglutination test, reverse passive haemagglutination test, enzyme linked immunosorbent assay (ELISA) methods can be adapted.

Lipoarabinomannan in urine can be detected by ELISA. According to study done by Sada et al the sensitivity of this test in patients with active pulmonary tuberculosis but negative for acid-fast bacilli in sputum was 67% with 100% sensitivity[48].

Tissue sections of organs suspected of tuberculosis can be subjected to immunohistochemical (IHC) staining with species specific monoclonal antibody to detect mycobacterial antigen. According to the study done by **Mukherjee** et al of the IHC test showed a sensitivity of 74% and was 100% specific [49].

Antibody detection

Serological methods like ELISA are used to detect mycobacterial antibodies. M.tuberculosis specific immunoglobulin G can be detected in tuberculous meningitis. Antibody detection has a role in diagnosing extra pulmonary tuberculosis like tubercular meningitis but not pulmonary tuberculosis. A study by Pottumarthy et al , evaluated seven commercially available serological tests, and found the

sensitivities of these tests in active tuberculosis patients ranged from 16 to 57% [50]. According to study conducted by Katoch et al. an ELISA kit (Pathozyme MYCO IgM, IgA, IgG) showed poor sensitivity . Recently it has been found that antibody detection is not of help in the diagnosis of tuberculosis and it is not recommended for the diagnosis of tuberculosis [51].

Nucleic acid amplification tests [52]

The amplification methods include PCR and Isothermal amplification methods. PCR methods include conventional DNA based PCR, Nested PCR and Real time (RT)-PCR. The sensitivity and specificity of PCR is 74.4% and 97.29% respectively. These amplification techniques can detect even 1-10 organisms/ml. It has an important role in early diagnosis in pauci-bacillary extra pulmonary tuberculosis. However the high costs limits the use of these tests.

Isothermal amplification techniques include strand displacement assay, gene probe amplified M.tuberculosis direct test and q-beta (QB) replicase based gene amplification. These tests are highly specific and very rapid test. The test are specific for MTB complex. It is an isothermal TMA (transcription-mediated amplification) test which targets mycobacterial 16SrRNA and IS6110. But these test are expensive and requires highly skilled personnel.

Newer techniques

1. LAMP (loop mediated isothermal amplification)

This is a method which amplifies TB DNA directly from clinical samples. It produces large amounts of amplified products and enables simple detection methods such as visual judgment by the turbidity or fluorescence of the reaction mixture, which is kept

in the reaction tube. A positive result is signalled by a colour reaction visible to the naked eye. LAMP amplicons in the reaction tube are directly detected with the naked eye by adding 1.0 _l of 1/10-diluted original SYBR Green I to the tube and observing the color of the solution. The solution turns green in the presence of a LAMP amplicon, while it remains orange with no amplification[53]. But the specificity and sensitivity is low as the sample is not decontaminated.

In a study conducted by 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% with a specificity of 99%[54].

2. Inno-LiPa assay

It is a commercialized assay (two types). The first is for diagnosis of tuberculosis (INNO-LiPA Mycobacteria Assay) and the second for detection of rifampicin resistance (INNO-LiPA Rif TB Assay). It is based on hybridization of amplified DNA (mycobacterial 16S- 23S rRNA spacer region) from cultured strains or clinical samples to 10 probes covering the core region of the rpoB gene of M.Tb, immobilized on a nitrocellulose strip[35]. In a study conducted by Ling et al Inno-LiPa assay showed a sensitivity of 98.1% and 98.7% of specificity for rifampicin resistance. The accuracy for detection of isoniazid resistance was variable, with a sensitivity of 84.3% and 99.5% of specificity[55].

Another study conducted by Viveiros et al showed a sensitivity of 82.2% and a specificity of 66.7% when compared to conventional methods. The sensitivity and specificity were 100.0% and 96.9%, respectively, for the detection of RIF resistance[56].

3. GenoTypes assay(two types)

The first is for tuberculosis diagnosis (GenoType Mycobacteria Assay), the second for detection of rifampicin and isoniazid resistance (GenoType MTBDR Assay). It is based on PCR amplification of the 16S-23S ribosomal DNA spacer region followed by hybridization of the biotinylated amplified DNA products with 16 specific oligonucleotide probes. The specific probes are immobilized as parallel lines on a membrane strip. The GenoType MTBDR detects resistance to isoniazid and rifampicin in culture samples, based on the detection of the most common mutations in the katG and rpoB genes respectively.

In a study conducted in Italy, the GenoType MTBDR assay showed a sensitivity of 91.5% for rifampin and 67.1% for isoniazid resistance [57].

4. Gene Xpert MTB/RIF assay

It is the Cepheid GeneXpert system, which is rapid and simple-to-use nucleic acid amplification test (NAAT). It detects DNA sequences specific for Mycobacterium tuberculosis and rifampicin resistance by polymerase chain reaction. The Xpert® MTB/RIF assay purifies, concentrates, amplifies (by real-time PCR) and identifies targeted nucleic acid sequences in the Mycobacterium tuberculosis genome, and thus provides results from unprocessed sputum samples in 90 minutes, with minimal biohazard. It does not require a trained technical staff to operate.

According to a study conducted by Boehme et al in culture-positive patients, a single, direct MTB/RIF test identified 98.2% of patients with smear-positive pulmonary tuberculosis 72.5% in patients with smear-negative pulmonary tuberculosis with a specificity of 99.2%. As compared with phenotypic drug-susceptibility testing, MTB/RIF assay identified 97.6% of patients with rifampin-resistant bacteria[58].

5. Interferon-Gamma Release Assays (IGRAs) [59]

Detection of the role of interferon-gamma (IFN-γ) to regulate cell-mediated immune responses to M. tuberculosis infection led to the development of IGRAs for the detection of M. tuberculosis infection. This assay is based on the principle that T-cells of individuals infected with *M. tuberculosis* release IFN-*γ* when they re-encounter TB-specific antigens. IGRAs have been developed to replace the tuberculin skin test (TST) for detection of latent TB infection (LTBI). Newer tests of IFN-*γ* assays use antigens such as the early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) which are encoded within the region of difference 1 (RD1) of the *M. tuberculosis* genome. They are specific to *M. tuberculosis* than PPD, as they are not shared with any BCG vaccine strains.

Two IFN- *γ* assays are available as commercial kits based on RD1 antigens: the QuantiFERON-TB Gold assay and the T SPOT-TB assay.The QuantiFERON-TB Gold assay is a whole-blood, ELISA-based test, whereas the T SPOT-TB test uses peripheral blood mononuclear cells.

Detection of Adenosine deaminase enzyme

It is a sensitive, specific and inexpensive - easy to perform test. It is a colorimetric test performed on body fluids. Adenosine deaminase is a catabolic enzyme produced as a part of the purine salvage metabolic pathway.The principal function of the enzyme may be related to the development of the immune response. Increased Adenosine deaminase activity in body fluids is classically associated with tuberculosis[60].

Drug susceptibility testing [35, 37]

Drug susceptibility testing for mycobacterium tuberculosis can be determined either by observation of growth or metabolic inhibition in a medium containing anti tubercular drugs or by detection of mutations. It includes,

1. CULTURE BASED METHODS
2. MOLECULAR METHODS

Culture based methods

1. **Absolute concentration method** [36]

It is similar to agar dilution technique performed for the detection of antibiotic susceptibility of bacterial isolates. A standardized inoculum of the test organism is inoculated onto both the control (without drug) and the test media containing various drug concentrations. A test organism is considered to be sensitive if it is inhibited by the lowest concentration of the drug, which is the minimum inhibitory concentration.

2. **Resistance ratio method** [36]

Here the resistance of the test organism is compared with the standard strain- H37RV. Both the strains are tested in parallel by inoculating a standard inoculum to media containing twofold serial dilutions of the drug. Resistance is expressed as the ratio of the MIC of the test strain divided by the MIC of the standard strain for each drug.

3. **Proportion method** [36]

For each drug to be tested, several dilutions of standardized inoculum are inoculated onto control and drug containing media. The isolate is considered to be resistant if the number of colonies formed on the drug-containing media is more than_1% of the colonies formed on the drug-free media.

Rapid culture-based techniques

1. BACTEC 460 [35]

BACTEC 460 TB is a radiometric system which detects the radioactive CO_2 produced by mycobacteria when it replicates and is expressed as "growth index". The ability of mycobacteria to grow in presence of anti-tubercular drugs incorporated into the media provides a rapid method to detect drug resistance. If there is increase in the growth index in drug containing medium when compared to the growth index of the control media, then the strain which is growing in presence of that drug is said to be resistant

2. MGIT [35,36]

It is a flurochrome based technique.The mycobacterial growth causes depletion of oxygen as a result of which there is fluorescence, which is detected visually under ultraviolet light or in the automated system (MGIT 960 system), by a sensor. For each drug tested a standardized inoculum is inoculated in to the test and control media. If there is growth of mycobhacteria even in the presence of drug, then the fluorescence can be seen under UV light.

3. Micro colony detection [35,36]

It is a solid media where the plate is examined microscopically on alternate days for the first two weeks to look for formation of microcolonies of M. Tuberculosis. The results for DST require 10-15 days.

4. Luciferase Reporter phages [35,36]

In this method viable bacteria are infected with reporter phages expressing luciferase gene. When the test mycobacterium strain is sensitive to the drug incubated with, then they fail to produce light after infection with luciferase reporter phages as there is growth of the organisms in the presence of the drug.

5. Phage assay [35,36]

It is a phage amplification-based assay which depends on the replication of MTB-specific phages in the presence of *M tuberculosis* in the clinical specimens. Phage assays can be directly used on sputum specimens and the direct drug susceptibility testing can also be performed through the incorporation of rifampicin into the assay – viable (rifampicin resistant) organisms support phage replication while dead (rifampicin susceptible) organisms do not.

Molecular detection

Mutations in katG for INH resistance, rpoB gene for rifampicin resistance, other genes like inhA, oxyR-ahpC and kasA gene can be detected by molecular methods. Genotypic assays based on amplification of a specific region of tuberculosis gene followed by its analysis of for specific mutations. The molecular methods include DNA microarray, molecular beacons and other PCR techniques[52].

INNO-LiPA Rif TB Assay

It is based on hybridization of amplified DNA (mycobacterial 16S- 23S rRNA spacer region) from cultured strains or clinical samples to 10 probes covering the core region of the rpoB gene of M.Tb, immobilized on a nitrocellulose strip [56].

GenoType MTBDR assay

It detects the resistance to isoniazid and rifampicin in culture samples, based on the detection of the most common mutations in the katG and rpoB genes respectively [57].

GeneXpert MTB/RIF assay

It is a simple nucleic acid amplification test (NAAT). It detects DNA sequences specific for Mycobacterium tuberculosis and rifampicin resistance by polymerase chain reaction [58].

DNA Microarrays

A new molecular method for detecting the drug resistance in MTB which is based on the hybridization of DNA obtained from clinical samples to oligonucleotides immobilized on a solid support, such as miniaturized glass slides. Microarrays have been mainly used to detect resistance to rifampicin [36].

Diagnosis of tuberculosis by rodents as detectors [61]

APOPO (Anti Persoonsmijnen Ontmijnende Product Ontwikkeling) (Anti-Personnel Landmine Detection Product Development) is a nonprofit humanitarian organization located in Morogoro, Tanzania which uses rats as mine-detection animals. This organization also has been experimenting the use of African giant pouched rats

(Cricetomys ngambianus) to detect the presence of TB. These rats are large and long lived rats, which have an excellent sense of smell, and are used to detect TB by sniffing. The sputum samples of the patients infected with tuberculosis has distinctive volatile organic compounds produced by MTB and these are detected are detected by Apopo's rodents. They are trained to respond consistently in one way (pause) if the sample contains the TB bacillus (is positive) and respond in another way (not pause) if the sample does not contain the bacillus (i.e., is negative). Each rat can test hundreds of samples each day, allowing inexpensive testing. According to a study 16 rats that were evaluated for the diagnosis of 2,597 sputum samples, of which 345 were smear positive showed a mean sensitivity of 87.9% and 93.3% of specificity[61].

Microscopy after Universal Sample Processing (USP) Technique [4]

Recently a new technique for processing the sputum sample to detect AFB, which is claimed to be more sensitive than the conventional direct smear microscopy has been introduced. This technique includes chemicals like guanidinium hydrochloride a Chaotropic agent, Tris chloride base, EDTA, B mercaptoethanol and Sarkosyl for processing the sputum sample with 6000rpm centrifugation. These chemicals are mucolytic and detergent in nature which renders the sputum sample liquefied and free from other cellular debris. The chaotropic agent have the ability to destabilize the hydrogen bonding and hydrophobic interactions damaging the cell wall of other cells, but the cell wall of mycobacterium which is made up of mycolic acid renders I,t resistant to the action of guanidinium hydrochloride. Thus only mycobacterium is seen in the smear without any cells or cellular debris.

A study conducted by Chakravory et al. claim that it can detect as low as 250-300 bacilli/ ml of sputum sample when compared to the 10,000 AFB/ ml by conventional ZN staining and USP smear microscopy showed a sensitivity and specificity of 98.2% and 91.4% respectively compared to the 68.6% and 92.6% by direct ZN staining method. In addition this test also detected 100 samples which were negative by direct smear microscopy, as positive for AFB after USP smear microscopy which were also positive by culture[4].

Anti-tubercular drugs and Drug resistance [62]

Mycobacterium tuberculosis are intracellular organisms which multiply in macrophages. Treatment includes the The treatment regimen for tuberculosis varies from months to years.

Isoniazid (INH), Rifampicin (R), Pyrazinamide (Z), Ethambutol (E) and Strptomycin are the first five line drugs used for the treatment of tuberculosis.

Amikacin, Ciprofloxacin, Capreomycin, Aminosalicyclic acid, Cycloserine, Ethionamide and Rifabutin forms the second line drugs.

In initial phase, during the first two weeks of therapy the actively multiplying tubercle bacilli are killed mainly by the action of Isoniazid and partly by Rifampicin and the

patient becomes non-infectious. Later on the less active bacilli in macrophages, caseous material are killed by Pyrazinamide and Rifampicin.

In the continuation phase, any remaining dormant bacilli are killed by rifampicin and any rifampicin resistant mutants that starts replicating is killed by isoniazid [47].

INH is a bactericidal agent. It penetrates into the macrophages and is active against both intracellular and extra cellular organisms. It acts by inhibiting the mycolic acids, the essential component of mycobacterial cell wall. INH occurs as inactive form, which is activated by mycobacterial catalase-peroxidase. The activated form combines with acyl carrier protein (AcpM& KasA) which blocks the mycolic acid synthesis resulting in cell death.

Rifampicin – a semisynthetic derivative of rifamycin an antibiotic produced by Streptomyces mediterranei. It is a bactericidal drug. Rifampicin binds to the B sub unit of bacterial DNA dependent RNA polymerase and inhibits RNA synthesis

Ethambutol, a synthetic drug acts by inhibiting mycobacterial arabinosyl transferases which blocks the synthesis of arabinoglycan an essential component of mycobacterial cell wall.

Pyrazinamide drug is active against the tubercle bacilli present in the macrophages. The drug action is not known.

Streptomycin isolated from Streptomyces griseus acts by binding to the ribosomes. The drug is active against extracellular tubercle bacilli due to its poor penetration in to the cells. Streptomycin is helpful in case of TB meningitis as it crosses the blood-brain-barrier.

Rifampicin forms the main stay in the treatment of tuberculosis. Its high level efficacy brought a new hope for the treatment of tuberculosis. But resistance to rifampicin was soon detected. The analysis of the laboratory data from different trials revealed that isoniazid resistance was seen among patients who received monotherapy and also they found that the resistance was uncommon when isoniazid was given in combination with streptomycin or para-aminosalicylic acid. These observations led to the use of multidrug treatment regimens. The British Medical Research Council conducted series of multi-country, clinical trials after which a four-drug regimen was recommended to treat patients who were newly diagnosed with tuberculosis. The backbone of such regimens was the combination of isoniazid and rifampin which is considered to be the most effective and well tolerated oral agents, given for 6 to 8 months. Thus, short-course chemotherapy was born.

The patients are classified into 3 different categories and are put on different treatment regimens [20].

Category I:

It includes patients who are sputum smear-positive (new), and patients who are seriously ill with smear negative pulmonary tuberculosis or extrapulmonary tuberculosis. The Patients in category I are treated with Isoniazide

(H), rifampicin (R), Pyrazinamide (Z) and Ethambutol (E) weekly thrice for a period of 2months followed by Isoniazide (H), rifampicin (R) weekly thrice for a period of 4months.

Category II

It includes patients who are sputum smear positive with history of relapse, treatment failure or default patients**.** These patients are treated with Isoniazide (H), rifampicin (R), Pyrazinamide (Z), Ethambutol (E) and Streptomycin (S) weekly thrice for a period of 2months followed by Isoniazide (H), rifampicin (R) Pyrazinamide (Z) , Ethambutol (E)weekly thrice for one month and Isoniazide (H), rifampicin (R), Ethambutol (E) weekly thrice for a period of 5months.

Category III

It includes patients who are suffering with either smear-negative pulmonary or Extra-pulmonary tuberculosis (new) with no serious illness. These patients are treated with Isoniazide (H), rifampicin (R) and Pyrazinamide (Z) weekly thrice for a period of 2months followed by Isoniazide (H), rifampicin (R) weekly thrice for a period of 4months

The emergence of resistant strains is due to the sequential accumulation of individual mutations in separate genes. A Mutation responsible for drug resistance occurs at a rate of about one mutation every 10^8 cell divisions [23]. Based on the specificity of anti-TB drugs, drug resistance mechanisms in M. tuberculosis are divided into specific and nonspecific mechanisms. The tuberculosis-specific drugs (isoniazid, pyrazinamide, ethambutol etc.) attack unique metabolic pathways in M. tuberculosis

Mechanisms of resistance to these drugs are unique to M. tuberculosis and so far only isoniazid (INH) resistance mechanisms have been characterized. The non-tuberculosis-specific drugs (streptomycin, rifampicin, fluoroquinolones) which are broad spectrum antibiotics. Resistance to INH is due to mutations, which results in overexpression of InhA, mutation or deletion of kat G, overexpression of ahpC and mutations in kas A gene. Rifampicin resistance develops as a result of point mutation in the rpoB gene. Pyrazinamide resistance may develop as a result of impaired uptake of pyrazinamide or mutations in pncA gene which interferes in conversion to its active form. Resistance to Streptomycin develops as a result of point mutation in rpsL and rrs gene which alters the ribosomal binding site.

Primary drug-resistance: "New Cases" [20]

Drug resistance in patients who have not been treated earlier for tuberculosis.

Secondary (acquired) drug-resistance: [20]

Drug resistance in a patient who has received at least one month of anti-TB therapy.

Multi-drug-resistant tuberculosis (MDR-TB) is defined as tuberculosis that is resistant to atleast one of the drug, isoniazid (H) or rifampicin (R), the two first-line anti-TB drugs. India accounts for 2.1% of new TB cases with MDR TB and 15% of retreatment TB cases with MDR TB [20].

Extensively drug resistant (XDR-TB) is defined as MDR plus resistance to fluoroquinolones and at least 1 of the 3 injectable drugs (amikacin, kanamycin, capreomycin).

These organisms have the ability to remain dormant and also have the ability to develop resistance by mutation to the drugs used in treatment by selection of resistant mutants which occurs due to inappropriate and irregular treatment.

4. Materials and Methods

Source of clinical material

Patients suspected of pulmonary tuberculosis, with history of cough for 2 weeks visiting the RNTCP screening laboratories situated at R.L. Jalappa hospital and SNR hospital, Kolar were included in the study.

Inclusion criteria:

Patients suspected of pulmonary tuberculosis, with history of cough for 2 weeks attending the above RNTCP screening laboratories, who were able to bring out well coughed up sputum sample were included in the study.

Exclusion criteria

Children who were unable to bring out satisfactory amount of sputum specimen and all those patients who were already on anti-tubercular treatment were excluded from the study.

Method of collection of samples

Sputum samples (both spot and early morning) as per the Revised National Tuberculosis Control Programme (RNTCP) guidelines[20] were collected.

The patients were advised to bring out well coughed up sputum samples. The sputum samples were collected in sterile wide mouthed plastic containers. The above collected samples were processed immediately. In case of any delay the specimens were stored at 4^{o}C in the refrigerator.

A total of 2000 (both spot and early morning) sputum samples from 1000 patients visiting the RNTCP screening laboratories were collected consecutively from November 2011 to May 2012.

Direct Ziehl-Neelsen staining [37]

Preparation of the smear

The staining technique followed was performed as per RNTCP guidelines.

The sputum sample was labeled with the name of the patient, age, sex, hospital number and an Universal Sample Processing (USP) number. The sputum sample was mixed well with a sterile loop and a loop full of sputum sample was placed on a clean labeled glass slide and the smear was made.

Procedure

1. The smear was heat fixed
2. The heat fixed smear was kept on a staining rack

Staining with concentrated Carbol fuchsin

3. Smear was flooded with 1% Carbol fuchsin stain reagent (Himedia). Gently heated until the vapors rouse, the smear was not allowed boil. Carbol fuchsin was left on the slide for 5 minutes.
4. The smear was gently washed in tap wate

Decolorisation

5. The slide was decolorized with 20% Sulphuric acid for 2 minutes. The slide was gently washed with tap water and the decolorisation step was repeated as above for 3 times.

Counter staining

6. The smear was counterstained with 1% Loeffler's methylene blue (Himedia) for 30 seconds.
7. The smear was gently washed with tap water and air dried.
8. The slide was examined under microscope using 40X objective to select the suitable area and then examined under 100X objective using a drop of immersion oil.

The smear was then graded as per the RNTCP guidelines [20] as follows

	Result	Grading	Number of fields examined
More than 10 AFB per oil immersion field	Positive	3+	20
1-10 AFB per oil immersion field	Positive	2+	50
10-99 AFB per oil immersion field	Positive	1+	100
1-9 AFB per oil immersion field	Scanty	Actual number	200
No AFB per oil immersion field	Negative	0	100

Preparation of USP solution[4]

The USP solution consists of the following chemicals Guanidinium hydrochloride (Sigma), Sarkosyl(Sigma), Tris –chloride(Sigma), EDTA(Sigma) and Beta-mercaptoethanol(Sigma).Tween 80 (Himedia).

USP Solution has two components: solution 1and solution 2.

Solution 1 consists of a mixture of stock solution A, stock solution B and stock solution C.

Preparation of individual components of USP solution 1

1. Preparation of Stock solution A.

Solution A consists of 25.392grams of Guanidinium hydrochloride in 44.3ml of sterile distilled water.

About 44.3ml of sterile (autoclaved) distilled water was measured in a measuring jar and poured into a sterile 200ml conical flask. In an electronic weighing machine, 25.392grams of guanidinium hydrochloride was weighed. Mixed well in the distilled water taken in a conical flask. The chemical dissolved completely in water and the resultant solution became cold due to endergonic reaction.

2. Preparation of stock solution B

Solution B consists of: 0.25grams of Sarkosyl in 1.25ml of sterile distilled water, 0.4625 grams of EDTA in 2.5ml of sterile distilled water and 0.303 grams of Tris chloride in 1.25ml of sterile distilled water.

Method of preparation

a. Preparation of Sarkosyl solution

Using an electronic weighing machine, 0.25grams of Sarkosyl was weighed. It was dissolved in 1.25ml of sterile distilled water taken in a sterile labeled Mc cartney bottle. On mixing, the resultant solution was not clear as the chemical did not

dissolve completely. The solution thus prepared was kept in a water bath at 65^{o}C overnight, after which the chemical was found to have dissolved completely.

b. Preparation of EDTA solution:

Using electronic weighing machine 0.4625 grams of EDTA was weighed. It was dissolved in 2.5ml of sterile distilled water taken in a sterile labeled Mc cartney bottle. On mixing the resultant solution was not clear as the chemical did not dissolve completely. The solution thus prepared was kept into the autoclave and sterilized at 121^{o} C for 15 min.

c. Preparation of Tris chloride solution:

Using an electronic weighing machine 0.303 gram of tris-chloride was weighed. It was dissolved in 1.25ml of sterile distilled water taken in a sterile labeled Mc cartney bottle. The solution thus prepared was kept in an autoclave and sterilized at 121^{o} C for 15 min.

Stock Solution C -

β-mercaptoethanol (sigma) stored at 4 °C.

Taken together the volumes of solution A and solution B made as above gives a volume of 49.3ml to this 700µl of β-mercaptoethanol-which is solution C is added. It gives a final volume 50ml of solution 1.

The USP solution prepared was used immediately within 24 hrs of reconstitution.

Preparation of solution 2

The working solution was made as follows:

Solution 2 was by made by adding 10µl of tween 80 into 100ml of sterile distilled water.

For this purpose about 10µl of Tween 80 (Himedia) was pipetted into 100ml of sterile distilled water taken in a conical flask and mixed well.

Processing the sputum sample[4]:

a. Treatment with USP solution

Sterile centrifuge tubes measuring 50ml were labeled with patient's name, hospital number and USP number (lab number) for each of the sample collected.

About 2-3ml of each of the two sputum sample collected from a patient was taken in a sterile 50ml centrifuge tube. About 2-3 volumes of USP solution depending on the tenacity and purulence of sputum was added; a maximum of 3 volumes.

The test tube containing the sample and USP solution mixture was mixed by shaking or by vortexing for 30-60 seconds until complete homogenization occurred, for about 2 minutes. Tenacious or excessively purulent samples which did not homogenize completely were allowed to stand for 10-15 minutes at room temperature then 5-15 ml of sterile water was added. Mixed well to obtain a clear homogenous solution.

b. Centrifugation of the prepared sputum sample

The above prepared solution was centrifuged at 6000rpm for 20 minutes at room temperature in a Remi centrifuge. The supernatant was decanted carefully so that the pellet was not lost with the supernatant. Using a sterile Pasteur pipette the pellet was suspended again in 2-5 ml of USP solution. When the pellet was very large appropriate amount of USP solution was used to suspend the pellet. The suspended pellet was centrifuged again at 6000rpm for 20 minutes at room temperature. Supernatant was decanted carefully.

About 5-10ml of sterile water was added to wash the pellet formed and was again centrifuged as before. The supernatant was decanted and the pellet was suspended in 500 µl of Solution 2.

c. Smear preparation with the deposit after USP:

The pellet suspended in solution 2 as above was mixed well. About 100µl of the suspension was pipetted onto a clean labeled glass slide and the smear was evenly spread with the help of an inoculating loop. The smear was air dried and heat fixed over low flame.

Ziehl-Neelsen staining was performed as described earlier.

USP smear screening.

The slide was examined under microscope using 40X objective to select the suitable area and then examined under 100X objective using a drop of immersion oil.

Grading of the USP smear could not be done as the acid fast bacilli was not evenly distributed. The acid fast bacilli were seen clumped together.

Culture of tubercle bacilli after subjecting to USP method

Preparation of Lowenstein Jensen (LJ) media [37]: for 100ml media

a. Ingredients consists of:

TB piezer media base – 4.47grams

0.85% sterile saline - 2.5ml

20% sterile Dextrose solution – 0.1ml

Malachite green - 1.3ml

Egg yolk - 1

Glycerol -4ml

b. Contents of TB Peizer medium base (Himedia), THIS

Casein acid hydrolysate,

Beef extract,

L-Asparagine,

Potato starch,

Ferric ammonium citrate,

Magnesium sulphate,

Dipotassium hydrogen phosphate,

Citric acid,

Agar.

c. Preparation of LJ medium

About 4.47 grams of TB Peizer media base is weighed in an electronic weighing machine. In a sterile conical flask containing 100ml of sterile distilled water, the base above weighed base was added and mixed well. To the above solution 4ml of glycerol was added**.** The above mixture in the conical flask was sterilized at 121^{o} C for 15 min in an autoclave.

The egg yolk collected aseptically was added into a sterile 100ml conical flask with glass beads. Mixed well. Then 2.5ml of 0.85% normal saline, 0.1 ml of

20% Dextrose solution, and 1.3ml of Malachite green was pipetted into the above conical flask containing egg yolk. Mixed well.

The above prepared mixture was added to the sterilized TB media base at 55^0C. Mixed carefully to avoid frothing. About 3ml of the mixture was dispensed into each Mc Cartney bottle. The bottles were then allowed to solidify in slants

Culture on LJ medium [4]

About 225µl of the final suspension after the USP technique was inoculated onto the LJ media and incubated at 37^0C. The tubes were observed at weekly intervals for growth and colonies suspicious of Mycobacterium tuberculosis were specially noted. The tubes were thus observed for 12 weeks and if there was no growth even after 12 weeks the Mc cartney bottles containing media were discarded. As a positive control the H37RV strain of tubercle bacillus was inoculated into a MC cartney bottle for each batch of the media made.

Niacin test[36] (Himedia)

1. **Extraction of the niacin from the Mycobacterium tuberculosis culture**

About 2ml of sterile saline was pipetted into the slant in LJ culture bottle with growth. Then the slant was stabbed with a needle. The slant was kept horizontally for 20min at room temperature, and then kept upright for 5min.

Criteria – Growth considered for niacin test was more than 3 weeks old.

1. **Test solution preparation**

There were two vials part A and part B 1ml each supplied by the manufacturer. The content of part A was transferred to part B vial. Then the test sample 1ml was transferred into the solution made as above using a syringe.

2. **Controls**

a. **Positive control**

The content of part A was transferred to part B. To this reagent solution, 1ml of R055 reagent was added using a syringe. The R055 reagent contains standard antigen. Development of yellow colour within 5min – positive control

a. Negative control

The content of part A was transferred to part B. To this reagen solution, 1ml of sterile saline was added. No development of yellow colour – negative control.

The direct ZN stained smears and smears after USP method were screened by two different technicians in two RNTCP centers to find whether there was any difference in the time taken to detect the AFB in the direct and USP smear microscopy.

A total of 24 stained smears of direct and USP smear microscopy each were subjected to this test. Of the 24 smears, 8 slides of grade1+, 8 slides of grade 2+ and 8 slides of 3+ to detect the AFB.

The direct and the USP smears were screened by the technicians and the time taken for them to detect the AFB in each slide was noted.

5.Results

Sputum samples from one thousand patients, who had cough for more than 2 weeks duration, were tested by direct smear microscopy and microscopy after USP method.

Among these samples 704 (70.4%) were from patients who visited the RNTCP centers situated at SNR hospital, the government district hospital, Kolar. The remaining 296 (29.6%) samples were from patients who visited the RNTCP center situated at R.L.Jalappa hospital, a tertiary care teaching hospital, Kolar.

Table 1- Sampling centers and the samples collected

Sampling centers	Number of samples (%)

SNR hospital, Kolar	704 (70.4%)
R.L.Jalappa Hospital, Kolar	296 (29.6%)
Total number of samples (n)	1000

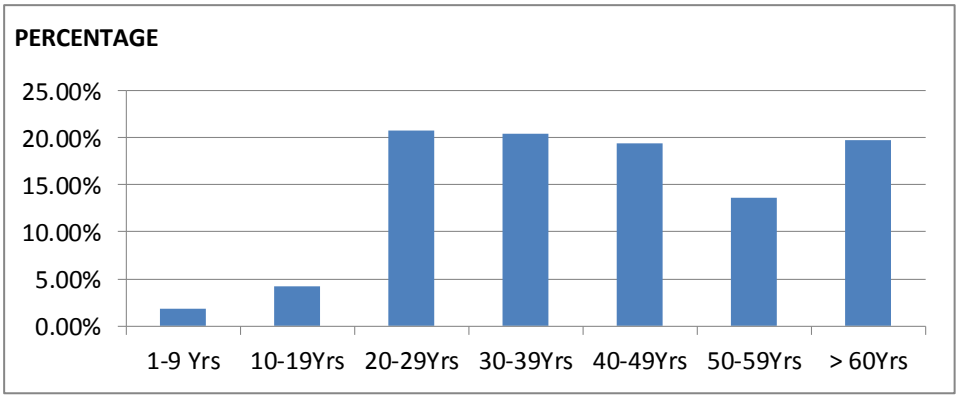
The age and sex distribution of the patients whose sputum (spot and early morning) samples were tested is shown in table 2 and age distribution is shown in figure 1.

Table 2-Age and sex distribution

Age	Males (%)	Females (%)	Total tested (%)
<10 years	11(1.1%)	7 (0.7%)	18 (1.8%)
10-19	25 (2.5%)	17 (1.7%)	42 (4.2%)
20-29	151 (15.1%)	45 (4.5%)	196 (19.6%)
30-39	147 (14.7%)	54 (5.4%)	201 (20.1%)
40-49	99 (9.9%)	87 (8.7%)	186 (18.6%)
50-59	85 (8.5%)	44 (4.4%)	129 (12.9%)
>60 years	123 (12.3%)	105 (10.5%)	228 (22.8%)

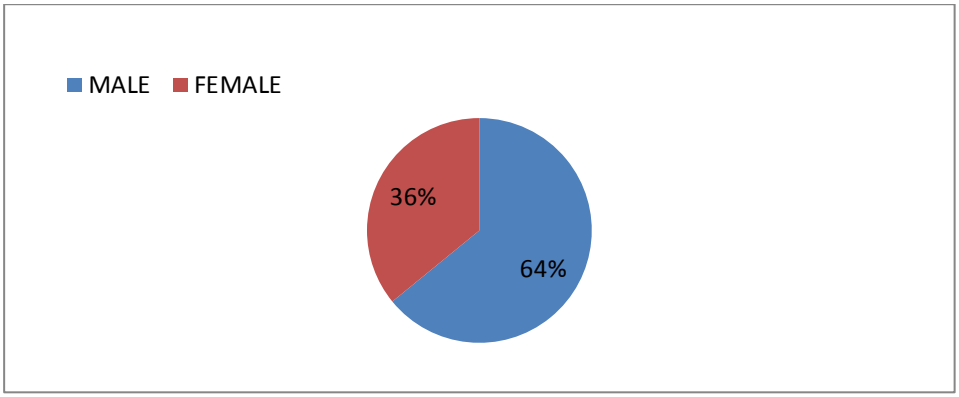
Total	641 (64.1%)	359 (35.9%)	1000

Figure 1 – Bar diagram showing the age distribution



Among the patients from whom sputum samples were studied, majority were adults in their 3rd, 4th and 5th decades of life, this was followed by those above 60 years of age.

Figure 2. Shows the percentage of gender distribution



Among the samples collected 64.1% were from males and 35.9% from females. The age-wise distribution of the genders is shown in table 2.

Table 3 - Shows the number of samples tested found to be positive in the direct smear examination and USP smear microscopy according to the age.

Age (in years)	Samples tested (%)	No. of positives in direct smear microscopy (%)	No. of positives after U microscopy (%)
<10	18	1 (5.5%)	1 (5.5%)
10-19	42	4(9.5%)	4(9.5%)
20-29	208	17 (8.17%)	17 (8.17%)
30-39	204	22 (10.7%)	22 (10.7%)
40-49	194	16 (8.2%)	16 (8.2%)
50-59	136	17 (12.5%)	17 (12.5%)
>60 years	198	17 (8.5%)	17 (8.5%)
Total	1000	94 (9.4%)	94 (9.4%)

Of the 1000 samples tested, 94 (9.4%) were positive for AFB by direct smear microscopy and 906 (90.6%) were negative. There was no difference between the direct smear microscopy and USP smear microscopy in the detection rates. The positivity for AFB ranged between 5.5% in the first decade and 12.5% in 6^{th} decade. However, the sputum positivity rate was not significantly more in any particular age group. ($p = 0.8290$, at 95% CI)

The positivity rate among the gender is shown in table 4.

Table 4. Number of samples positive for AFB in each gender

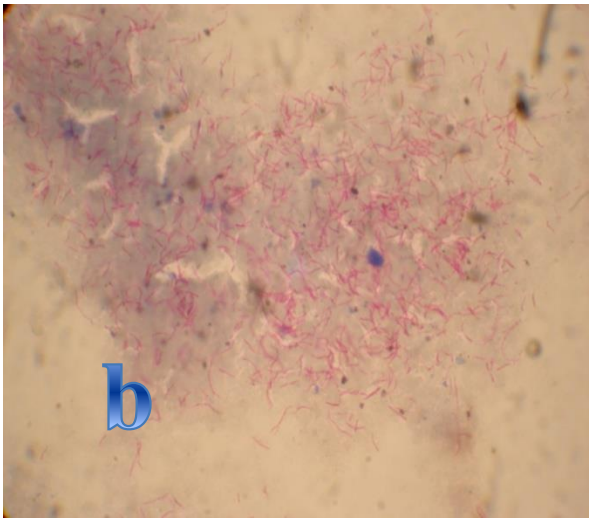
Gender	Total number of samples tested	Total number of positives
Male	641	66 (10.2%)
Female	359	28 (7.7%)

Among the sputum samples collected from 641 males, 66 (10.2%) were positive for AFB while among the 359 samples collected from female patients, 28 (7.7%) were positive for AFB.

The positivity rates compared among the genders showed no statistically significant difference among them (x^2= 1.526, p = 0.2167).

Though there was no difference in the positivity rates detected between the direct smear microscopy and USP smear microscopy, there was difference in the microscopic picture of the stained smear observed at the end of the tests.

Figure 3. Microscopic pictures of USP and direct smears after ZN stain



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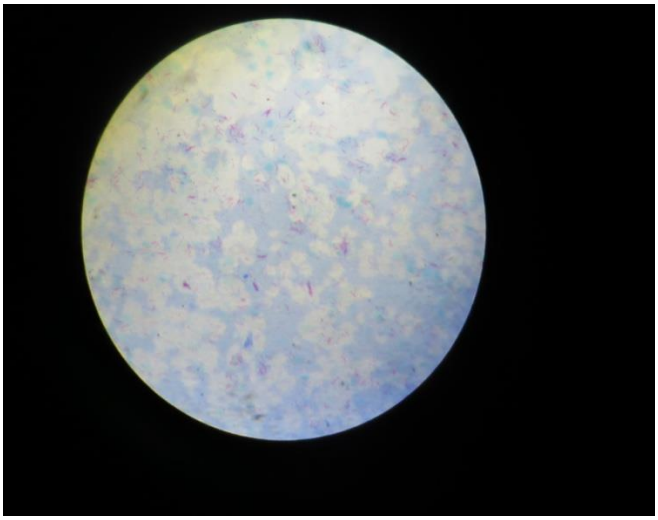


The USP smear microscopy showed a clear background without any cells and cellular debris (figure 3.a) and the bacilli were seen clumped together at one or more sites in contrast to the direct smear microscopy (figure 3.b) which showed acid fast bacilli distributed in singles or small groups of two or three on thick, heavily counterstained blue background with numerous pus cells, macrophages and lymphocytes. The USP smear microscopy could not be graded as the AFB were seen clumped together. The smear required to be focused in low power initially to identify the muck which acted as an indicator for the presence of bacilli in the vicinity and then focused under the oil

immersion lens for observing the details. In contrast the bacilli in direct smear microscopy were identified under oil immersion lens directly.

Table 5. Presents the grading of the positive samples for AFB in direct smear microscopy according to RNTCP guidelines.

Table 5. Grading of AFB by direct smear microscopy

Grading for AFB	Total number of samples
Scanty	12 (12.7%)
1+	24 (25.53%)
2+	28 (29.7%)
3+	30 (31.9%)
Total number of positives	94

Among the 94 samples positive for AFB, most were grade 3+ (31.9%), followed by grade 2+ (29.7%). There was no difference in the appearance of positives by direct and USP smear microscopy based on grading in the direct smear microscopy.

The number of sputum samples collected from each sampling center and the positivity rate among them is shown in table 6.

Table 6. Total number of samples positive in each RNTCP center

RNTCP centers	Total number of samples tested	Total number of positives by direct smear microscopy	Total number of positives by USP smear microscopy
SNR hospital, Kolar	704	65 (9.2%)	65 (9.2%)
R.L. Jalappa	296	29 (9.7%)	29 (9.7%)

hospital, Kolar			

Among the 704 sputum samples collected from SNR hospital, 65(9.2%) were positive for AFB and among the 296 samples collected from R.L. Jalappa hospital, 29 (9.7%) were positive for AFB.

There was no statistically significant difference ($p > 0.75$) in the proportion of positives detected at SNR and R.L.Jalappa hospitals.

The culture outcome of the smear positive samples inoculated onto LJ medium is shown in table 7.

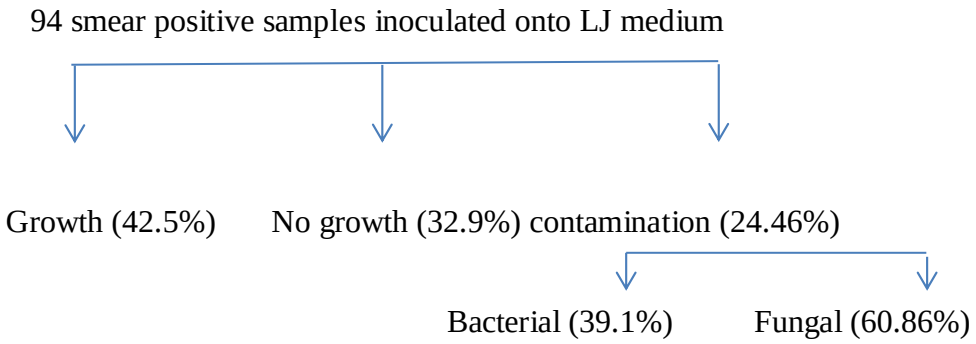
Table 7. Growth on LJ medium

Total number of smear positive samples inoculated	**N=94**
Growth on LJ medium	40 (42.5%)
No growth	31 (32.9%)
Contamination	23 (24.46%)

The mean time taken for Mycobacterial growth on LJ medium was 22 days.

Figure 4. Culture on LJ medium showing Mycobacterial growth and fungal contamination respectively.

Figure 5. Flow chart showing the growth and contamination rates on LJ medium



None of the patients included in our study were on treatment. Of the 94 smear positive samples inoculated onto LJ medium, 42.5% showed Mycobacterial growth and contamination was observed in 23 (24.46%) samples.

The type and rate of contamination of the smear positive samples inoculated onto LJ medium is shown in table 8.

Table 8. Results of the observations culture on LJ medium

Time period	**Bacterial contamination n=9 (39.1%)**	**Fungal contamination n=14 (60.86%)**
First week	9	2 (14.2%)
Second week	-	7 (50%)
Third week	-	5 (35.7%)
Fourth week	-	-

Among the 23 samples inoculated onto LJ medium which got contaminated, fungal contamination predominated. Fungi accounted for 14 (60.86%) of the contaminants which grew most often in the second and third weeks after inoculation. Bacteria accounted for 9 (39.1%) of the contaminants which grew mostly with in first week.

The time taken to detect the AFB in direct smear and USP smear microscopy by two different RNTCP technicians in the two RNTCP centers is shown in table 9.

Table 9. Time taken to detect AFB in direct smear and USP smear microscopy

Grade	Time taken to detect AFB in Direct smear microscopy in seconds	Time taken to detect AFB after USP smear microscopy in seconds	p value at 95% CI
1+	43.44	42.94	0.8769
2+	28.38	26.94	0.1698
3+	14.00	12.19	0.1840
Total	28.40	27.40	0.7236

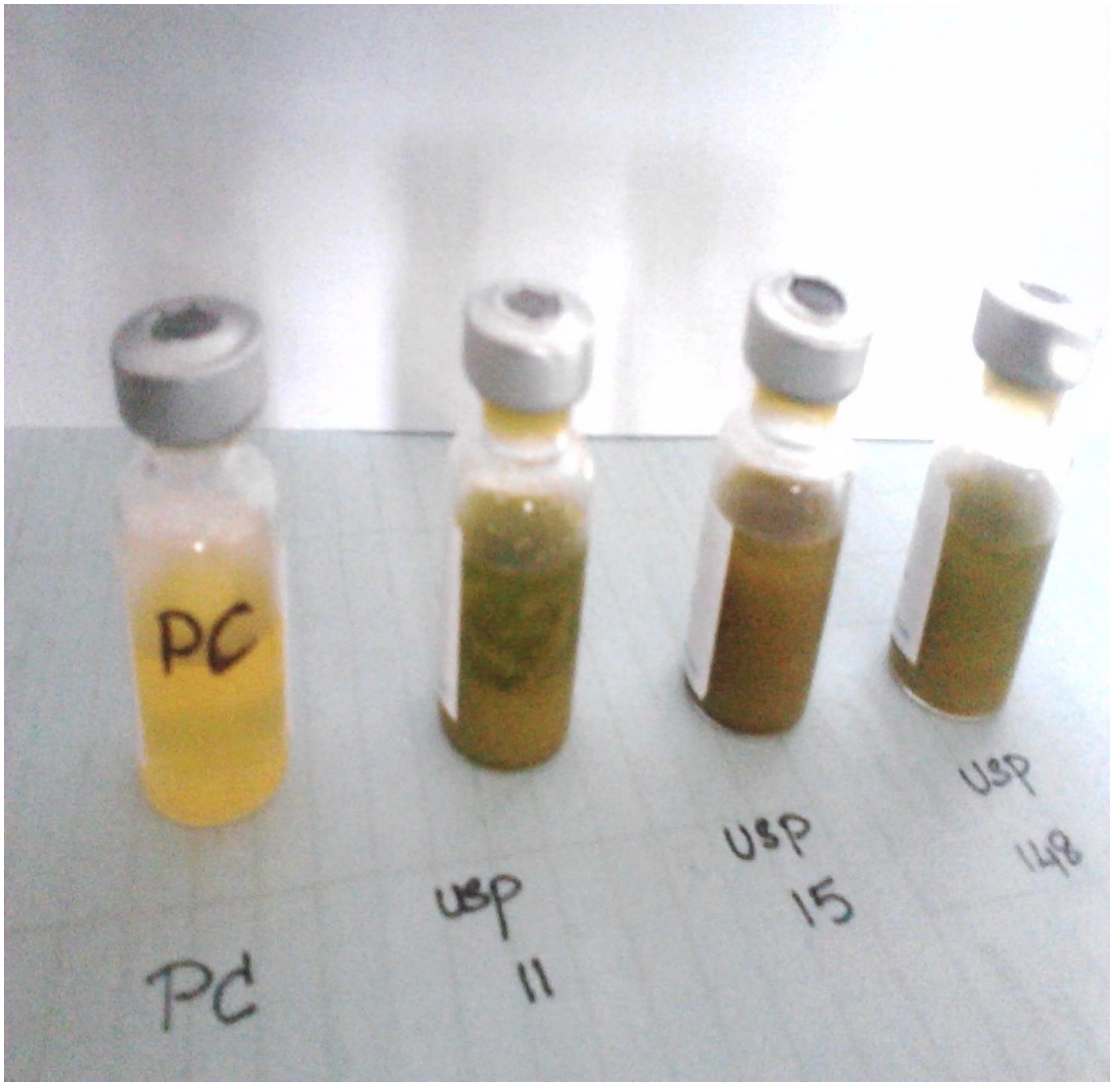
There was no statistically significant difference in the time taken to detect the AFB in direct and USP smear microscopy methods (p= 0.7236, at 95% CI).

Niacin test

The test was performed on all 42 samples positive for AFB which grew on LJ medium. All were niacin test positive. The development of yellow or yellowish green colour is interpreted as positive test.

Figure 6. Shows the Positive niacin test (yellowish green in colour) performed on the positive samples which grew on LJ medium with the Positive control

The positive niacin test is yellowish green in colour as the LJ media was scraped during the test.



Discussion

Tuberculosis is one of the most common airborne infectious diseases and is rampant in developing countries like India. It is a chronic, intracellular infection which is treatable with anti-tubercular drugs given over a period of 6 months to 1 year.

The infection remains asymptomatic most often or may manifest with clinical illness. The patient with tuberculosis suffers from fever, cough with expectoration, and weight loss. The patient shedding tubercle bacilli in his sputum is an open case who is responsible for the spread of infection. The main stay in diagnosing this infection is ZN staining which is inexpensive, easy to perform and also the technicians can be trained easily to perform the test. ZN staining can detect 10,000 AFB/ml [36]. A newer technique called Universal Sample Processing (USP) has been claimed to detect as low as 250-300 bacilli/ml and also to detect positives from smear negative pulmonary tuberculosis. However there is no study under the RNTCP set up which has evaluated the USP technique for the detection of AFB.

Among the 1000 sputum samples processed 704 (70.4%) were from patients who visited the RNTCP center at SNR hospital, the government district hospital, Kolar and 296 (29.6%) sample were from R.L.Jalappa hospital, Kolar.

The study received more number of samples from SNR hospital, Kolar compared to R.L. Jalappa hospital, Kolar, as SNR hospital is a government set up and it gets more referrals for evaluation of TB from surrounding Primary health centers.

In the study carried out here we included subjects of all age groups and both the sexes with suspected tuberculosis. The youngest patient included in our study was 7 year old and oldest patient was of 90 years. Mean age of the patients from whom sputum samples were collected was 42.36 years. Maximum number of patients with history of cough for 2 weeks duration, who were studied by us were in the age group of 20-40 years (39.2%) followed by more than 60 years (23%) . Thus the predominate group which was included in our study forms economically most productive age group in any society.

Our findings are similar to those of Shivaraman et al who have reported that 40.8% of their study subjects belonged to this age group [63]. In a study by Narang et al. at Wardha, 26.30% belonged to this age group [64]. A study by S.B.Richards et al. has reported that 61% of their study population was in this age group [65]. The reasons that make this age group more vulnerable are that they are socially more active and may be they are exposed to an open case of tuberculosis more when compared to other age groups. There were very less pediatric patients in our study; this could be due to the well-known fact that children cannot bring out adequate sputum for microscopic testing.

In our study there were 641 (64.1%) males and 359 (35.9%) females. Male to female ratio was 1.7:1. Narang P et al. have reported 61.03% of their subjects were males while 38.97 % females [64]. Peter Eriki et al. and Fandinho et al. have reported male to female ratio of 1.8:1 and 1.6:1 respectively [66, 67]. All these findings are comparable to our study. The likely reasons for male preponderance is that the males are the usual breadwinners of the family, especially in India, who go out for work and hence more likely to come in contact with open cases. Males are also more likely get exposed to risk factors like smoking and alcoholism.

The spot and the early sputum samples collected were subjected to direct smear microscopy and USP smear microscopy. Of the 1000 samples processed, 94 (9.4%) were positive by both direct ZN staining and ZN staining after USP processing. The specimens which were positive by direct smear microscopy were also positive for AFB after USP smear microscopy. Since, the sensitivity of direct sputum microscopy is low, it is expected to miss the diagnosis of some pulmonary TB patients, and USP smear microscopy was expected to detect at least some of these as it was claimed to have a better sensitivity. But, in our study, none of the sample which were negative by

direct smear microscopy were positive for AFB after USP smear microscopy. But in contrast according to a study conducted by Chakravorty et al . USP smear microscopy showed a sensitivity and specificity of 98.2% and 91.4% respectively compared to 68.6% and 92.6%, by direct ZN staining method [4]. In addition they also detected 100 samples which were negative by direct smear microscopy, as positive for AFB after USP smear microscopy which were also positive by culture [4]. Another study conducted by Chakravorty et al on extra-pulmonary specimens showed that sensitivity of USP smear microscopy was 21.1% (16 of 76), whereas that of the conventional method was only 3.9% (3 of 76) [68]. The explanation offered for the higher sensitivity of the USP smear microscopy is that it can detect as low as 300-500 bacilli/ml of the specimen, the efficient removal of the pus cells and other cellular debris without any deleterious effect on *M. tuberculosis*, the centrifugation step, during processing of the sample aggregates the bacilli in the sediment and also the amount of sample for making the smear consisted of about 10% of the processed material whereas very minute sample is used in direct smear microscopy.

In contrast to the results by Chakravorty et al. a study conducted by Cattamanchi et al. in Uganda showed that the USP method did not significantly improve the overall diagnostic performance of smear microscopy whose results were similar to our study [69]. The reasons explained by Chakravorty et al. for the poor performance of USP technique is that, in the study by Cattamanchi et al the samples were sedimented at a lower centrifugation speed 3,000rpm instead of the recommended 5,000 to 6,000 rpm. The culture positivity could be compromised by the inefficient bacterial sedimentation, incomplete removal of guanidinium hydrochloride (GuHCl), or the use of 4 to 6 M GuHCl [70].

A study conducted by Daley et al. at Christian Medical College, Vellore showed that short duration of sputum pretreatment with bleach and USP centrifugation did not increase the yield when compared to direct sputum smears [71].

In our study the smear microscopy after USP method showed pink colored AFB on a clear background with no other cells. This is due to the chemical guanidinium hydrochloride, a chaotropic agent used in the processing of sputum samples. It disrupts the cell wall of other cells/organisms present in the specimen by interfering with the stabilizing intra-molecular interactions mediated by non-covalent forces such as hydrogen bonds, vanderwaals forces and hydrophobic effects. But the unique characteristic of mycobacterial cell wall which is made up of mycolic acid, resists the action of guanidinium hydrochloride. Thus AFB is seen on a clear background in USP smear microscopy.

Grading of smears for AFB gives an idea regarding the bacterial load. It is an important indicator of patient's response and adherence to treatment, which also gives an idea about developing resistance. In our study, the smear microscopy after USP treatment could not be graded for AFB. The AFB bacilli were seen clumped together in one or more sites which made the smear ineffective for grading. This is due to the fact that during the processing of sputum samples, the USP method involves a step of centrifugation at 5000-6000 rpm for 20 min which aggregates the AFB into small sediment and 10% of this sediment is used for making the smear. Thus one cannot grade the slide after processing the sputum sample by USP method.

Of the 94 samples positive for AFB, 40 (42.5%) samples were positive for growth on LJ media, no growth was seen in 31 (32.9%) samples and 23 (24.46%) samples showed contamination. According to a study conducted by Cattamanchi et al. in Uganda, 31% of the samples showed growth compared to 46% after NALC method, 58% of mycobacterial cultures were negative after USP method, compared to 43% after NALC method. The proportions of contaminated cultures were 11.2% versus 11.7 for the NALC and USP methods respectively [69]. The lower mycobacterial growth rate and the high contamination rate in culture on LJ medium after USP technique was also seen in the study conducted by Cattamanchi et al.

All the samples put up for culture in our study were positive by smear microscopy but only 42.5% of them grew on LJ medium. The reason could be that a proportion of AFB might have been killed during the processing of the sputum samples by USP technique.

In our study 24.46% of the positives samples inoculated onto LJ medium showed contamination. Fungi (60.86%) were the predominant contaminants followed by bacterial contaminants (39.1%). Most of the fungal contaminants were grown by 2nd week followed by 3rd week and the bacterial contaminants were seen grown by first week. The higher fungal contamination rate on LJ medium may be due to decreased action of guanidinium hydrochloride on fungus and also the presence of Malachite green in LJ medium which suppresses bacterial growth but not the fungal growth.

In our study the mean duration of isolation of mycobacteria on LJ medium was 22 days. The period of maximum isolation was 3rd week followed by 4th week.

The time taken for the detection of AFB by two different RNTCP technicians in SNR hospital and R.L.Jalappa hospital did not show any significant difference (p=0.7236) in the speed of detection of AFB in direct and USP smear microscopy.

The processing of the sputum samples by USP method involves centrifugation at a higher speed producing aerosal, which is potentially hazardous for the person performing the test and also others.

Thus our study found that USP smear microscopy did not confer any advantage over direct smear microscopy in the diagnosis of pulmonary tuberculosis under the RNTCP set up. There was no sputum sample which was negative by direct smear microscopy that turned out to be positive by USP smear microscopy. Our study also showed that USP procedure was inefficient in containing fungal contaminations when the processed samples were cultured on LJ medium and also the USP method showed low positive culture rate

Summary

Our study included the processing of sputum samples collected from patients with suspected tuberculosis visiting two RNTCP centers situated at SNR hospital and R.L.Jalappa hospital, Kolar. The objective of our study was to compare the detection rates of AFB after USP method with that of direct smear microscopy to diagnose pulmonary tuberculosis under the RNTCP set up.

Sputum samples (both spot and early morning) collected from 1000 patients with history of cough for more than 2 weeks were subjected to direct smear microscopy and USP technique in parallel. The samples which were positive for AFB was put up for culture on LJ medium and the mycobacterial growth was confirmed by niacin test.

Of the samples processed 704 (70.4%) were from SNR hospital and 296 (29.6%) samples from R.L.Jalappa hospital, Kolar.

Majority of the patients from whom the sputum samples were collected, were adults belonging to 3rd, 4th and 5th decades of life, followed by those above 60years of age.

Among the sputum samples collected from 641 males, 66 (10.2%) were positive for AFB while among the 359 samples collected from female patients, 28 (7.7%) were positive for AFB.

Of the 1000 sputum samples processed for AFB 94 (9.4%) were positive for AFB in direct and USP smear microscopy. There was no difference in the detection rate of AFB by USP method when compared to direct smear microscopy. The USP smear showed AFB clumped together in a clear background and hence could not be graded.

Only 40 (42.5%) of the smear positive samples inoculated onto the LJ medium showed growth and the mycobacterial growth was confirmed by niacin test. Contamination was seen in 23 (24.46%) of the samples inoculated. Fungal contamination 14 (60.86%) was predominant, mostly seen in 2nd week and bacterial contamination 9 (39.1%) in the first week.

There was no difference in the time taken to detect the AFB in direct smear and USP smear microscopy as screened by the two RNTCP technicians. The USP technique could not detect any positives for AFB which were negative by direct smear microscopy and also culture after USP technique showed high contamination rates.

Thus our study showed that USP technique did not provide any additional advantage over direct smear microscopy to detect AFB under RNTCP set up.

Conclusion

In our study there was no difference in the detection rate of AFB after USP smear microscopy when compared to the direct smear microscopy. The sputum samples which were positive for AFB in the direct smear microscopy were also positive by USP smear microscopy. None of the sputum samples which were negative for AFB by direct smear microscopy were positive after USP smear microscopy.

Microscopic picture of the direct and USP smear microscopy differed substantially. The USP smear microscopy showed AFB clumped together against a clear background in contrast to the direct smear microscopy where AFB were seen against blue counterstained background with pus cells.

The USP smear microscopy could not be graded because of the clumping of AFB.

Among the smear positive samples processed by USP method, only 40 (42.5%) grew on LJ medium Contamination was seen in 24.46% (23) of the samples inoculated. Fungal contamination [14 (60.86%)] was predominant followed by bacterial contamination [9 (39.1%)].

There was no difference in the time taken to detect AFB by USP smear microscopy when compared to direct smear microscopy by experienced RNTCP technicians.

Thus USP smear microscopy did not yield any additional advantage over conventional direct smear microscopy to detect AFB in the diagnosis of pulmonary tuberculosis under RNTCP set up. Also mycobacterial culture after USP technique showed high contamination rates and low growth rate.

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1. Introduction

Tuberculosis is an ancient disease causing immense damage to the human race from time immemorial. It has been and continues to be a major public health problem in India & other developing countries[1]. With urbanization, industrialization, overcrowding facilitating its spread, the emergence of HIV infection and also the spread of multi drug resistant (MDR) TB, the situation of tuberculosis has worsened to such an extent that it has been declared a global emergency[2].

Tuberculosis caused by the acid fast bacilli (AFB), Mycobacterium tuberculosis (MTB) infects one third of the world's population. Pulmonary tuberculosis is the most common condition caused by MTB however it can affect any organ system in the body. It usually gets transmitted by air-borne droplet nuclei, produced by patients who are open cases of pulmonary tuberculosis. Therefore an important step to prevent this infection is to diagnose early and treat the patients appropriately.

Clinical diagnosis of TB has always played an important role in the control of TB even before the discovery of its causative agent. Hence, the diagnosis of TB was for a very long time, only 'clinical' and such a diagnosis was made mainly to identify and isolate, and to a small extent to attempt a cure of such patients. Robert Koch opened a new avenue in TB diagnosis, which along with the discovery of the anti -TB drugs, has given new hopes to put an end to the reign of this dreaded disease.

'Early diagnosis & treatment' was found to be an effective way to reduce the spread of TB. It is one of the mainstays in the strategy of TB control throughout the world even today, as evidenced by the World Health Organization (WHO) & Revised National Tuberculosis Control Programme

(RNTCP) recommendations. Today the techniques of diagnosis have reached such an advanced stage that, even as research in TB diagnosis continues at a rapid pace, advancing the techniques to new heights, the practical applicability of most such advanced techniques is a challenge for the TB control programmes in developing countries. In India, RNTCP runs the TB diagnostic tests to lakhs of patients annually. Hence, the programmes need a test which has good sensitivity and specificity, as well as being cost effective.

Conventional TB diagnosis mainly depends upon the bacteriological examination. Even today Ziehl-Neelsen staining remains the main stay to detect the AFB and to diagnose tuberculosis, which is recommended by RNTCP. Direct smear microscopy has a poor sensitivity of 60%–70%, and detects AFB only at concentrations of 10,000 bacilli per mL of the sample[3]. Methods to improve the smear microscopy by using fluorescent microscopy, bleach method, LED microscopy have been explored. However, smear microscopy after USP technique is claimed to detect AFB as low as 250 -300 bacilli per mL[4]. Here we conducted a study to evaluate the utility of ZN staining after Universal Sample Processing (USP) technique on the sputum samples collected from two RNTCP centers, one situated at Sri Narasimha Raja Wadeyar (SNR) hospital and the other at R.L.Jalappa hospital, Kolar for the detection of AFB.

2. Objectives

1. To compare the detection rate of microscopy done after USP with that of direct microscopy.

2. To perform microscopic examination after subjecting the sputum to Universal Sample Processing (USP) methodology on sputum samples of patients found to be negative by direct smear microscopy under RNTCP.

3. The positives detected by the above method will be confirmed by culturing Mycobacterium on LJ medium.

3. Review of literature

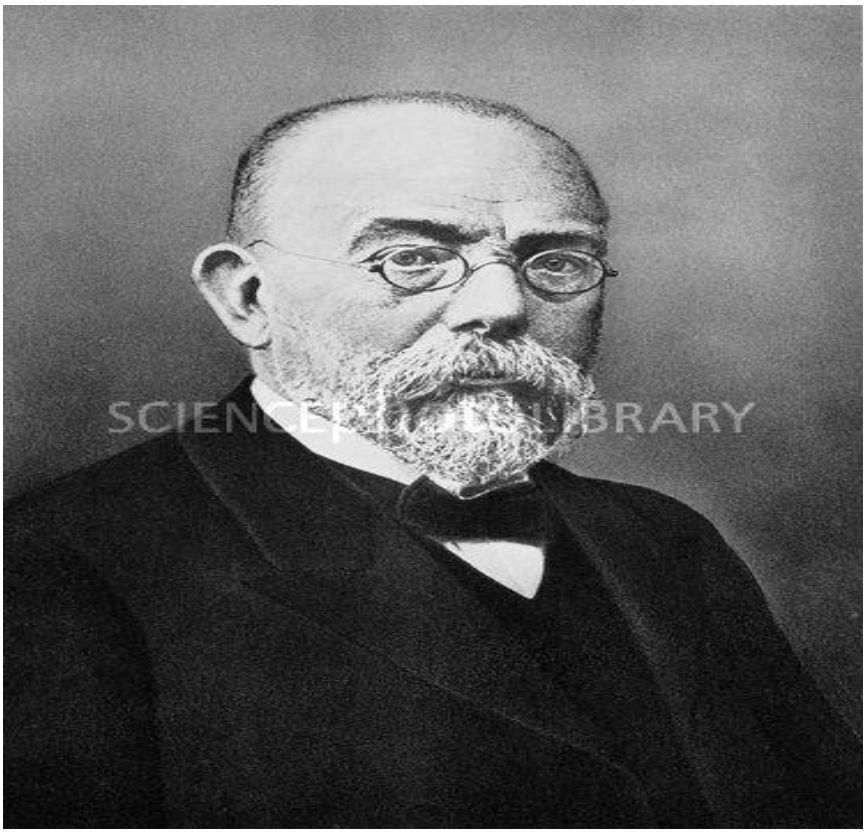
3.1 History

In India tuberculosis is a common disease caused by Mycobacterium tuberculosis, an acid fast bacillus. The disease tuberculosis has been present in the human population since antiquity. The evidence for the existence of this disease has been found since 4000BC, by the skeletal examination of the mummies with spinal tuberculosis and from Egyptian tomb paintings [5]. The first reference to tuberculosis in Asian civilization is found in the Vedas. The oldest of them (Rig-Veda, 1500 BCE) calls the disease *yaksma*[6]. One of the prayers says 'God give me a life without tuberculosis'[7]. The Atharvaveda calls it by another name: *balasa*. The first description of scrofula is given in Atharvaveda.

Tuberculosis may have emerged as a human disease during Neolithic times when human populations increased aggregated and cattle were domesticated. Examination of a skeletons dating back to fourth millennium BC, which were excavated from Arene Candida Cave in Liguria, Italy, demonstrated the evidence of tuberculosis [8].

Around 460 B.C Hippocrates and Aristotle identified tuberculosis described as 'phthisis'(consumption) as the most widespread disease of the times, and noted that it was almost always fatal. In 17th century, the tuberculosis epidemic occurred in Europe, which lasted for two hundred years, was known as the Great White Plague [9]. John Bunyan the 17th century writer referred to tuberculosis as the 'captain of all the men of death'.

Sylvius (1614-1672) was the first to identify the characteristic nodules at autopsy as 'tubercles' in the lungs and other organs [10]. He also described their progression to abscesses and cavities. Villemin (1868), a French military surgeon, succeeded in transmitting the disease to animals [11].



ROBERT KOCH (1843-1910)

In 1882, Robert Koch a Prussian physician identified a new staining method and applied it to the sputum of tuberculosis patients, revealing the causal agent of the disease for the first time which came to be known as Mycobacterium tuberculosis, or Koch's bacillus. He also cultivated the tubercle bacilli on inspissated serum. Robert Koch reported the isolation of tubercle bacilli to the Berlin Phthisiological society on 24 March 1882 where he quoted, *"I have no business to live*

this life if I cannot eradicate this horrible scourge from the mankind". Since then, 24th March has been known as World Tuberculosis Day. Robert Koch received the Nobel Prize in physiology or medicine for this discovery in 1905 [12].

The improvement of the staining technique by Paul Ehrilch in 1885 and the subsequent modification by Ziehl-Neelsen were important landmarks in the diagnosis of the disease. Paul Ehrlich (1854-1915), an assistant Professor at the Charite Hospital, Berlin, who was present at Koch's lecture on 24 March 1882 recalled his observation of tubercle bacilli in various clinical materials. Ehrlich who had already devised staining technique for mast cells, using aniline water, fuchsin and gentian-violet, experimented the same technique to demonstrate tubercle bacilli but used a shorter staining time (15 to 30 minutes, instead of Koch's 24 hours) and he also applied 30% nitric acid and alcohol for a few seconds to decolorize the surrounding tissues, while the tubercle bacilli retained the stain[12]. On counterstaining with a yellow or blue dye, the red tubercle bacilli showed up more clearly. In 1887, Ehrlich tested his own sputum, in which he found tubercle bacilli, was diagnosed to have pulmonary tuberculosis[12].

Later Ziehl introduced carbolic instead of aniline, while Neelsen advocated the use of sulphuric instead of nitric acid which was later on called as "Ziehl-Neelsen" (ZN) stain [12]. The Ziehl-Neelsen staining is an important technique followed even today in diagnosing tuberculosis. In 1890 Koch developed tuberculin, a purified protein derivative (PPD) of the bacteria for immunization but it was declared ineffective. In 1908, Charles Mantoux found it was an effective intradermal test for diagnosing tuberculosis. The invention of X-rays by Roentgen in 1895 further helped in the confirmation of clinical diagnosis of tuberculosis. Fluorescent microscopy was introduced by Hageman in 1937 for the diagnosis of tuberculosis.

In 1907 A.S.Griffith and F.Griffith working for Royal commission of tuberculosis, reported dorset egg as the most satisfactory medium for cultivation of Mycobacterium tuberculosis (MTB). In 1930 Lowenstein modified the dorset egg medium. In 1946 Copper and Cohn introduced the use of malachite green for the suppression of contaminants. Jensen in 1955 further modified it by using glycerol as carbon source instead of starch. To date culture on Lowenstein-Jensen medium is an important technique followed for the isolation of Mycobacterium tuberculosis. Canetti et al (1963) described the criteria and techniques for the reliable testing of Mycobacterial resistance to tuberculosis[13]. Deland and Wager (1969) developed techniques for automated detection of bacterial metabolism by measuring radioactive carbon dioxide liberated during decarboxylation of C^{14} labelled substrates in the medium[14]. Middlebrook et al (1977) later modified this technique by using 7H12 medium. Drug susceptibility testing with the above principle was introduced by Snider and colleagues (1981).

The idea of sanatoriums aroused as a result of scientific understanding and the contagious nature of the disease. George Bodington (1840) made the first proposal for a tuberculosis facility in his paper titled '*An essay on the treatment and cure of pulmonary consumption, on principles natural, rational and successful* '. In this paper, he proposed a dietary, rest, and medical care program for the patients[15].

MAMMOTH CAVE (USA)

In United States Dr. John Croghan (1842), the owner of Mammoth Cave, brought 15 tuberculosis sufferers into the cave in the hope of curing the disease with the constant temperature and purity of the cave air[15]. Patients were lodged in stone huts, and each patient was attended by servant to bring meals and other needs. John Croghan himself died of tuberculosis in 1849.

The first anti-tuberculosis sanatorium was established in 1854, 650 meters above sea level, at Görbersdorf. Brehmer and one of his patients, Peter Dettweiler, started the sanatorium movement, and by 1877, sanatoriums began to spread beyond Germany and throughout Europe. Specialized tuberculosis clinics began to develop in other major metropolitan areas. In India, the first sanatorium for the treatment and isolation of patients suffering from tuberculosis was founded in 1906 in Tiluania, near Ajmer. In 1912 the United Mission Tuberculosis Sanatorium (UMTS) was built at Madanapalle, south India[16]. Dr Frimodt Moller, the first Medical

superintendent during that period played an important role in India's fight against tuberculosis through training the TB workers, conducting TB surveys and by introducing BCG vaccination [16].

POSTAGE STAMP - Albert Calmette and Camille Guérin

Albert Calmette and Camille Guérin (1908) developed the vaccine against tuberculosis from attenuated bovine-strain. It was called "BCG" (*Bacille Calmette-Guérin*). Albert Calmette, a French bacteriologist, and his assistant Camille Guérin, a veterinarian, working at the Pasteur institute, France (1908), noted that tubercle bacilli grown in glycerin-bile-potato mixture seemed less virulent. This changed the course of their research, to see if repeated sub culturing would produce a strain that was attenuated enough to be considered for use as a vaccine. The BCG vaccine was first used in humans in 1921 in France. In Lübeck (1930), 240 infants were vaccinated in the first 10 days of life; almost all developed tuberculosis and 72 infants died. It was then discovered that the BCG vaccine that had been administered was contaminated with a virulent strain that was being stored in the same incubator, and led to legal action being taken against the manufacturers of BCG. In 1928, BCG was adopted by the Health Committee of the League of Nations (predecessor to the WHO)[17,18]. Because of opposition, however, it did not become widely used until after World War II.

Rene Jules Dubos (1901-1982) a French born American Microbiologist studied the physiology and immunology of the tubercle bacillus and tuberculosis infection—an investigation which was stimulated by the illness and the death of his wife due to tuberculosis. He introduced wetting agents into the culture medium to enable the diffuse growth of tubercle bacilli which brought about a revolution in tuberculosis research. The wetting agents used were Tween and other factors like albumin, fatty acids, which led to diffuse growth of tubercle bacilli. This culture method helped the researchers to make accurate, quantitative studies of various strains of tubercle bacilli and also to study the virulence and pathogenic properties of tubercle bacilli. He also pioneered international standards for the BCG vaccination against tuberculosis and described the social aspects of the disease in "The White Plague" (1952)[9]. Later he investigated how the environmental effects of crowding, malnutrition, pesticides, toxins, and stress increased the susceptibility to tuberculosis.

Streptomycin, the first antibiotic and first bacterial agent effective against M. tuberculosis was discovered by Albert Schatz, Elizabeth Bugie, and Selman Waksman in 1944. In 1952 Isoniazid, the first oral Mycobactericidal drug was discovered. In 1950 para-aminosalisylic acid (PAS) was added to the therapy. In 1956 it was shown that pyrazinamide was active within the macrophage against the tubercle bacilli and by mid I970s this agent was also included in the anti-tuberculosis regimen. The advent of Rifampicin in the 1970s significantly reduced the number of tuberculosis cases until the 1980s[19]. With the discovery of anti-tuberculosis drugs, sanatorium based treatment became unpopular.

In India, the National Tuberculosis Programme (NTP) was started in 1962 in view of reducing the burden of tuberculosis, which was based on the research by Tuberculosis research centre (TRC), Chennai and National TB Institute (NTI), Bangalore[20]. At first the National programme was designed for domiciliary treatment using self-administered standard drug regimens. Though a large network of District TB centers were created with trained staff and infrastructure, the impact on the TB burden till 1992 was very minimal. A review of this programme in 1992 revealed that the NTP did not achieve the objectives because of low priority, managerial weaknesses, over dependence on X-rays for diagnosis, inadequate funding and incomplete treatment due to low rates of treatment adherence and lack of supervision.

Based on the recommendations of an expert committee, a revised strategy to control TB, Revised National Tuberculosis Control Programme (RNTCP), a state-run tuberculosis control programme was initiated by the Government of India in 1992. It incorporates the principles of directly observed treatment-short course (DOTS), the global TB control strategy of the World Health Organization. The program provides, free anti-tubercular drugs across the country through the numerous Primary Health Centers .It was pilot-tested in 1993 in a population of 2.35 million which was later increased in phases. A full-fledged revised programme was started in 1997 which rapidly expanded with good results[20].

By June 2005 the RNTCP had covered more than 1 billion population (more than 90% of the country was covered). Since the introduction of RNTCP, up to June 2005, more than 4.5 million patients were initiated on treatment and about 750,000 additional lives were saved.

Directly observed short course chemotherapy (DOTS) is the internationally recommended strategy for TB control with the objective of improving patient compliance and ensuring complete treatment and also decrease the prevalence of drug resistance in tuberculosis [20].

With the emergence of human immunodeficiency virus (HIV) infection and acquired immunodeficiency syndrome (AIDS), there was a resurgence of tuberculosis in the late 1980s in the developed countries while the poor socioeconomic conditions and increased immigrant population contributed to the situation in the developing countries which were already burdened with tuberculosis[21]. Noncompliance by the patients and incomplete and inadequate treatment further aggravated the problem with the emergence of multi drug resistance strains. Multi drug resistant strains of Mycobacterium tuberculosis made current chemotherapy regimens ineffective. In response to the resurgence of tuberculosis, the World Health Organization issued a declaration of a global health emergency in 1993.

In 1994, WHO an International Union Tuberculosis and Lung Disease (IUALTD) proposed a global tuberculosis surveillance programme to collect data on the global extent of severity of anti TB drug resistance in a standardized manner at country or regional level. In 2006, WHO launched the STOP TB strategy, which is to be implemented from 2006-2015[22].

Table I: Historical milestones in Tuberculosis

Year	Scientist	Discovery
460 BC	Hippocrates and Aristotle	Identified tuberculosis as 'phthisis'(consumption).
1670	Sylvius	Identified 'tubercles'
1854	Sanatorium	First tuberculosis sanatorium was established , at Görbersdorf
1868	Villemin	Succeeded in transmitting the disease to animals.
1882	Robert Koch	new staining method cultivated the tubercle bacilli Koch's bacillus
1885	Paul Ehrlich	Improvement in staining technique
1890	Robert Koch	Tuberculin – purified protein derivative
1895	Roentgen	X- rays for diagnosis
1907	A.S.Griffith and F.Griffith	dorset egg medium for cultivation of MTB
1908	Albert Calmette and Camille Guérin	BCG vaccine
1930	Lowenstein	Modified dorset egg media
1937	Hageman	Fluorescent microscopy
1944	Rene Jules Dubo	Introduced wetting agents into the culture medium to

		enable the diffuse growth of tubercle bacilli which helped in quantification and to study the virulence and pathogenic properties of tubercle bacilli
1946	copper and Cohn	Malachite green to inhibit the contaminants in LJ medium
1955	Canetti et al	glycerol as carbon source instead of starch
1962		National Tuberculosis programme by government of India
1963	Canetti et al	Tests for mycobacterial resistance
1969	Deland and Wager	techniques for automated detection of bacterial metabolism
1992	TRC, Chennai and NTI, Bangalore	DOTS therapy India
2006	Government of India	STOP TB strategy

3.2 Genus definition:

M.tuberculosis belongs to Order – Actinomycetales **Family –** Mycobacteriaceae **Genus –** Mycobacterium

The Greek prefix *myco*= "fungus" is used, as mycobacteria when cultured in liquid medium shows mould-like growth on the surface. The genus includes pathogens known to cause serious

diseases in mammals, including tuberculosis (Mycobacterium tuberculosis) and leprosy(Mycobacterium leprae).

The genus characteristics are,

1. Mycobacteria are non-motile, non-spore forming, weakly Gram positive, aerobic and micro-aerophilic. Straight or slightly curved rod -shaped bacteria, measuring 0.2-0.6x 1-10 micrometer in size[23].

2. Acid fastness (resistance to decolourisation by acid being stained with a basic fuchsin dye)

3. The cell wall consists of the hydrophobic mycolate layer and a peptidoglycan layer held together by a polysaccharide, arabinogalactan.

4. Mycolic acid is made up of 70 carbon atoms

5. These are oxygen loving organisms

6. G+C content of the DNA of 6I-70mol%

Genomics of H37Rv strain of Mycobacterium tuberculosis

The strain H37 was originally isolated in 1905. Unlike some clinical isolates that often lose virulence after repeated passages in the laboratory, this strain has maintained its full virulence in animals experimented since its isolation. In 1934, H37 was dissociated into "virulent" (Rv) and "avirulent" (Ra) strains). The original 1905 H37 isolate was then discontinued, and the H37Rv and H37Ra isolates have been maintained at the Trudeau Institute. Its size is 4 million base pairs, with 3959 genes; 40% of these genes have had their function characterized. There are also six pseudogenes.

The genome consists of 250 genes which are involved in fatty acid metabolism, with 39 of these involved in the polyketide metabolism generating the waxy coat. About 10% of the coding capacity is taken up by two clustered gene families that encode acidic, glycine-rich proteins. These proteins have a conserved N-terminal motif, deletion of which impairs growth in macrophages and granulomas. Nine noncoding sRNAs have been characterized in M. tuberculosis, with a further 56 predicted in a bioinformatics screen.

3.3 Epidemiology

It is estimated that one third of the world's population is infected with M.TB, and that each year, about 9 million people develop TB, one in ten of whom is HIV positive, equivalent to about 1.1 million (13%) in 2010[24].

In countries worldwide, the reported percentage of all TB cases occurring in children varies from 3% to more than 25%. About 5 per cent of those infected are likely to develop disease in the first year after infection and the remaining 5 per cent during their lifetime. These rates increase about six-fold in HIV infected individuals.

According to the Global TB report 2011, there were 8.8 million (range, 8.5–9.2 million) cases of TB, 1.1 million (range, 0.9–1.2 million) deaths from TB among HIV-negative people and an additional 0.35 million (range, 0.32–0.39 million) deaths from HIV-associated TB in 2010. Most of these occurred in Asia (59%) and Africa (26%)[24].

The five countries with the largest number of incident cases in 2010 were, developing countries like India (2.0 million–2.5 million), China (0.9 million–1.2 million), South Africa (0.40 million–0.59 million), Indonesia (0.37 million–0.54 million) and Pakistan (0.33 million–0.48 million). India ranks number 1 among all countries in terms of the total number of incident cases of TB & harbors one fifth (20%) of global TB burden. Of the 9 million annual TB cases, about 1 million (11%) occur in children (under 15 years of age). Of these childhood cases, 75% occur annually in 22 high-burden countries that together account for 80% of the world's estimated incident cases[25].

India alone accounted for an estimated one quarter (26%) of all TB cases worldwide, and China and India combined accounted for 38%.

The proportion of TB cases co-infected with HIV is highest in countries in the African Region; overall, the African Region accounted for 82% of TB cases among people living with HIV. Globally, incidence rates fell slowly from 1990 to around 1997, and then increased up around 2001 as the number of TB cases in Africa was driven upwards by the HIV epidemic.

The tuberculosis mortality is decreasing globally and the Stop TB Partnership target of a 50% reduction by 2015 compared with 1990 can be met if the current trend is sustained. The target could also be achieved in all WHO regions with the exception of African Region.

India has more new TB cases annually than any other country. In 2009, out of the estimated global annual incidence of 9.4 million TB cases, 2 million were estimated to have occurred in India, thus contributing to a fifth of the global burden of TB. It is estimated that about 40% of Indian population is infected with tuberculosis. The high burden of tuberculosis in India is illustrated by the estimation that TB accounts for 17.6% of deaths of all communicable diseases and 3.5% of all causes of mortality.

The prevalence of MDR-TB is less than 3% amongst new cases and 12-17% in re-treatment cases. These surveys also indicate that the prevalence of MDR-TB is stable in the country as similar prevalence results were obtained in studies conducted by TRC, Chennai and NTI, Bangalore.

The risk factors for tuberculosis infection include poverty, overcrowding, poor ventilation, immune suppressed status and extreme age group.

3.4 Pathogenesis [23]

The mode of infection in primary pulmonary tuberculosis is by direct inhalation of the aerosolized bacilli present in droplet nuclei of expectorated sputum. An open case whose sputum sample contains more than 10,000 bacilli per ml has more chances of spreading the disease. Most of the inhaled bacilli are arrested by the natural defenses of upper respiratory tract. On reaching the lungs the bacilli are ingested by the alveolar macrophages. The virulence of tubercle bacilli depends on its ability to survive within the macrophages and also to inhibit the fusion of phagosomes with lysosomes. The bacteria are disseminated by the lymphatic circulation to regional lymph nodes in the lung, forming the primary or Ghon complex. There is hematogenous circulation of the bacteria to many organs including Central nervous system (CNS) and other parts of the lung, where the disease may manifest as fatal tubercular meningitis or miliary (disseminated) tuberculosis respectively.

The outcome of infection is influenced by many other factors like genetic susceptibility of an individual, age, immune status, stress, nutrition, and co existing diseases.

The Mycobacterial antigens are presented by the antigen presenting cells to the antigen specific T-lymphocytes which undergo clonal proliferation. The activated T cells release cytokines, notably interferon gamma, which, together with calcitriol, activate macrophages and cause them to form a compact cluster, or granuloma, around the foci of infection. The center of the granuloma contains a mixture of necrotic tissue and dead macrophages and is called as caseation due to its cheese like appearance. Though most the tubercle bacilli are killed some remain in dormant form, which gets reactivated and cause post-primary tuberculosis[26].

3.5 Immunology

Cell mediated immunity plays an important role in the host defense against the MTB, and the first step in this process is recognition of MTB by cells of the innate immune system. Since the main route of entry is the respiratory route, the alveolar macrophages are the important cell type that fights the pathogen. They form the main component of the innate immune response. MTB initially infects macrophages and dendritic cells. The macrophage-Mycobacterium interaction include the binding of M.tuberculosis to macrophages via the surface receptors, mycobacterial growth inhibition or killing through free radical based mechanisms (such as oxygen and nitrogen intermediates) and cytokine- mediated mechanisms. They are also involved in mycobacterium escaping these mechanisms and its persistence. Macrophages are involved in recruitment of accessory immune cells for local inflammatory response and presentation of antigens to T cells for development of acquired immunity. Other components of the innate immune response include neutrophils, natural killer cells and natural resistance associated macrophage protein (NRAMP)[27]. MTB is surrounded by lipid-rich outer molecules as a capsule which protects it from the toxic radicals and hydrolytic enzymes produced as a defense by macrophages and inflammatory cells[28].

The specific acquired Th1 response is characterized by the production of TNF-α, IL-2 and INF-γ. These cytokines stimulate macrophages and there by cell-mediated immunity which is important against the intracellular pathogens. Cytokines like interleukins 4, 5 and 10 induce delayed type of hypersensitivity, tissue destruction and progressive disease. Studies have shown that defects in the generation of the Th1 cell effector INF-γ results in susceptibility to TB infection[29]. Robert Koch (1891) noticed a necrotic lesion at the site of inoculation of MTB

bacillus in a previously infected guinea pig which came to be known as Koch's phenomenon. It is a delayed type of hypersensitivity reaction which demonstrates the cell mediated immunity and also forms the basis for Tuberculin test.

Tuberculin reaction

The development of DTH reaction is an attempt at preventing reinfection by same the organism. Sub cutaneous inoculation of tubercle bacilli in an normal individual produces no immediate response, but a nodule develops at the site of inoculation in 10 to 14 days which ulcerates. Regional lymph adenitis is seen. In contrast, inoculation into the previously infected individual results in indurated area at the site of injection within 1 to 2 day with no lymphadenitis. The bacterial cell wall component lipopolysaccharide-protein complex is the responsible antigen. This reaction is permanently / transiently negative in individuals in whom CMI are transiently or permanently impaired which is due to anergy.

Anergy is diminished or absent DTH or type IV hypersensitivity reaction which occurs as a result of lack of responsiveness to commonly used skin test antigens like PPD, histoplasmin. Here there is decreased capapcity of T.lymphocytes to secrete lymphokines when their T cell receptors interact with specific antigens. Anergy is often associated with lack of co stimulation[30].

3.6 Pathology [28, 31]

MTB infection produces characteristic lesions called 'Tubercle' in the infected tissue. These are avascular granulomas composed of a central zone containing giant cells, with or without caseation, and a peripheral zone of lymphocytes and fibroblasts. Depending on the time of infection and the type of response, tuberculosis may be classified as 'primary' and 'post primary'.

Primary tuberculosis is the form of disease that develops in a previously unexposed and therefore unsentisized individuals. The bacilli, which are engulfed by alveolar macrophages multiply and give rise to a sub pleural focus of tuberculous pneumonia, commonly located in the upper part of the lower lobe or lower part of the upper lobe. The involved area is called the Ghon focus. The Ghon focus together with the enlarged hilar lymph node and intervening lymphangitis constitutes 'the primary complex'. This occurs 3-8 weeks from the time of infection and is associated with the development of tuberculin hypersensitivity. In most cases, the lesion heals spontaneously in 2-6 months leaving behind a calcified node. The implications of primary tuberculosis are hypersensitivity reaction which causes tissue damage, scar formation that may harbor viable bacilli for years which later on may get reactivated and progressive primary tuberculosis which is most commonly seen in immunocompromised individuals. A few bacilli may survive in the healed lesion and remain latent.

The post-primary tuberculosis, also called secondary or adult type of tuberculosis arises in a previously sensitized host. It is either due to the reactivation of latent infection, (in which case it is called post-primary progression or endogenous reactivation) or due to exogenous re-infection. It affects mainly the upper lobes of the lungs and the lesions undergo necrosis and tissue destruction, leading to cavitations. Lymph node involvement is unusual. The necrotic materials break down into the airways, leading to expectoration of

bacteria-laden sputum, which is the main source of infection to others. With the dissemination of infection tubercle bacilli can cause meningitis, genital tuberculosis leading to infertility, intestinal tuberculosis leading to fibrosis and perforation. In immunodeficient individuals, instead of cavity formation there is widespread dissemination of lesions in the lungs and other organs.

3.7 Clinical features [32]

Tuberculosis can manifest as pulmonary or extra pulmonary tuberculosis. Pulmonary tuberculosis manifest usually as asymptomatic in early stage of the disease. The disease tuberculosis, according to the status of the patient's immune system may develop differently in each patient. The disease can manifest as latent infection, primary disease and extra pulmonary disease. Each stage has different clinical manifestations

Persons with latent tuberculosis though infected, do not show any signs or symptoms of the disease. But, the viable bacilli can persist in the necrotic material for years or even *lifelong*, and if the immune system of an individual *becomes* compromised later the disease gets reactivated. Co-infection with human immunodeficiency virus is the most common cause for progression to active disease; other factors include uncontrolled diabetes mellitus, chemotherapy, organ transplantation, long-term corticosteroid usage.

Primary pulmonary tuberculosis is often asymptomatic. Lymphadenopathy occurs as the bacilli spreads from the lungs through the lymphatic system. Patient develops pleural effusion because the tubercle bacilli infiltrate the pleural space. The effusion may remain small and resolve

spontaneously, or it may become large to induce symptoms such as fever, pleuritic chest pain, and dyspnea. Dyspnea occurs as a result of poor gas exchange in the areas of affected lung tissue[33].

Active tuberculosis develops in only 5% to 10% of persons exposed to M tuberculosis. The early signs and symptoms are often nonspecific as the patient progresses to active tuberculosis. Manifestations often include progressive fatigue, malaise, weight loss, and a low-grade fever usually associated with evening rise of temperature. Wasting is a classic feature of tuberculosis. It is due to the lack of appetite and the altered metabolism associated with the inflammatory and immune responses. Wasting involves the loss of both fat and lean tissue; the decreased muscle mass contributes to the fatigue. The patient may develop cough which is non-productive initially, but later develops productive cough expectorating thick sometimes blood streaked sputum. The patient also suffers from anemia which causes fatigue and weakness. Leukocytosis occurs in response to the infection and inflammatory process.

Though pulmonary tuberculosis is the most common form, extrapulmonary tuberculosis occurs in more than 20% of immunocompetent individuals , and the risk for extrapulmonary disease increases further in immune suppressed individuals. Lymphatic tuberculosis is the most common extrapulmonary tuberculosis, and cervical adenopathy is involved most commonly. The most serious extra pulmonary tuberculosis is the involvement of central nervous system, where infection may result in meningitis or space occupying tuberculomas which is often fatal. Another fatal form of extra-pulmonary tuberculosis is disseminated or miliary tuberculosis. The bacilli can then spread throughout the body, leading to multi organ involvement. Miliary tuberculosis progresses rapidly and can be difficult to diagnose because of its systemic and

nonspecific signs and symptoms. Other possible locations include bones, joints, pleura, and genitourinary system[33].

3.8. HIV infection and Tuberculosis

Tuberculosis is the most common opportunistic infection amongst HIV-infected individuals. The rapid surge of HIV infection in developing countries like Africa, India has resulted in the increased incidence of tuberculosis. The co-existence of HIV infection with tuberculosis is called as 'The cursed duet'[34]. HIV infection is the most important factor responsible for developing tuberculosis and tuberculosis is a leading cause of HIV - related mortality and morbidity.

People once infected by tubercle bacilli have about, 10% chance of developing tuberculosis during the remainder of their lives, whereas an HIV positive person, already infected by tubercle bacillus has a 5-15% chance of developing overt disease annually or up to 50% related morbidity and mortality. According to the sentinel surveillance, India accounts for one eighth of all HIV infections in the world. TB occurs in 60% to 70% of HIV positive patients in India, majority of who are in the economically active age groups. It is being predicted that if India does not cap the HIV/AIDS epidemic, it would affect the economy of the nation in the coming years.

The CD4+ T cell depletion due to HIV infection results in dissemination of M.TB. There is reduced immune response in HIV infected patients, which contributes to their susceptibility to tuberculosis. The susceptibility to get infected as well as to develop tuberculosis disease is higher among HIV infected patients. Also these patients are increasingly susceptible to re-infection even after treatment. At the site of active TB infection, macrophages infected with M.

tuberculosis expresses tumour necrosis factor-α (TNF-α), which along with Monocyte Chemotactic Protein 1(MCP 1), transcriptionally activates HIV-1 replication. The tubercle bacillus also enhances the HIV replication by inducing nuclear factor kappa-B, the cellular factor that binds to promoter regions of HIV. Thereby, the immune response to TB bacilli and the bacilli themselves increase HIV replication (viral load increases by 6 to 7 fold), as a result there is rapid progression of HIV infection [35].

The clinical presentation of tuberculosis in most patients with HIV is indistinguishable from those who do not have HIV infection. The clinical manifestation of TB in HIV infected patients depends on the degree of immunosuppression. Post primary TB (classical reactivation-type disease) is the most common among People living with HIV/AIDS (PLHAs). In the presence of severe immune deficiency, the disease presents atypically. Cavity formation is less common in patients with pulmonary disease. Most commonly the lower lobes are involved when compared to the apical lobe, this leads to smear negativity but culture could be positive. Induced sputum or bronchoscopy specimens may be useful in diagnosis. Pleural effusions also occur more frequently with HIV infection

More than 50% of cases with PTB occur in patients with CD4 counts more than 200cells/microL. Patients with low CD4 T-cell counts(<200/μL) are associated with an increased frequency extra pulmonary TB (EPTB) , disseminated TB(DTB), positive mycobacterial blood cultures, with atypical chest radiographic findings. The overall prevalence of EPTB is more among people living with HIV/AIDS (being more frequent in severely immunocompromised) when compared to HIV- uninfected.

3.9 Laboratory diagnosis of mycobacterium tuberculosis

The diagnosis of pulmonary tuberculosis involves demonstration of mycobacterium tuberculosis in the sputum sample of a patient with compatible clinical picture by smear examination, culture or by molecular methods.

India has four National Reference Laboratories (NRLs), one Intermediate Reference Laboratory (IRL) for mycobacterial culture and drug susceptibility testing in each state and almost 13,000 designated microscopy centers (DMCs) which serves a population of 1,00,000 or 50,000 in tribal or difficult/mountainous areas.

At the level of Primary Health centre (PHC) - Sputum testing for mycobacterium is done as per the guidelines of RNTCP. Community health centre (CHC) provides diagnostic services through the microscopy centres and treatment services as per the Technical and Operational Guidelines for Tuberculosis Control.

Sample collection: Presently two sputum samples, one early morning sample and another spot sample, are collected from each patient with history of cough for 2 weeks. The patient is asked to collect well coughed-up sputum sample into a clean wide mouthed container[20].

Laboratory diagnosis includes,

I. Smear examination

II. Culture and identification

III. Molecular techniques

I. Smear examination

Smear examination is the most common method followed to diagnose pulmonary and extra pulmonary tuberculosis. This method is rapid and inexpensive. To detect acid fast bacilli by microscopy the clinical specimen should contain more than 10,000 organisms per ml[36].

Acid fast staining

In 1882 Ehrilch demonstrated that Mycobacteria resist decolourisation with mineral acids. This property of Mycobacteria is due to the presence of mycolic acid in its cell wall[35].

Though Ehrilch used Aniline oil as a mordant to demonstrate acid fastness, Frank Ziehl a German Bacteriologist changed the mordant to carbolic acid. In 1883 Neelsen a German Pathologist modified the concentration of the mordant Carbolic acid and incorporated it with the dye to form carbolfuchsin which is now used as Ziehl-Neelsen stain.

a. Ziehl-Neelsen staining [37]

Is the most common technique followed to demonstrate the acid fastness of Mycobacteria. The primary stain used is concentrated carbolfuchsin. Heating with the flame along with phenol present in carbolfuchsin helps in penetration of the dye, 25% sulphuric acid acts as a decolorizing agent. This is counterstained with 1% Loeffler's methylene blue.

The acid fast bacilli are stained pink and the background including pus cells and other organisms appear blue. Ziehl-Neelsen staining method has 33.79% of sensitivity and 100% specificity.

Grading is a technique followed as per the RNTCP guidelines based on the number of AFB. It is an important indicator which gives an idea about bacterial laod, whether the patient is on regular treatment or not and also about the development of resistance to the anti-tubercular drugs.

The RNTCP grading for AFB as follows [20]

	Result	Grading	Number of fields examined
More than 10 AFB per oil immersion field	Positive	3+	20
1-10 AFB per oil immersion field	Positive	2+	50
10-99 AFB per oil immersion field	Positive	1+	100
1-9 AFB per oil immersion field	Scanty	Actual number	200
No AFB per oil immersion field	Negative	0	100

Factors influencing the results of smear examination[37] are:

a. Thickness of the smear
b. Extent of decolorization
c. Type of counterstain used
d. The person examining the smear

b. Fluorescent Microscopy [36,37]

In this method flurochrome stains are used which have the property to absorb the shorter wavelength light rays and emit light rays of longer wavelength. This results in fluorescence. Auramine or Rhodamine is used as the flurochrome where in the organism appears as bright yellow-orange rods.

It is a popular screening method and is also less laborious for the technician. For positive results the specimen should contain 10,000 bacilli per ml. However as fluorescent microscope are expensive they are not be available in all laboratories.

c. LED microscopy [38]

Recently World health organization (WHO) has implemented the use of Light emitting diode (LED) microscope for the diagnosis of tuberculosis instead of conventional ZN staining. LED microscope is a newly developed technique to diagnose tuberculosis in resource-limited settings. Compared to conventional fluorescent microscopes, LED microscopes are less

expensive, require less power and are able to run on batteries with bulbs having long half-life and also do not have the risk of releasing toxic products.

LED microscopy showed a sensitivity of 84% and specificity of 98% against culture as reference standard. LED microscopy showed a statistically significant increase in sensitivity of 6% when compared to direct ZN microscopy, 5% more sensitive and 1% more specific than conventional fluorescent microscopy[38].

II. Culture

Culture is more sensitive than the smear examination and is the gold standard. The clinical specimen should contain at least 100 bacilli per ml for the culture to become positive[36]. The samples collected is digested and decontaminated before culture. The most common method followed is Petroff's method, in which 4% sodium hydroxide (NaoH) is used for digestion and decontamination. Other agents used are N-acetyl-cystiene along with 2% NaoH, 5% Oxalic acid, 13% trisodium phosphate and 1% Cetylpyridium chloride along with 2% NaoH.

There are 3 Types of media used [36]:

Egg based: LJ, Petragnani, Dorset's egg medium.

Agar based: Middlebrook 7H10 or 7H11.

Liquid based: Dubo's medium, Kirschner's, Middlebrook 7H9.

Lowenstien-Jensen (LJ) media is the commonest medium used for culture of mycobacteria. It is an egg based medium, with malachite green as a selective agent. Culture of Mycobacteria on LJ media provides a definitive diagnosis. It is more sensitive and is considered gold standard. The main drawback is the prolonged time for the organism to grow, about 6-8 weeks [37, 39].

Rapid slide culture (RSC) [40]

Robert Koch was the first person to use RSC technique which consists of human blood medium. The major drawback was the high rate of contamination. Recently it has been modified by adding antimicrobial drugs like Amphotericin B, Polymixin B, Carbenicillin and Trimethoprim to overcome contamination by bacteria and fungus. The growth in this medium is obtained in 7 days. Recently there is renewed interest in RSC as it gives faster results than the growth on LJ medium. But it could detect only an additional 1.3% more for the detection of positive over that detected by direct microscopy under the RNTCP.

Newer culture methods

BACTEC system [35]

The BACTEC 460 TB Radiometric system was first developed by Becton Dickinson in 1980. This technique includes Middle brook 7H12 or 7H13 liquid media for the growth of the mycobacteria which contains ^{14}C – labelled palmitic acid. The growth of the Mycobacteria utilizes the acid and releases radioactive CO_2 which is measured by BACTEC instrument and expressed as "growth index". The time required to detect the growth averages 9-14 days. To distinguish tuberculosis from other mycobacteria, NAP(Beta nitro alpha acetylamine beta hydroxyl propiophenone) is used. M.tuberculosis is inhibited by NAP but not by other mycobacteria. This system can be adapted for antimicrobial sensitivity testing also. The limitations of this method are that, it cannot be used to study the colony morphology, high cost of the instruments and the disposal of radioactive material used.

According to a study conducted by Mendoza et al. the BACTEC culture system detected mycobacterial growth earlier than the conventional LJ culture by 1-2 weeks and also the culture positivity rate was also more when compared to the conventional LJ medium [42].

MGIT (Mycobacteria Growth Indicator Tube) [36, 37]

MGIT is a non-radiometric, flourochrome based method for the detection of mycobacterial growth. It consists of a glass tube with modified 7H12 broth base, with basic nutrients and antibiotics. An oxygen sensitive fluorescent compound embedded at the bottom of the tube does not fluoresce in the presence of oxygen. The mycobacterial growth causes depletion of oxygen as a result of which there is fluorescence, which is detected visually under ultraviolet light or in the automated system (MGIT 960 system), by a sensor. According to a study conducted by Rodrigues et al. MGIT 960 TB system showed a positivity of 41% when compared to 24% *by* the conventional LJ medium[43].

MB/BaCT [37]

Is an automated system for colorimetric detection of mycobacterial growth. This system utilizes a colorimetric sensor and reflected light to monitor the concentration of carbon dioxide in the medium. The mycobacterial growth causes increase in the concentration of carbon dioxide in the media. This is indicated by change in the color from green to yellow. The colorimetric method does not require careful handling and disposal as required in case of BACTEC system. According to a study conducted by Brunello et al. the MB/BacT and BACTEC 460 TB systems detected 96.5 and 99.4% of all isolates, respectively, when compared to 95.9% detected by LJ medium[44].

Septi-check [37]

This is a biphasic medium system which consists of an enriched selective broth and a slide with non-selective Middle brook agar on one side and two sections on the other side, one with NAP and egg containing agar, the other with chocolate agar for detection of contamination. The biphasic nature of the system and the advantage gained by repeated exposure of the agar media from beginning to organisms if any in the broth makes it more sensitive than other methods. According to study conducted by Sewell et al. Septi-Chek detected 95% of the positive specimens, BACTEC detected all the isolates and LJ detected 75% of the 4 positive isolates[45].

MB redox

It is a system based on the reduction of tetrazolium salt indicator in liquid medium which forms red to violet particles when reduced. The growth of the mycobacteria allows an easy macroscopic visualization of the bacterial growth. This system does not require specialized machine (can be read by eye or simple spectrophotometer) but cannot be used to measure DST[37].

Thin layer agar culture (TLA)

It is a solid media which includes plates with a thin layer of 7H11 agar medium incubated in Co2 incubator and examined microscopically on alternate days for the first two weeks to look for formation of micro colonies of M. tuberculosis. The results are obtained within 5-10 days but for DST it requires 10-15 days. A study conducted by Silva et al showed that the time taken for the

positive result by thin layer 7H11 method was significantly less when compared to the conventional methods [46].

Reporter genes assay [35, 36]

Mycobacterial growth can be detected by using specific reporter genes like luciferase genes. The mycobacterial growth is indicated by the emission of light due to the replication of luciferase gene. The results can be obtained in 48 hours. These genes can also be used for anti-tubercular drug susceptibility tests.

Phage assay [35,36]

It is a phage amplification-based assay where in presence of M. tuberculosis in the clinical samples is detected by the replication of MTB-specific phages. Replication of the phages is identified by the production of plaques which can be visualized. Advantage of this assay is that it can be directly done on the sputum specimens. Drug susceptibility testing can also be performed by incorporating rifampicin into the assay – viable (rifampicin resistant) organisms support phage replication whilst dead (rifampicin susceptible) organisms do not. The assay is available as a commercial kit or as an in-house method.

Identification of MTB [37]

1. Colony morphology

Mycobacterium tuberculosis appears as dry, rough, raised, wrinkled, buff coloured colonies on solid media, hence known as "rough, buff & tough" colonies compared to the bread crumbs [47]. In liquid media the growth begins at the bottom, creeps up the sides, and forms a pellicle on the top. Virulent strains form long serpentine cords whereas the avirulent forms grow in a dispersed manner; this is due to the presence of cord factor.

2. Culture smear

Positive cultures are confirmed by acid fast staining

3. Rate of growth

MTB is slow growing & requires 3 to 8 weeks to form visible colonies on solid media. The generation time of MTB is 15-20 hours.

4. Pigment production

MTB does not produce any pigment. Produces ruff, buff, tuff colonies

5. Nitrate test

MTB reduces nitrates to nitrites.

6. Niacin test

Niacin is accumulates when MTB is grown on 'egg containing' medium.

7. Growth on paranitrobenzoic acid (PNB containing medium)

MTB being sensitive to PNB and does not grow in PNB containing media, whereas non tuberculous mycobacteria grow in the presence of PNB.

Rapid identification techniques

a. Chemical methods based on lipid profile
b. Hybridization with specific gene probes
c. Gene amplification methods

1. Analysis of the Lipid Profile :

Mycobacteria have characteristic lipid profile, which is analyzed by High performance liquid chromatography (HPLC). HPLC detects the mycolic acid 6,7 dimethyl oxycoumarin esters of mycobacterium tuberculosis[36].

2. Identification based on cell wall associated proteins

The cell free extracts prepared from mycobacterial growth are subjected to polyacrylamide gel electrophoresis and the resulting electrophoretic patterns of mycobacterial proteins are studied.

3. Nucleic acid probes[36]

These are well defined oligonucleotide probes which are developed based on the information about specific gene sequences. M.tuberculosis sequences can be detected directly from clinical specimens. This test is based on IS6110 and also on genes encoding 38kDa protein antigen and 65kDa antigen. This test can detect 100 organisms/ml. Ribosomal RNA based probes are also available. These are more sensitive than DNA probes ref. Restriction fragment length polymorphism (RFLP) methods such gene for hsp 65kDa protein, KatG and r RNA genes and sequencing of 16sRNA have been described. The RFLP technique using IS6110 repetitive sequence as a probe is considered as the gold standard for typing Mycobacterium tuberculosis complex.

4. Biomarkers

M.tuberculosis specific biomarkers are Urine tuberculosis DNA, Anti alanine dehydrogenase, Sputum Ag 85 B-RNA and volatile organic compounds. These biomarkers are measurable, which indicates the pathogenic process or pharmacological response to therapeutic interventions[36].

Non Culture methods [35, 36, 37]

1. antigen detection

2. antibody detection

3. Nucleic Acid Amplification (NAA)

1. Antigen detection

Tuberculous meningitis can be diagnosed by detecting the mycobacterial antigen in cerebrospinal fluid (CSF). Latex agglutination test, reverse passive haemagglutination test, enzyme linked immunosorbent assay (ELISA) methods can be adapted.

Lipoarabinomannan in urine can be detected by ELISA. According to study done by Sada et al the sensitivity of this test in patients with active pulmonary tuberculosis but negative for acid-fast bacilli in sputum was 67% with 100% sensitivity[48].

Tissue sections of organs suspected of tuberculosis can be subjected to immunohistochemical (IHC) staining with species specific monoclonal antibody to detect mycobacterial antigen. According to the study done by **Mukherjee** et al of the IHC test showed a sensitivity of 74% and was 100% specific [49].

Antibody detection

Serological methods like ELISA are used to detect mycobacterial antibodies. M.tuberculosis specific immunoglobulin G can be detected in tuberculous meningitis. Antibody detection has a role in diagnosing extra pulmonary tuberculosis like tubercular meningitis but not pulmonary tuberculosis. A study by Pottumarthy et al , evaluated seven commercially available serological tests, and found the sensitivities of these tests in active tuberculosis patients ranged from 16 to 57% [50]. According to study conducted by Katoch et al. an ELISA kit (Pathozyme MYCO IgM, IgA, IgG) showed poor sensitivity . Recently it has been found that antibody detection is not of help in the diagnosis of tuberculosis and it is not recommended for the diagnosis of tuberculosis [51].

Nucleic acid amplification tests [52]

The amplification methods include PCR and Isothermal amplification methods. PCR methods include conventional DNA based PCR, Nested PCR and Real time (RT)-PCR. The sensitivity and specificity of PCR is 74.4% and 97.29% respectively. These amplification techniques can detect even 1-10 organisms/ml. It has an important role in early diagnosis in pauci-bacillary extra pulmonary tuberculosis. However the high costs limits the use of these tests.

Isothermal amplification techniques include strand displacement assay, gene probe amplified M.tuberculosis direct test and q-beta (QB) replicase based gene amplification. These tests are highly specific and very rapid test. The test are specific for MTB complex. It is an isothermal TMA (transcription-mediated amplification) test which targets mycobacterial 16SrRNA and IS6110. But these test are expensive and requires highly skilled personnel.

Newer techniques

1. LAMP (loop mediated isothermal amplification)

This is a method which amplifies TB DNA directly from clinical samples. It produces large amounts of amplified products and enables simple detection methods such as visual judgment by the turbidity or fluorescence of the reaction mixture, which is kept in the reaction tube. A positive result is signalled by a colour reaction visible to the naked eye. LAMP amplicons in the reaction tube are directly detected with the naked eye by adding 1.0 _l of 1/10-diluted original SYBR Green I to the tube and observing the color of the solution. The solution turns green in the presence of a LAMP amplicon, while it remains orange with no amplification[53]. But the specificity and sensitivity is low as the sample is not decontaminated.

In a study conducted by 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% with a specificity of 99%[54].

2. Inno-LiPa assay

It is a commercialized assay (two types). The first is for diagnosis of tuberculosis (INNO-LiPA Mycobacteria Assay) and the second for detection of rifampicin resistance (INNO-LiPA Rif TB Assay). It is based on hybridization of amplified DNA (mycobacterial 16S- 23S rRNA spacer region) from cultured strains or clinical samples to 10 probes covering the core region of the rpoB gene of M.Tb, immobilized on a nitrocellulose strip[35]. In a study conducted by Ling et al Inno-LiPa assay showed a sensitivity of 98.1% and 98.7% of specificity for rifampicin resistance. The accuracy for detection of isoniazid resistance was variable, with a sensitivity of 84.3% and 99.5% of specificity[55].

Another study conducted by Viveiros et al showed a sensitivity of 82.2% and a specificity of 66.7% when compared to conventional methods. The sensitivity and specificity were 100.0% and 96.9%, respectively, for the detection of RIF resistance[56].

3. GenoTypes assay(two types)

The first is for tuberculosis diagnosis (GenoType Mycobacteria Assay), the second for detection of rifampicin and isoniazid resistance (GenoType MTBDR Assay). It is based on PCR amplification of the 16S-23S ribosomal DNA spacer region followed by hybridization of the biotinylated amplified DNA products with 16 specific oligonucleotide probes. The specific probes are immobilized as parallel lines on a membrane strip. The GenoType MTBDR detects

resistance to isoniazid and rifampicin in culture samples, based on the detection of the most common mutations in the katG and rpoB genes respectively.

In a study conducted in Italy, the GenoType MTBDR assay showed a sensitivity of 91.5% for rifampin and 67.1% for isoniazid resistance [57].

4. Gene Xpert MTB/RIF assay

It is the Cepheid GeneXpert system, which is rapid and simple-to-use nucleic acid amplification test (NAAT). It detects DNA sequences specific for Mycobacterium tuberculosis and rifampicin resistance by polymerase chain reaction. The Xpert® MTB/RIF assay purifies, concentrates, amplifies (by real-time PCR) and identifies targeted nucleic acid sequences in the Mycobacterium tuberculosis genome, and thus provides results from unprocessed sputum samples in 90 minutes, with minimal biohazard. It does not require a trained technical staff to operate.

According to a study conducted by Boehme et al in culture-positive patients, a single, direct MTB/RIF test identified 98.2% of patients with smear-positive pulmonary tuberculosis 72.5% in patients with smear-negative pulmonary tuberculosis with a specificity of 99.2%. As compared with phenotypic drug-susceptibility testing, MTB/RIF assay identified 97.6% of patients with rifampin-resistant bacteria[58].

5. Interferon-Gamma Release Assays (IGRAs) [59]

Detection of the role of interferon-gamma (IFN-γ) to regulate cell-mediated immune responses to M. tuberculosis infection led to the development of IGRAs for the detection of M. tuberculosis infection. This assay is based on the principle that T-cells of individuals infected with *M.*

tuberculosis release IFN-γ when they re-encounter TB-specific antigens. IGRAs have been developed to replace the tuberculin skin test (TST) for detection of latent TB infection (LTBI). Newer tests of IFN-γ assays use antigens such as the early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) which are encoded within the region of difference 1 (RD1) of the *M. tuberculosis* genome. They are specific to *M. tuberculosis* than PPD, as they are not shared with any BCG vaccine strains.

Two IFN- γ assays are available as commercial kits based on RD1 antigens: the QuantiFERON-TB Gold assay and the T SPOT-TB assay.The QuantiFERON-TB Gold assay is a whole-blood, ELISA-based test, whereas the T SPOT-TB test uses peripheral blood mononuclear cells.

Detection of Adenosine deaminase enzyme

It is a sensitive, specific and inexpensive - easy to perform test. It is a colorimetric test performed on body fluids. Adenosine deaminase is a catabolic enzyme produced as a part of the purine salvage metabolic pathway.The principal function of the enzyme may be related to the development of the immune response. Increased Adenosine deaminase activity in body fluids is classically associated with tuberculosis[60].

Drug susceptibility testing [35, 37]

Drug susceptibility testing for mycobacterium tuberculosis can be determined either by observation of growth or metabolic inhibition in a medium containing anti tubercular drugs or by detection of mutations. It includes,

1. CULTURE BASED METHODS

2. MOLECULAR METHODS

Culture based methods

1. Absolute concentration method [36]

It is similar to agar dilution technique performed for the detection of antibiotic susceptibility of bacterial isolates. A standardized inoculum of the test organism is inoculated onto both the control (without drug) and the test media containing various drug concentrations. A test organism is considered to be sensitive if it is inhibited by the lowest concentration of the drug, which is the minimum inhibitory concentration.

2. Resistance ratio method [36]

Here the resistance of the test organism is compared with the standard strain- H37RV. Both the strains are tested in parallel by inoculating a standard inoculum to media containing twofold serial dilutions of the drug. Resistance is expressed as the ratio of the MIC of the test strain divided by the MIC of the standard strain for each drug.

3. Proportion method [36]

For each drug to be tested, several dilutions of standardized inoculum are inoculated onto control and drug containing media. The isolate is considered to be resistant if the number of colonies formed on the drug-containing media is more than_1% of the colonies formed on the drug-free media.

Rapid culture-based techniques

1. BACTEC 460 [35]

BACTEC 460 TB is a radiometric system which detects the radioactive CO_2 produced by mycobacteria when it replicates and is expressed as "growth index". The ability of mycobacteria to grow in presence of anti-tubercular drugs incorporated into the media provides a rapid method to detect drug resistance. If there is increase in the growth index in drug containing medium when compared to the growth index of the control media, then the strain which is growing in presence of that drug is said to be resistant

2. MGIT [35,36]

It is a flurochrome based technique.The mycobacterial growth causes depletion of oxygen as a result of which there is fluorescence, which is detected visually under ultraviolet light or in the automated system (MGIT 960 system), by a sensor. For each drug tested a standardized inoculum is inoculated in to the test and control media. If there is growth of mycobhacteria even in the presence of drug, then the fluorescence can be seen under UV light.

3. Micro colony detection [35,36]

It is a solid media where the plate is examined microscopically on alternate days for the first two weeks to look for formation of microcolonies of M. Tuberculosis. The results for DST require 10-15 days.

4. Luciferase Reporter phages [35,36]

In this method viable bacteria are infected with reporter phages expressing luciferase gene. When the test mycobacterium strain is sensitive to the drug incubated with, then they fail to produce light after infection with luciferase reporter phages as there is growth of the organisms in the presence of the drug.

5. Phage assay [35,36]

It is a phage amplification-based assay which depends on the replication of MTB-specific phages in the presence of *M tuberculosis* in the clinical specimens. Phage assays can be directly used on sputum specimens and the direct drug susceptibility testing can also be performed through the incorporation of rifampicin into the assay – viable (rifampicin resistant) organisms support phage replication while dead (rifampicin susceptible) organisms do not.

Molecular detection

Mutations in katG for INH resistance, rpoB gene for rifampicin resistance, other genes like inhA, oxyR-ahpC and kasA gene can be detected by molecular methods. Genotypic assays based on amplification of a specific region of tuberculosis gene followed by its analysis of for specific mutations. The molecular methods include DNA microarray, molecular beacons and other PCR techniques[52].

INNO-LiPA Rif TB Assay

It is based on hybridization of amplified DNA (mycobacterial 16S- 23S rRNA spacer region) from cultured strains or clinical samples to 10 probes covering the core region of the rpoB gene of M.Tb, immobilized on a nitrocellulose strip [56].

GenoType MTBDR assay

It detects the resistance to isoniazid and rifampicin in culture samples, based on the detection of the most common mutations in the katG and rpoB genes respectively [57].

GeneXpert MTB/RIF assay

It is a simple nucleic acid amplification test (NAAT). It detects DNA sequences specific for Mycobacterium tuberculosis and rifampicin resistance by polymerase chain reaction [58].

DNA Microarrays

A new molecular method for detecting the drug resistance in MTB which is based on the hybridization of DNA obtained from clinical samples to oligonucleotides immobilized on a solid support, such as miniaturized glass slides. Microarrays have been mainly used to detect resistance to rifampicin [36].

Diagnosis of tuberculosis by rodents as detectors [61]

APOPO (Anti Persoonsmijnen Ontmijnende Product Ontwikkeling) (Anti-Personnel Landmine Detection Product Development) is a nonprofit humanitarian organization located in Morogoro, Tanzania which uses rats as mine-detection animals. This organization also has been experimenting the use of African giant pouched rats (Cricetomys ngambianus) to detect the presence of TB. These rats are large and long lived rats, which have an excellent sense of smell, and are used to detect TB by sniffing. The sputum samples of the patients infected with tuberculosis has distinctive volatile organic compounds produced by MTB and these are detected are detected by Apopo's rodents. They are trained to respond consistently in one way

(pause) if the sample contains the TB bacillus (is positive) and respond in another way (not pause) if the sample does not contain the bacillus (i.e., is negative). Each rat can test hundreds of samples each day, allowing inexpensive testing. According to a study 16 rats that were evaluated for the diagnosis of 2,597 sputum samples, of which 345 were smear positive showed a mean sensitivity of 87.9% and 93.3% of specificity[61].

Microscopy after Universal Sample Processing (USP) Technique [4]

Recently a new technique for processing the sputum sample to detect AFB, which is claimed to be more sensitive than the conventional direct smear microscopy has been introduced. This technique includes chemicals like guanidinium hydrochloride a Chaotropic agent, Tris chloride base, EDTA, B mercaptoethanol and Sarkosyl for processing the sputum sample with 6000rpm centrifugation. These chemicals are mucolytic and detergent in nature which renders the sputum sample liquefied and free from other cellular debris. The chaotropic agent have the ability to destabilize the hydrogen bonding and hydrophobic interactions damaging the cell wall of other cells, but the cell wall of mycobacterium which is made up of mycolic acid renders I,t resistant to the action of guanidinium hydrochloride. Thus only mycobacterium is seen in the smear without any cells or cellular debris.

A study conducted by Chakravory et al. claim that it can detect as low as 250-300 bacilli/ ml of sputum sample when compared to the 10,000 AFB/ ml by conventional ZN staining and USP smear microscopy showed a sensitivity and specificity of 98.2% and 91.4% respectively compared to the 68.6% and 92.6% by direct ZN staining method. In addition this test also

detected 100 samples which were negative by direct smear microscopy, as positive for AFB after USP smear microscopy which were also positive by culture[4].

Anti-tubercular drugs and Drug resistance [62]

Mycobacterium tuberculosis are intracellular organisms which multiply in macrophages. Treatment includes the The treatment regimen for tuberculosis varies from months to years.

Isoniazid (INH), Rifampicin (R), Pyrazinamide (Z), Ethambutol (E) and Strptomycin are the first five line drugs used for the treatment of tuberculosis.

Amikacin, Ciprofloxacin, Capreomycin, Aminosalicyclic acid, Cycloserine, Ethionamide and Rifabutin forms the second line drugs.

In initial phase, during the first two weeks of therapy the actively multiplying tubercle bacilli are killed mainly by the action of Isoniazid and partly by Rifampicin and the patient becomes non-infectious. Later on the less active bacilli in macrophages, caseous material are killed by Pyrazinamide and Rifampicin.

In the continuation phase, any remaining dormant bacilli are killed by rifampicin and any rifampicin resistant mutants that starts replicating is killed by isoniazid [47].

INH is a bactericidal agent. It penetrates into the macrophages and is active against both intracellular and extra cellular organisms. It acts by inhibiting the mycolic acids, the essential component of mycobacterial cell wall. INH occurs as inactive form, which is activated by

mycobacterial catalase-peroxidase. The activated form combines with acyl carrier protein (AcpM& KasA) which blocks the mycolic acid synthesis resulting in cell death.

Rifampicin – a semisynthetic derivative of rifamycin an antibiotic produced by Streptomyces mediterranei. It is a bactericidal drug. Rifampicin binds to the B sub unit of bacterial DNA dependent RNA polymerase and inhibits RNA synthesis

Ethambutol, a synthetic drug acts by inhibiting mycobacterial arabinosyl transferases which blocks the synthesis of arabinoglycan an essential component of mycobacterial cell wall.

Pyrazinamide drug is active against the tubercle bacilli present in the macrophages. The drug action is not known.

Streptomycin isolated from Streptomyces griseus acts by binding to the ribosomes. The drug is active against extracellular tubercle bacilli due to its poor penetration in to the cells. Streptomycin is helpful in case of TB meningitis as it crosses the blood-brain-barrier.

Rifampicin forms the main stay in the treatment of tuberculosis. Its high level efficacy brought a new hope for the treatment of tuberculosis. But resistance to rifampicin was soon detected. The analysis of the laboratory data from different trials revealed that isoniazid resistance was seen among patients who received monotherapy and also they found that the resistance was uncommon when isoniazid was given in combination with streptomycin or para-aminosalicylic acid. These observations led to the use of multidrug treatment regimens. The British Medical Research Council conducted series of multi-country, clinical trials after which a four-drug regimen was recommended to treat patients who were newly diagnosed with tuberculosis. The backbone of such regimens was the combination of isoniazid and rifampin which is considered to

be the most effective and well tolerated oral agents, given for 6 to 8 months. Thus, short-course chemotherapy was born.

The patients are classified into 3 different categories and are put on different treatment regimens [20].

Category I:

It includes patients who are sputum smear-positive (new), and patients who are seriously ill with smear negative pulmonary tuberculosis or extrapulmonary tuberculosis. The Patients in category I are treated with Isoniazide (H), rifampicin (R), Pyrazinamide (Z) and Ethambutol (E) weekly thrice for a period of 2months followed by Isoniazide (H), rifampicin (R) weekly thrice for a period of 4months.

Category II

It includes patients who are sputum smear positive with history of relapse, treatment failure or default patients**.** These patients are treated with Isoniazide (H), rifampicin (R), Pyrazinamide (Z), Ethambutol (E) and Streptomycin (S) weekly thrice for a period of 2months followed by

Isoniazide (H), rifampicin (R) Pyrazinamide (Z) , Ethambutol (E)weekly thrice for one month and Isoniazide (H), rifampicin (R), Ethambutol (E) weekly thrice for a period of 5months.

Category III

It includes patients who are suffering with either smear-negative pulmonary or Extra-pulmonary tuberculosis (new) with no serious illness. These patients are treated with Isoniazide (H), rifampicin (R) and Pyrazinamide (Z) weekly thrice for a period of 2months followed by Isoniazide (H), rifampicin (R) weekly thrice for a period of 4months

The emergence of resistant strains is due to the sequential accumulation of individual mutations in separate genes. A Mutation responsible for drug resistance occurs at a rate of about one mutation every 10^8 cell divisions [23]. Based on the specificity of anti-TB drugs, drug resistance mechanisms in M. tuberculosis are divided into specific and nonspecific mechanisms. The tuberculosis-specific drugs (isoniazid, pyrazinamide, ethambutol etc.) attack unique metabolic pathways in M. tuberculosis

Mechanisms of resistance to these drugs are unique to M. tuberculosis and so far only isoniazid (INH) resistance mechanisms have been characterized. The non-tuberculosis-specific drugs (streptomycin, rifampicin, fluoroquinolones) which are broad spectrum antibiotics. Resistance to INH is due to mutations, which results in overexpression of InhA, mutation or deletion of kat G, overexpression of ahpC and mutations in kas A gene. Rifampicin resistance develops as a result of point mutation in the rpoB gene. Pyrazinamide resistance may develop as a result of impaired uptake of pyrazinamide or mutations in pncA gene which interferes in conversion to its active

form. Resistance to Streptomycin develops as a result of point mutation in rpsL and rrs gene which alters the ribosomal binding site.

Primary drug-resistance: "New Cases" [20]

Drug resistance in patients who have not been treated earlier for tuberculosis.

Secondary (acquired) drug-resistance: [20]

Drug resistance in a patient who has received at least one month of anti-TB therapy.

Multi-drug-resistant tuberculosis (MDR-TB) is defined as tuberculosis that is resistant to atleast one of the drug, isoniazid (H) or rifampicin (R), the two first-line anti-TB drugs. India accounts for 2.1% of new TB cases with MDR TB and 15% of retreatment TB cases with MDR TB [20].

Extensively drug resistant (XDR-TB) is defined as MDR plus resistance to fluoroquinolones and at least 1 of the 3 injectable drugs (amikacin, kanamycin, capreomycin).

These organisms have the ability to remain dormant and also have the ability to develop resistance by mutation to the drugs used in treatment by selection of resistant mutants which occurs due to inappropriate and irregular treatment.

4. Materials and Methods

Source of clinical material

Patients suspected of pulmonary tuberculosis, with history of cough for 2 weeks visiting the RNTCP screening laboratories situated at R.L. Jalappa hospital and SNR hospital, Kolar were included in the study.

Inclusion criteria:

Patients suspected of pulmonary tuberculosis, with history of cough for 2 weeks attending the above RNTCP screening laboratories, who were able to bring out well coughed up sputum sample were included in the study.

Exclusion criteria

Children who were unable to bring out satisfactory amount of sputum specimen and all those patients who were already on anti-tubercular treatment were excluded from the study.

Method of collection of samples

Sputum samples (both spot and early morning) as per the Revised National Tuberculosis Control Programme (RNTCP) guidelines[20] were collected.

The patients were advised to bring out well coughed up sputum samples. The sputum samples were collected in sterile wide mouthed plastic containers. The above collected samples were processed immediately. In case of any delay the specimens were stored at $4^{o}C$ in the refrigerator.

A total of 2000 (both spot and early morning) sputum samples from 1000 patients visiting the RNTCP screening laboratories were collected consecutively from November 2011 to May 2012.

Direct Ziehl-Neelsen staining [37]

Preparation of the smear

The staining technique followed was performed as per RNTCP guidelines.

The sputum sample was labeled with the name of the patient, age, sex, hospital number and an Universal Sample Processing (USP) number. The sputum sample was mixed well with a sterile loop and a loop full of sputum sample was placed on a clean labeled glass slide and the smear was made.

Procedure

1. The smear was heat fixed
2. The heat fixed smear was kept on a staining rack

Staining with concentrated Carbol fuchsin

3. Smear was flooded with 1% Carbol fuchsin stain reagent (Himedia). Gently heated until the vapors rouse, the smear was not allowed boil. Carbol fuchsin was left on the slide for 5 minutes.
4. The smear was gently washed in tap wate

Decolorisation

5. The slide was decolorized with 20% Sulphuric acid for 2 minutes. The slide was gently washed with tap water and the decolorisation step was repeated as above for 3 times.

Counter staining

6. The smear was counterstained with 1% Loeffler's methylene blue (Himedia) for 30 seconds.
7. The smear was gently washed with tap water and air dried.

8. The slide was examined under microscope using 40X objective to select the suitable area and then examined under 100X objective using a drop of immersion oil.

The smear was then graded as per the RNTCP guidelines [20] as follows

	Result	Grading	Number of fields examined
More than 10 AFB per oil immersion field	Positive	3+	20
1-10 AFB per oil immersion field	Positive	2+	50
10-99 AFB per oil immersion field	Positive	1+	100
1-9 AFB per oil immersion field	Scanty	Actual number	200
No AFB per oil immersion field	Negative	0	100

Preparation of USP solution[4]

The USP solution consists of the following chemicals Guanidinium hydrochloride (Sigma), Sarkosyl(Sigma), Tris –chloride(Sigma), EDTA(Sigma) and Beta-mercaptoethanol(Sigma).Tween 80 (Himedia).

USP Solution has two components: solution 1and solution 2.

Solution 1 consists of a mixture of stock solution A, stock solution B and stock solution C.

Preparation of individual components of USP solution 1

1. Preparation of Stock solution A.

Solution A consists of 25.392grams of Guanidinium hydrochloride in 44.3ml of sterile distilled water.

About 44.3ml of sterile (autoclaved) distilled water was measured in a measuring jar and poured into a sterile 200ml conical flask. In an electronic weighing machine, 25.392grams of guanidinium hydrochloride was weighed. Mixed well in the distilled water taken in a conical flask. The chemical dissolved completely in water and the resultant solution became cold due to endergonic reaction.

2. Preparation of stock solution B

Solution B consists of: 0.25grams of Sarkosyl in 1.25ml of sterile distilled water, 0.4625 grams of EDTA in 2.5ml of sterile distilled water and 0.303 grams of Tris chloride in 1.25ml of sterile distilled water.

Method of preparation

a. Preparation of Sarkosyl solution

Using an electronic weighing machine, 0.25grams of Sarkosyl was weighed. It was dissolved in 1.25ml of sterile distilled water taken in a sterile labeled Mc cartney bottle. On mixing, the resultant solution was not clear as the chemical did not dissolve completely. The solution thus prepared was kept in a water bath at 65^{o}C overnight, after which the chemical was found to have dissolved completely.

b. Preparation of EDTA solution:

Using electronic weighing machine 0.4625 grams of EDTA was weighed. It was dissolved in 2.5ml of sterile distilled water taken in a sterile labeled Mc cartney bottle. On mixing the resultant solution was not clear as the chemical did not dissolve completely. The solution thus prepared was kept into the autoclave and sterilized at 121^{o} C for 15 min.

c. Preparation of Tris chloride solution:

Using an electronic weighing machine 0.303 gram of tris-chloride was weighed. It was dissolved in 1.25ml of sterile distilled water taken in a sterile labeled Mc cartney bottle. The solution thus prepared was kept in an autoclave and sterilized at 121^{o} C for 15 min.

Stock Solution C -

β-mercaptoethanol (sigma) stored at 4 ℃.

Taken together the volumes of solution A and solution B made as above gives a volume of 49.3ml to this 700µl of β-mercaptoethanol-which is solution C is added. It gives a final volume 50ml of solution 1.

The USP solution prepared was used immediately within 24 hrs of reconstitution.

Preparation of solution 2

The working solution was made as follows:

Solution 2 was by made by adding 10µl of tween 80 into 100ml of sterile distilled water.

For this purpose about 10µl of Tween 80 (Himedia) was pipetted into 100ml of sterile distilled water taken in a conical flask and mixed well.

Processing the sputum sample[4]:

a. Treatment with USP solution

Sterile centrifuge tubes measuring 50ml were labeled with patient's name, hospital number and USP number (lab number) for each of the sample collected.

About 2-3ml of each of the two sputum sample collected from a patient was taken in a sterile 50ml centrifuge tube. About 2-3 volumes of USP solution depending on the tenacity and purulence of sputum was added; a maximum of 3 volumes.

The test tube containing the sample and USP solution mixture was mixed by shaking or by vortexing for 30-60 seconds until complete homogenization occurred, for about 2 minutes. Tenacious or excessively purulent samples which did not homogenize completely were allowed to stand for 10-15 minutes at room temperature then 5-15 ml of sterile water was added. Mixed well to obtain a clear homogenous solution.

b. Centrifugation of the prepared sputum sample

The above prepared solution was centrifuged at 6000rpm for 20 minutes at room temperature in a Remi centrifuge. The supernatant was decanted carefully so that the pellet was not lost with the

supernatant. Using a sterile Pasteur pipette the pellet was suspended again in 2-5 ml of USP solution. When the pellet was very large appropriate amount of USP solution was used to suspend the pellet. The suspended pellet was centrifuged again at 6000rpm for 20 minutes at room temperature. Supernatant was decanted carefully.

About 5-10ml of sterile water was added to wash the pellet formed and was again centrifuged as before. The supernatant was decanted and the pellet was suspended in 500 µl of Solution 2.

c. Smear preparation with the deposit after USP:

The pellet suspended in solution 2 as above was mixed well. About 100µl of the suspension was pipetted onto a clean labeled glass slide and the smear was evenly spread with the help of an inoculating loop. The smear was air dried and heat fixed over low flame.

Ziehl-Neelsen staining was performed as described earlier.

USP smear screening.

The slide was examined under microscope using 40X objective to select the suitable area and then examined under 100X objective using a drop of immersion oil.

Grading of the USP smear could not be done as the acid fast bacilli was not evenly distributed. The acid fast bacilli were seen clumped together.

Culture of tubercle bacilli after subjecting to USP method

Preparation of Lowenstein Jensen (LJ) media [37]: for 100ml media

a. Ingredients consists of:

TB piezer media base – 4.47grams

0.85% sterile saline - 2.5ml

20% sterile Dextrose solution – 0.1ml

Malachite green - 1.3ml

Egg yolk - 1

Glycerol -4ml

b. Contents of TB Peizer medium base (Himedia), THIS

Casein acid hydrolysate,

Beef extract,

L-Asparagine,

Potato starch,

Ferric ammonium citrate,

Magnesium sulphate,

Dipotassium hydrogen phosphate,

Citric acid,

Agar.

c. Preparation of LJ medium

About 4.47 grams of TB Peizer media base is weighed in an electronic weighing machine. In a sterile conical flask containing 100ml of sterile distilled water, the base

above weighed base was added and mixed well. To the above solution 4ml of glycerol was added**.** The above mixture in the conical flask was sterilized at 121^{o} C for 15 min in an autoclave.

The egg yolk collected aseptically was added into a sterile 100ml conical flask with glass beads. Mixed well. Then 2.5ml of 0.85% normal saline, 0.1 ml of 20% Dextrose solution, and 1.3ml of Malachite green was pipetted into the above conical flask containing egg yolk. Mixed well.

The above prepared mixture was added to the sterilized TB media base at 55^{0}C. Mixed carefully to avoid frothing. About 3ml of the mixture was dispensed into each Mc Cartney bottle. The bottles were then allowed to solidify in slants

Culture on LJ medium [4]

About 225µl of the final suspension after the USP technique was inoculated onto the LJ media and incubated at 37^{0}C. The tubes were observed at weekly intervals for growth and colonies suspicious of Mycobacterium tuberculosis were specially noted. The tubes were thus observed for 12 weeks and if there was no growth even after 12 weeks the Mc cartney bottles containing media were discarded. As a positive control the H37RV strain of tubercle bacillus was inoculated into a MC cartney bottle for each batch of the media made.

Niacin test[36] (Himedia)

1. **Extraction of the niacin from the Mycobacterium tuberculosis culture**

About 2ml of sterile saline was pipetted into the slant in LJ culture bottle with growth. Then the slant was stabbed with a needle. The slant was kept horizontally for 20min at room temperature, and then kept upright for 5min.

Criteria – Growth considered for niacin test was more than 3 weeks old.

1. Test solution preparation

There were two vials part A and part B 1ml each supplied by the manufacturer. The content of part A was transferred to part B vial. Then the test sample 1ml was transferred into the solution made as above using a syringe.

2. Controls

a. **Positive control**

The content of part A was transferred to part B. To this reagent solution, 1ml of R055 reagent was added using a syringe. The R055 reagent contains standard antigen. Development of yellow colour within 5min – positive control

a. Negative control

The content of part A was transferred to part B. To this reagen solution, 1ml of sterile saline was added. No development of yellow colour – negative control.

The direct ZN stained smears and smears after USP method were screened by two different technicians in two RNTCP centers to find whether there was any difference in the time taken to detect the AFB in the direct and USP smear microscopy.

A total of 24 stained smears of direct and USP smear microscopy each were subjected to this test. Of the 24 smears, 8 slides of grade1+, 8 slides of grade 2+ and 8 slides of 3+ to detect the AFB.

The direct and the USP smears were screened by the technicians and the time taken for them to detect the AFB in each slide was noted.

5.Results

Sputum samples from one thousand patients, who had cough for more than 2 weeks duration, were tested by direct smear microscopy and microscopy after USP method.

Among these samples 704 (70.4%) were from patients who visited the RNTCP centers situated at SNR hospital, the government district hospital, Kolar. The remaining 296 (29.6%) samples were from patients who visited the RNTCP center situated at R.L.Jalappa hospital, a tertiary care teaching hospital, Kolar.

Table 1- Sampling centers and the samples collected

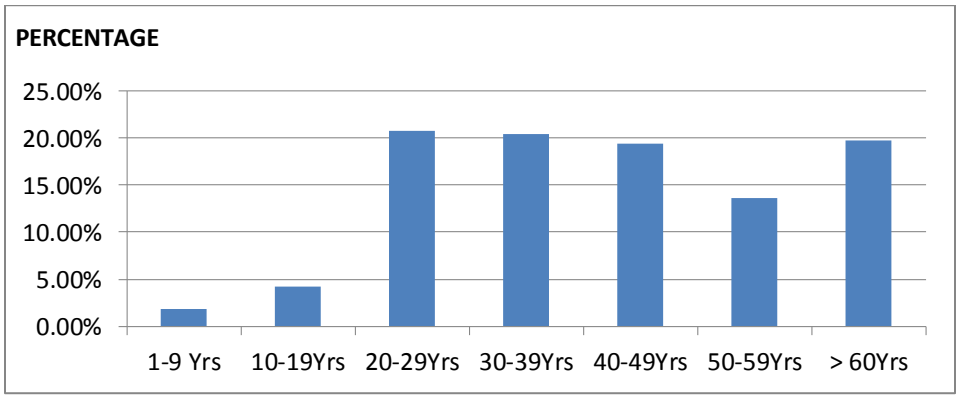
Sampling centers	Number of samples (%)
SNR hospital, Kolar	704 (70.4%)
R.L.Jalappa Hospital, Kolar	296 (29.6%)
Total number of samples (n)	1000

The age and sex distribution of the patients whose sputum (spot and early morning) samples were tested is shown in table 2 and age distribution is shown in figure 1.

Table 2-Age and sex distribution

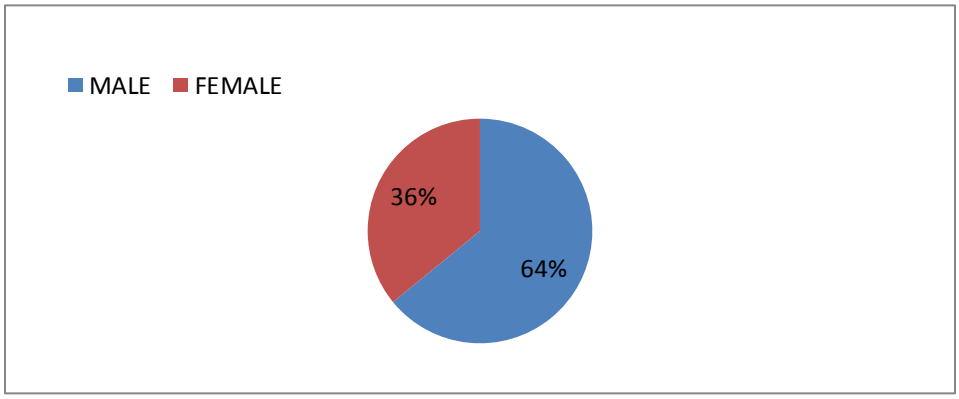
Age	Males (%)	Females (%)	Total tested (%)
<10 years	11(1.1%)	7 (0.7%)	18 (1.8%)
10-19	25 (2.5%)	17 (1.7%)	42 (4.2%)
20-29	151 (15.1%)	45 (4.5%)	196 (19.6%)
30-39	147 (14.7%)	54 (5.4%)	201 (20.1%)
40-49	99 (9.9%)	87 (8.7%)	186 (18.6%)
50-59	85 (8.5%)	44 (4.4%)	129 (12.9%)
>60 years	123 (12.3%)	105 (10.5%)	228 (22.8%)
Total	641 (64.1%)	359 (35.9%)	1000

Figure 1 – Bar diagram showing the age distribution



Among the patients from whom sputum samples were studied, majority were adults in their 3rd, 4th and 5th decades of life, this was followed by those above 60 years of age.

Figure 2. Shows the percentage of gender distribution



Among the samples collected 64.1% were from males and 35.9% from females. The age-wise distribution of the genders is shown in table 2.

Table 3 - Shows the number of samples tested found to be positive in the direct smear examination and USP smear microscopy according to the age.

Age (in years)	Samples tested (%)	No. of positives in direct smear microscopy (%)	No. of positives after USP microscopy (%)
<10	18	1 (5.5%)	1 (5.5%)
10-19	42	4(9.5%)	4(9.5%)
20-29	208	17 (8.17%)	17 (8.17%)

30-39	204	22 (10.7%)	22 (10.7%)
40-49	194	16 (8.2%)	16 (8.2%)
50-59	136	17 (12.5%)	17 (12.5%)
>60 years	198	17 (8.5%)	17 (8.5%)
Total	1000	94 (9.4%)	94 (9.4%)

Of the 1000 samples tested, 94 (9.4%) were positive for AFB by direct smear microscopy and 906 (90.6%) were negative. There was no difference between the direct smear microscopy and USP smear microscopy in the detection rates. The positivity for AFB ranged between 5.5% in the first decade and 12.5% in 6^{th} decade. However, the sputum positivity rate was not significantly more in any particular age group. ($p = 0.8290$, at 95% CI)

The positivity rate among the gender is shown in table 4.

Table 4. Number of samples positive for AFB in each gender

Gender	Total number of samples tested	Total number of positives
Male	641	66 (10.2%)

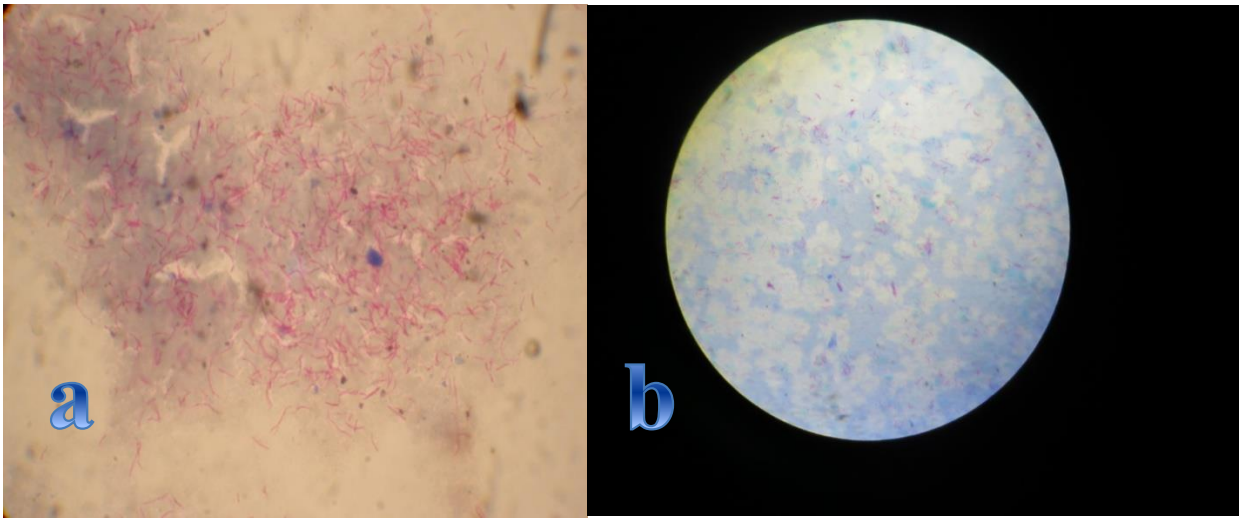
Female	359	28 (7.7%)

Among the sputum samples collected from 641 males, 66 (10.2%) were positive for AFB while among the 359 samples collected from female patients, 28 (7.7%) were positive for AFB.

The positivity rates compared among the genders showed no statistically significant difference among them (x^2= 1.526, p = 0.2167).

Though there was no difference in the positivity rates detected between the direct smear microscopy and USP smear microscopy, there was difference in the microscopic picture of the stained smear observed at the end of the tests.

Figure 3. Microscopic pictures of USP and direct smears after ZN stain



The USP smear microscopy showed a clear background without any cells and cellular debris (figure 3.a) and the bacilli were seen clumped together at one or more sites in contrast to the direct smear microscopy (figure 3.b) which showed acid fast bacilli distributed in singles or small groups of two or three on thick, heavily counterstained blue background with numerous

pus cells, macrophages and lymphocytes. The USP smear microscopy could not be graded as the AFB were seen clumped together. The smear required to be focused in low power initially to identify the muck which acted as an indicator for the presence of bacilli in the vicinity and then focused under the oil immersion lens for observing the details. In contrast the bacilli in direct smear microscopy were identified under oil immersion lens directly.

Table 5. Presents the grading of the positive samples for AFB in direct smear microscopy according to RNTCP guidelines.

Table 5. Grading of AFB by direct smear microscopy

Grading for AFB	Total number of samples
Scanty	12 (12.7%)
1+	24 (25.53%)
2+	28 (29.7%)
3+	30 (31.9%)
Total number of positives	94

Among the 94 samples positive for AFB, most were grade 3+ (31.9%), followed by grade 2+ (29.7%). There was no difference in the appearance of positives by direct and USP smear microscopy based on grading in the direct smear microscopy.

The number of sputum samples collected from each sampling center and the positivity rate among them is shown in table 6.

Table 6. Total number of samples positive in each RNTCP center

RNTCP centers	Total number of samples tested	Total number of positives by direct smear microscopy	Total number of positives by USP smear microscopy
SNR hospital, Kolar	704	65 (9.2%)	65 (9.2%)
R.L. Jalappa hospital, Kolar	296	29 (9.7%)	29 (9.7%)

Among the 704 sputum samples collected from SNR hospital, 65(9.2%) were positive for AFB and among the 296 samples collected from R.L. Jalappa hospital, 29 (9.7%) were positive for AFB.

There was no statistically significant difference ($p > 0.75$) in the proportion of positives detected at SNR and R.L.Jalappa hospitals.

The culture outcome of the smear positive samples inoculated onto LJ medium is shown in table 7.

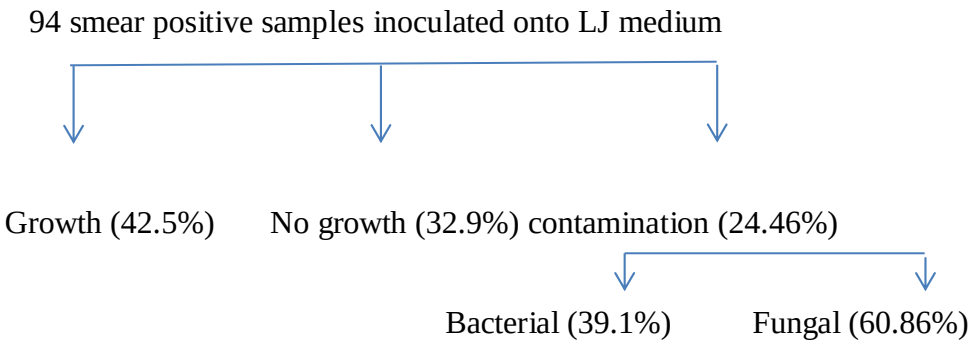
Table 7. Growth on LJ medium

Total number of smear positive samples inoculated	**N=94**
Growth on LJ medium	40 (42.5%)
No growth	31 (32.9%)
Contamination	23 (24.46%)

The mean time taken for Mycobacterial growth on LJ medium was 22 days.

Figure 4. Culture on LJ medium showing Mycobacterial growth and fungal contamination respectively.

Figure 5. Flow chart showing the growth and contamination rates on LJ medium



None of the patients included in our study were on treatment. Of the 94 smear positive samples inoculated onto LJ medium, 42.5% showed Mycobacterial growth and contamination was observed in 23 (24.46%) samples.

The type and rate of contamination of the smear positive samples inoculated onto LJ medium is shown in table 8.

Table 8. Results of the observations culture on LJ medium

Time period	**Bacterial contamination n=9 (39.1%)**	**Fungal contamination n=14 (60.86%)**
First week	9	2 (14.2%)
Second week	-	7 (50%)
Third week	-	5 (35.7%)
Fourth week	-	-

Among the 23 samples inoculated onto LJ medium which got contaminated, fungal contamination predominated. Fungi accounted for 14 (60.86%) of the contaminants which grew most often in the second and third weeks after inoculation. Bacteria accounted for 9 (39.1%) of the contaminants which grew mostly with in first week.

The time taken to detect the AFB in direct smear and USP smear microscopy by two different RNTCP technicians in the two RNTCP centers is shown in table 9.

Table 9. Time taken to detect AFB in direct smear and USP smear microscopy

Grade	Time taken to detect AFB in Direct smear microscopy in seconds	Time taken to detect AFB after USP smear microscopy in seconds	p value at 95% CI
1+	43.44	42.94	0.8769
2+	28.38	26.94	0.1698
3+	14.00	12.19	0.1840
Total	28.40	27.40	0.7236

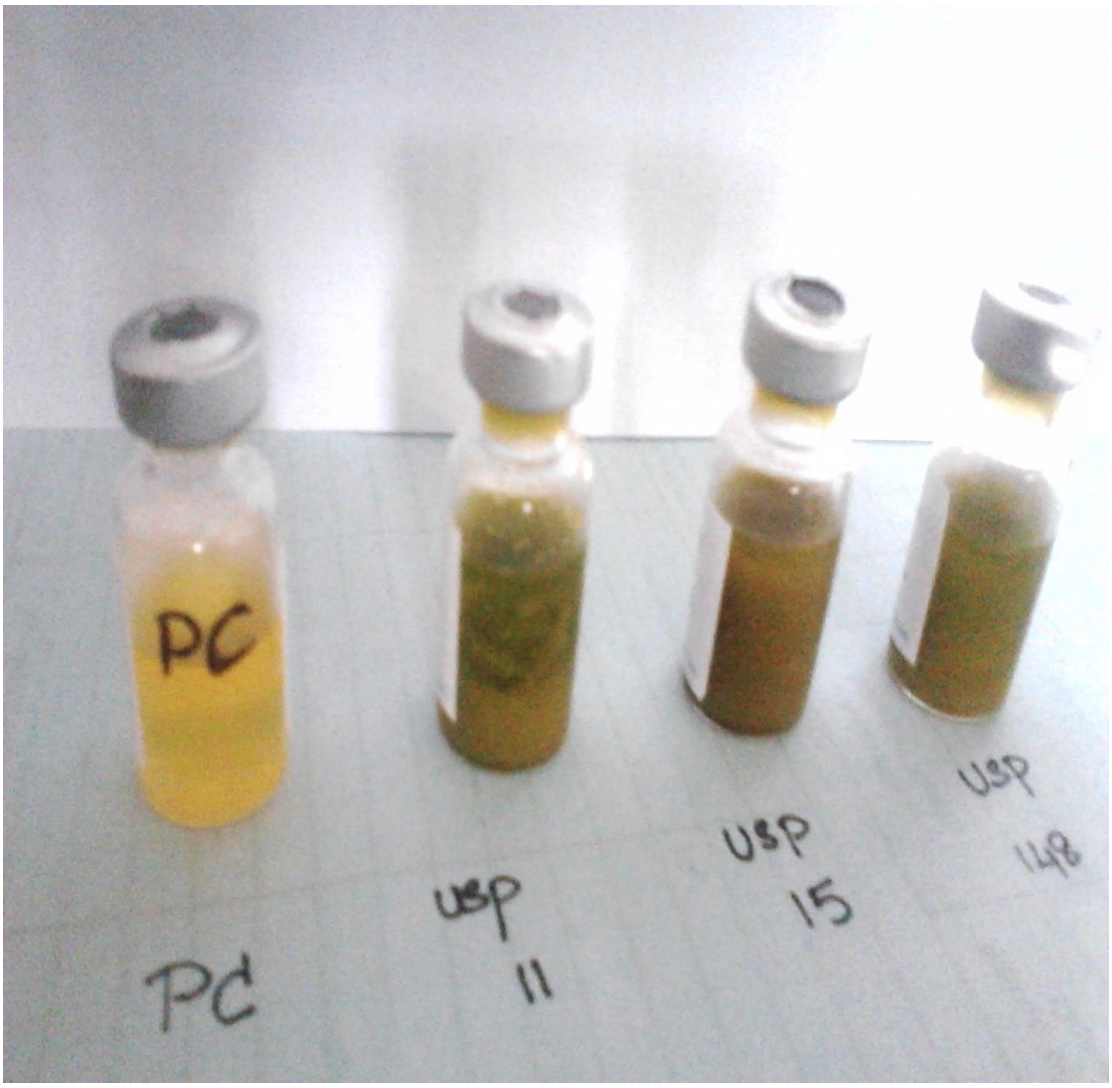
There was no statistically significant difference in the time taken to detect the AFB in direct and USP smear microscopy methods (p= 0.7236, at 95% CI).

Niacin test

The test was performed on all 42 samples positive for AFB which grew on LJ medium. All were niacin test positive. The development of yellow or yellowish green colour is interpreted as positive test.

Figure 6. Shows the Positive niacin test (yellowish green in colour) performed on the positive samples which grew on LJ medium with the Positive control

The positive niacin test is yellowish green in colour as the LJ media was scraped during the test.



Discussion

Tuberculosis is one of the most common airborne infectious diseases and is rampant in developing countries like India. It is a chronic, intracellular infection which is treatable with anti-tubercular drugs given over a period of 6 months to 1 year.

The infection remains asymptomatic most often or may manifest with clinical illness. The patient with tuberculosis suffers from fever, cough with expectoration, and weight loss. The patient shedding tubercle bacilli in his sputum is an open case who is responsible for the spread of infection. The main stay in diagnosing this infection is ZN staining which is inexpensive, easy to perform and also the technicians can be trained easily to perform the test. ZN staining can detect 10,000 AFB/ml [36]. A newer technique called Universal Sample Processing (USP) has been claimed to detect as low as 250-300 bacilli/ml and also to detect positives from smear negative pulmonary tuberculosis. However there is no study under the RNTCP set up which has evaluated the USP technique for the detection of AFB.

Among the 1000 sputum samples processed 704 (70.4%) were from patients who visited the RNTCP center at SNR hospital, the government district hospital, Kolar and 296 (29.6%) sample were from R.L.Jalappa hospital, Kolar.

The study received more number of samples from SNR hospital, Kolar compared to R.L. Jalappa hospital, Kolar, as SNR hospital is a government set up and it gets more referrals for evaluation of TB from surrounding Primary health centers.

In the study carried out here we included subjects of all age groups and both the sexes with suspected tuberculosis. The youngest patient included in our study was 7 year old and oldest patient was of 90 years. Mean age of the patients from whom sputum samples were collected was 42.36 years. Maximum number of patients with history of cough for 2 weeks duration, who were studied by us were in the age group of 20-40 years (39.2%) followed by more than 60 years (23%) . Thus the predominate group which was included in our study forms economically most productive age group in any society.

Our findings are similar to those of Shivaraman et al who have reported that 40.8% of their study subjects belonged to this age group [63]. In a study by Narang et al. at Wardha, 26.30% belonged to this age group [64]. A study by S.B.Richards et al. has reported that 61% of their study population was in this age group [65]. The reasons that make this age group more vulnerable are that they are socially more active and may be they are exposed to an open case of tuberculosis more when compared to other age groups. There were very less pediatric patients in our study; this could be due to the well-known fact that children cannot bring out adequate sputum for microscopic testing.

In our study there were 641 (64.1%) males and 359 (35.9%) females. Male to female ratio was 1.7:1. Narang P et al. have reported 61.03% of their subjects were males while 38.97 % females [64]. Peter Eriki et al. and Fandinho et al. have reported male to female ratio of 1.8:1 and 1.6:1 respectively [66, 67]. All these findings are comparable to our study. The likely reasons for male preponderance is that the males are the usual breadwinners of the family, especially in India, who go out for work and hence more likely to come in contact with open cases. Males are also more likely get exposed to risk factors like smoking and alcoholism.

The spot and the early sputum samples collected were subjected to direct smear microscopy and USP smear microscopy. Of the 1000 samples processed, 94 (9.4%) were positive by both direct ZN staining and ZN staining after USP processing. The specimens which were positive by direct smear microscopy were also positive for AFB after USP smear microscopy. Since, the sensitivity of direct sputum microscopy is low, it is expected to miss the diagnosis of some pulmonary TB patients, and USP smear microscopy was expected to detect at least some of these as it was claimed to have a better sensitivity. But, in our study, none of the sample which were negative by direct smear microscopy were positive for AFB after USP smear microscopy. But in contrast according to a study conducted by Chakravorty et al . USP smear microscopy showed a sensitivity and specificity of 98.2% and 91.4% respectively compared to 68.6% and 92.6%, by direct ZN staining method [4]. In addition they also detected 100 samples which were negative by direct smear microscopy, as positive for AFB after USP smear microscopy which were also positive by culture [4]. Another study conducted by Chakravorty et al on extra-pulmonary specimens showed that sensitivity of USP smear microscopy was 21.1% (16 of 76), whereas that of the conventional method was only 3.9% (3 of 76) [68]. The explanation offered for the higher sensitivity of the USP smear microscopy is that it can detect as low as 300-500 bacilli/ml of the specimen, the efficient removal of the pus cells and other cellular debris without any deleterious effect on *M. tuberculosis*, the centrifugation step, during processing of the sample aggregates the bacilli in the sediment and also the amount of sample for making the smear consisted of about 10% of the processed material whereas very minute sample is used in direct smear microscopy.

In contrast to the results by Chakravorty et al. a study conducted by Cattamanchi et al. in Uganda showed that the USP method did not significantly improve the overall diagnostic performance of smear microscopy whose results were similar to our study [69]. The reasons explained by Chakravorty et al. for the poor performance of USP technique is that, in the study by Cattamanchi et al the samples were sedimented at a lower centrifugation speed 3,000rpm instead of the recommended 5,000 to 6,000 rpm. The culture positivity could be compromised by the inefficient bacterial sedimentation, incomplete removal of guanidinium hydrochloride (GuHCl), or the use of 4 to 6 M GuHCl [70].

A study conducted by Daley et al. at Christian Medical College, Vellore showed that short duration of sputum pretreatment with bleach and USP centrifugation did not increase the yield when compared to direct sputum smears [71].

In our study the smear microscopy after USP method showed pink colored AFB on a clear background with no other cells. This is due to the chemical guanidinium hydrochloride, a chaotropic agent used in the processing of sputum samples. It disrupts the cell wall of other cells/organisms present in the specimen by interfering with the stabilizing intra-molecular interactions mediated by non-covalent forces such as hydrogen bonds, vanderwaals forces and hydrophobic effects. But the unique characteristic of mycobacterial cell wall which is made up of mycolic acid, resists the action of guanidinium hydrochloride. Thus AFB is seen on a clear background in USP smear microscopy.

Grading of smears for AFB gives an idea regarding the bacterial load. It is an important indicator of patient's response and adherence to treatment, which also gives an idea about developing

resistance. In our study, the smear microscopy after USP treatment could not be graded for AFB. The AFB bacilli were seen clumped together in one or more sites which made the smear ineffective for grading. This is due to the fact that during the processing of sputum samples, the USP method involves a step of centrifugation at 5000-6000 rpm for 20 min which aggregates the AFB into small sediment and 10% of this sediment is used for making the smear. Thus one cannot grade the slide after processing the sputum sample by USP method.

Of the 94 samples positive for AFB, 40 (42.5%) samples were positive for growth on LJ media, no growth was seen in 31 (32.9%) samples and 23 (24.46%) samples showed contamination. According to a study conducted by Cattamanchi et al. in Uganda, 31% of the samples showed growth compared to 46% after NALC method, 58% of mycobacterial cultures were negative after USP method, compared to 43% after NALC method. The proportions of contaminated cultures were 11.2% versus 11.7 for the NALC and USP methods respectively [69]. The lower mycobacterial growth rate and the high contamination rate in culture on LJ medium after USP technique was also seen in the study conducted by Cattamanchi et al.

All the samples put up for culture in our study were positive by smear microscopy but only 42.5% of them grew on LJ medium. The reason could be that a proportion of AFB might have been killed during the processing of the sputum samples by USP technique.

In our study 24.46% of the positives samples inoculated onto LJ medium showed contamination. Fungi (60.86%) were the predominant contaminants followed by bacterial contaminants (39.1%). Most of the fungal contaminants were grown by 2nd week followed by 3rd week and the bacterial contaminants were seen grown by first week. The higher fungal contamination rate on LJ

medium may be due to decreased action of guanidinium hydrochloride on fungus and also the presence of Malachite green in LJ medium which suppresses bacterial growth but not the fungal growth.

In our study the mean duration of isolation of mycobacteria on LJ medium was 22 days. The period of maximum isolation was 3rd week followed by 4th week.

The time taken for the detection of AFB by two different RNTCP technicians in SNR hospital and R.L.Jalappa hospital did not show any significant difference (p=0.7236) in the speed of detection of AFB in direct and USP smear microscopy.

The processing of the sputum samples by USP method involves centrifugation at a higher speed producing aerosal, which is potentially hazardous for the person performing the test and also others.

Thus our study found that USP smear microscopy did not confer any advantage over direct smear microscopy in the diagnosis of pulmonary tuberculosis under the RNTCP set up. There was no sputum sample which was negative by direct smear microscopy that turned out to be positive by USP smear microscopy. Our study also showed that USP procedure was inefficient in containing fungal contaminations when the processed samples were cultured on LJ medium and also the USP method showed low positive culture rate

Summary

Our study included the processing of sputum samples collected from patients with suspected tuberculosis visiting two RNTCP centers situated at SNR hospital and R.L.Jalappa hospital, Kolar. The objective of our study was to compare the detection rates of AFB after USP method with that of direct smear microscopy to diagnose pulmonary tuberculosis under the RNTCP set up.

Sputum samples (both spot and early morning) collected from 1000 patients with history of cough for more than 2 weeks were subjected to direct smear microscopy and USP technique in parallel. The samples which were positive for AFB was put up for culture on LJ medium and the mycobacterial growth was confirmed by niacin test.

Of the samples processed 704 (70.4%) were from SNR hospital and 296 (29.6%) samples from R.L.Jalappa hospital, Kolar.

Majority of the patients from whom the sputum samples were collected, were adults belonging to 3^{rd}, 4^{th} and 5^{th} decades of life, followed by those above 60years of age.

Among the sputum samples collected from 641 males, 66 (10.2%) were positive for AFB while among the 359 samples collected from female patients, 28 (7.7%) were positive for AFB.

Of the 1000 sputum samples processed for AFB 94 (9.4%) were positive for AFB in direct and USP smear microscopy. There was no difference in the detection rate of AFB by USP method when compared to direct smear microscopy. The USP smear showed AFB clumped together in a clear background and hence could not be graded.

Only 40 (42.5%) of the smear positive samples inoculated onto the LJ medium showed growth and the mycobacterial growth was confirmed by niacin test. Contamination was seen in 23 (24.46%) of the samples inoculated. Fungal contamination 14 (60.86%) was predominant, mostly seen in 2nd week and bacterial contamination 9 (39.1%) in the first week.

There was no difference in the time taken to detect the AFB in direct smear and USP smear microscopy as screened by the two RNTCP technicians. The USP technique could not detect any positives for AFB which were negative by direct smear microscopy and also culture after USP technique showed high contamination rates.

Thus our study showed that USP technique did not provide any additional advantage over direct smear microscopy to detect AFB under RNTCP set up.

Conclusion

In our study there was no difference in the detection rate of AFB after USP smear microscopy when compared to the direct smear microscopy. The sputum samples which were positive for AFB in the direct smear microscopy were also positive by USP smear microscopy. None of the sputum samples which were negative for AFB by direct smear microscopy were positive after USP smear microscopy.

Microscopic picture of the direct and USP smear microscopy differed substantially. The USP smear microscopy showed AFB clumped together against a clear background in contrast to the direct smear microscopy where AFB were seen against blue counterstained background with pus cells.

The USP smear microscopy could not be graded because of the clumping of AFB.

Among the smear positive samples processed by USP method, only 40 (42.5%) grew on LJ medium Contamination was seen in 24.46% (23) of the samples inoculated. Fungal contamination [14 (60.86%)] was predominant followed by bacterial contamination [9 (39.1%)].

There was no difference in the time taken to detect AFB by USP smear microscopy when compared to direct smear microscopy by experienced RNTCP technicians.

Thus USP smear microscopy did not yield any additional advantage over conventional direct smear microscopy to detect AFB in the diagnosis of pulmonary tuberculosis under RNTCP set up. Also mycobacterial culture after USP technique showed high contamination rates and low growth rate.

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ANNEXURE 1: MASTER CHART OF SAMPLES POSITIVE FOR AFB BY DIRECT AND USP SMEAR MICROSCOPY, CULTURE ON LJ MEDIUM AND NIACIN TEST

SL.NO	Hospital	Lab no	USP NO	Sex	Age	Direct smear	USP smear	Grade	Growth in days	Niacin test
1	RLJH	1171	11	M	40	positive	positive	2	15	Positive
2	SNR	941	15	M	41	positive	positive	2	18	Positive
3	RLJH	1079	16	M	45	positive	positive	2	contamination	
4	SNR	3004	25	F	60	positive	positive	1	24	Positive
5	RLJH	1086	48	M	26	positive	positive	3	22	Positive
6	SNR	2709	132	F	28	positive	positive	1	19	Positive
7	SNR	2710	133	F	45	positive	positive	2	contamination	
8	SNR	2711	134	M	40	positive	positive	2	No growth	
9	RLJH	2127	148	M	45	positive	positive	1	18	Positive
10	SNR	2660	156	M	55	positive	positive	3	No growth	
11	RLJH	1153	175	F	35	positive	positive	2	contamination	
12	RLJH	1148	200	M	48	positive	positive	3	contamination	
13	SNR	2738	201	M	47	positive	positive	2	19	Positive
14	SNR	2749	212	M	32	positive	positive	1	contamination	
25	SNR	2757	220	M	36	positive	positive	3	24	Positive
16	SNR	2760	223	M	60	positive	positive	3	22	Positive
17	SNR	2768	231	F	35	positive	positive	1	contamination	
18	SNR	2775	238	M	35	positive	positive	1	No growth	
19	SNR	2783	246	M	60	positive	positive	1	No growth	
20	SNR	2803	266	F	50	positive	positive	2	19	Positive
21	SNR	2806	269	M	45	positive	positive	3	19	Positive
22	SNR	2814	277	M	45	positive	positive	3	contamination	
23	SNR	2831	294	M	55	positive	positive	3	20	Positive
24	SNR	2842	305	M	19	positive	positive	1	No growth	
25	SNR	2876	339	M	40	positive	positive	1	contamination	
26	SNR	2828	346	F	69	positive	positive	2	17	Positive
27	SNR	2870	353	M	26	positive	positive	1	32	Positive
28	SNR	2645	363	M	60	positive	positive	1	No growth	
29	SNR	2674	364	M	46	positive	positive	3	contamination	
30	SNR	2920	383	F	60	positive	positive	2	contamination	
31	SNR	2950	414	M	60	positive	positive	3	28	Positive
32	SNR	2952	416	M	55	positive	positive	3	21	Positive
33	SNR	2975	439	F	38	positive	positive	3	No growth	

34	SNR	2689	448	F	45	positive	positive	2	No growth	
35	SNR	2684	475	F	40	Positive	Positive	1	32	Positive
36	SNR	2710	482	F	40	Positive	Positive	1	contamination	
37	SNR	907	489	F	23	Positive	Positive	3	29	Positive
38	SNR	2803	535	F	65	Positive	Positive	3	19	Positive
39	SNR	2814	537	M	65	Positive	Positive	3	21	Positive
40	SNR	2789	544	M	45	Positive	Positive	3	No growth	
41	RLJH	411	572	M	60	Positive	Positive	3	19	Positive
42	RLJH	412	573	M	50	Positive	Positive	1	No growth	
43	RLJH	414	575	M	60	Positive	Positive	1	29	Positive
44	RLJH	427	588	M	18	Positive	Positive	1	No growth	
45	RLJH	432	593	M	39	Positive	Positive	3	No growth	
46	SNR	2984	595	M	55	Positive	Positive	2	contamination	
47	RLJH	438	599	M	60	Positive	Positive	2	contamination	
48	RLJH	442	603	F	58	Positive	Positive	3	No growth	
49	RLJH	447	608	M	30	Positive	Positive	1	No growth	
50	RLJH	448	609	M	35	Positive	Positive	2	18	Positive
51	RLJH	415	612	M	48	Positive	Positive	1	No growth	
52	RLJH	1236	625	M	41	Positive	Positive	1	22	Positive
53	RLJH	1158	627	F	55	Positive	Positive	2	No growth	
54	RLJH	1167	636	F	25	Positive	Positive	1	No growth	
55	RLJH	1180	638	M	55	Positive	Positive	1	18	Positive
56	RLJH	376	642	M	60	Positive	Positive	2	14	Positive
57	RLJH	350	655	F	37	Positive	Positive	1	17	Positive
58	SNR	881	667	F	45	Positive	Positive	1	No growth	
59	SNR	2987	683	M	28	Positive	Positive	1	No growth	
60	SNR	1000	684	M	60	Positive	Positive	1	No growth	
61	RLJH	1256	709	F	60	Positive	Positive	3	14	Positive
62	RLJH	896	718	F	15	Positive	Positive	1	19	Positive
63	SNR	1341	722	M	45	Positive	Positive	1	contamination	
64	RLJH	1197	727	F	45	Positive	Positive	1	19	Positive
65	SNR	1354	738	M	45	Positive	Positive	1	22	Positive
66	SNR	1305	748	M	65	Positive	Positive	3	No growth	
67	SNR	1363	754	M	72	Positive	Positive	3	contamination	
68	SNR	1235	761	F	30	Positive	Positive	3	19	Positive

69	SNR	1187	763	M	34	Positive	Positive	3	no growth	
70	SNR	2869	774	M	55	Positive	Positive	3	18	Positive
71	RLJH	1228	776	M	45	Positive	Positive	2	24	Positive
72	RLJH	471	792	M	50	Positive	Positive	1	contamination	
73	RLJH	497	818	M	70	Positive	Positive	3	contamination	
74	SNR	114	826	M	40	Positive	Positive	1	contamination	
75	SNR	116	832	M	26	Positive	Positive	3	12	Positive
76	SNR	2932	850	M	30	Positive	Positive	2	contamination	
77	SNR	1187	869	M	35	Positive	Positive	1	21	Positive
78	RLJH	501	882	M	45	Positive	Positive	2	19	Positive
79	SNR	1206	888	M	34	Positive	Positive	2	22	Positive
80	SNR	2962	890	M	70	Positive	Positive	3	18	Positive
81	SNR	1238	920	F	28	Positive	Positive	1	16	Positive
82	SNR	1242	924	M	45	Positive	Positive	1	no growth	
83	SNR	1246	928	M	40	Positive	Positive	3	no growth	
84	SNR	1253	935	M	69	Positive	Positive	1	no growth	
85	SNR	1346	939	M	76	Positive	Positive	2	contamination	
86	SNR	983	940	F	38	Positive	Positive	2	no growth	
87	SNR	971	941	F	25	Positive	Positive	2	no growth	
88	SNR	1269	951	M	35	Positive	Positive	1	contamination	
89	SNR	1285	967	M	60	Positive	Positive	2	no growth	
90	SNR	1290	972	M	25	Positive	Positive	2	no growth	
91	SNR	1295	977	M	34	Positive	Positive	2	no growth	
92	SNR	1313	994	F	20	Positive	Positive	2	no growth	
93	SNR	999	995	M	42	Positive	Positive	1	no growth	
94	SNR	1141	998	F	29	Positive	Positive	1	no growth	