AMERICAN JOURNAL OF PHARMTECH RESEARCH

Journal home page: http://www.ajptr.com/

Examination of Sputum for Detection of *Mycobacterium Tuberculosis*

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ABSTRACT

Tuberculosis is one of the main public health problems worldwide. Nearly one-third of the global population, i.e. two billion people, are infected with *Mycobacterium tuberculosis* and accordingly at risk of developing the disease. More than nine million people develop active TB every year and about two million die. More than 90% of global TB cases and deaths occur in the developing world, where 75% of cases are in the most economically productive age group (15-54 years). At every outpatient clinic, hospitals and health amenities, together in the public and private sectors, all patients have to to be thoroughly screened for cough by health officers manning the health facilities. In addition, within medical colleges and hospitals, in-patients also need to be screened for detection of TB suspects. People with cough for 2 weeks, or more, with or without extra symptoms indicative of TB, should be punctually recognized as pulmonary TB suspects and steps taken to question them to sputum smear microscopy for acid-fast bacilli, for diagnosis of TB.

Keywords: *Mycobacterium tuberculosis*, Diagnosis, Healthcare, Acid fast.

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Received 29 January 2015, Accepted 06 February 2015

Please cite this article as: Bhat JA *et al.*, Examination of Sputum for Detection of *Mycobacterium Tuberculosis*. American Journal of PharmTech Research 2015.

INTRODUCTION

Tuberculosis, a disease of great archeological find is known to mankind since ages. Among all the infectious diseases, tuberculosis is accountable for highest number of deaths[1]. Despite availability of effective chemotherapy it continues to be a major health problem all over the world, especially developing nations. In the last decade there has been a dramatic reappearance in the incidence of TB all around the globe, fueled in part by harsh population growth, poor implementation and execution of public health measures and the human immunodeficiency virus (HIV) / AIDS pandemic[2]. Every year 1.7 million people die of tuberculosis, which averages around 6000 deaths per day, with India alone contributing 40% of the total global burden. Irony being that tuberculosis is the most cost effective of all grown diseases to treat[3]. The vast majority of TB patients live in low and middle income countries, where the diagnosis of tuberculosis relies primarily on identifying acid fast bacilli in sputum smears using a simple light microscope[4]. The need of sensitivity of the only diagnostic test in many parts of the world results in delays in diagnosis, enabling the disease to progress and enhancing the prospective for swell of *Mycobacterium tuberculosis*[5]. A universal threat to TB control programme is the appearance of multi drug resistant *Mycobacterium tuberculosis* (MDR- TB), defined as resistance to slightest isoniazid (INH) and rifampicin (RIF), with resistance to the latter being more severe. In the Revised National Tuberculosis Control Programme (RNTCP), under performance since 1993, the diagnosis of pulmonary tuberculosis is also mainly sputum based in agreement with the WHO and IUALD guidelines[6,7]. Each chest symptomatic is compulsory to give three sputum specimens (first spot, second early morning and third spot specimen). To make easy this requirement, sputum testing centers have been provided, one per 1,00,000 populations in the community, and these microscopy centers have been entirely upgraded in terms of equipment and well trained staff.

Biology of Mycobcateria

M. tuberculosis is a slim, sturdily acid-alcohol-fast rod. It frequently shows uneven beading in its staining, appearing as connected series of acid-fast granules. It grows at 37^0C but not at room temperature, and it requires enriched or complex media for primary growth. Growth is improved by 5-10% carbon dioxide but is still very slow, with a mean generation time of 12-24 hrs. The classic medium, Lowenstein-Jensen, contains homogenized egg in nutrient base with dyes to inhibit the growth of other bacterial contaminants. The dehydrated, rough, buff-colored colonies typically appear after 3-6 weeks of incubation. Mycobacterial growth is swifter in two semi-synthetic oleic acid-albumin media. Virulent strains grown in the latter display “cording” in which

multiplying organisms stay attached in parallel bundles to form long intertwining cords or ropes as shown in figure 1. Of particular significance is the ability of *M. tuberculosis* to produce large quantities of niacin, which is rare in other mycobacteria. Due to its hydrophobic lipid surface, *M. tuberculosis* is abnormally resistant to drying to most ordinary disinfectants, and to acids and alkalis. Tubercle bacilli are susceptible to heat, including pasteurization, and individual organisms in droplet nuclei are prone to inactivation by ultraviolet light. As with other mycobacterium, the *M. tuberculosis* cell wall structure is conquered by mycolic acids and LAM. Its antigenic makeup includes numerous protein and polysaccharide antigens of which tuberculin is the most studied. It consists of heat-stable proteins enlightened into liquid culture media. A purified protein derivative (PPD) of tuberculin is used for skin testing for hypersensitivity and is identical in tuberculin units according to skin test activity.

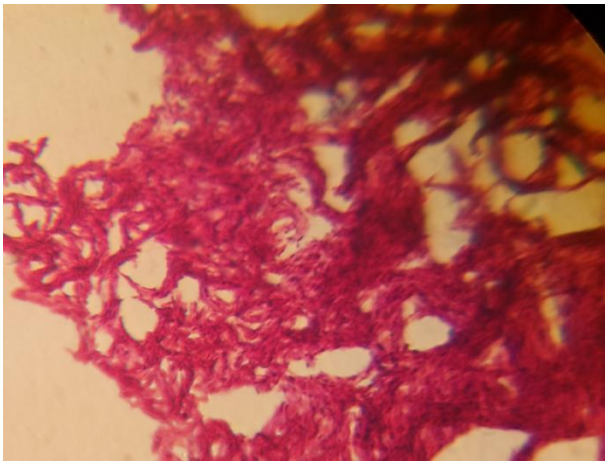

Figure 1: Shows cording of *Mycobacterium tuberculosis* in sputum.

Epidemiology

Tuberculosis (TB) continues to be a chief public health problem, mostly in developing countries. The WHO estimates that one third of the world's population is infected with *Mycobacterium tuberculosis*, the causative agent of TB. There were an estimated 8.3 million new vigorous cases and 1.8 million deaths from TB in the year 2000, making it the second greatest destroyer among infectious diseases globally [8]. The occurrence of multidrug-resistant TB (MDR-TB), defined as resistance to at least rifampicin (RIF) and isoniazid (INH), is increasing in a number of geographic regions. According to a recent WHO report[8]. A well-known disease of ancient times, tuberculosis first reached epidemic proportions in the western world in the major periods of urbanization in the 18th and 19th centuries. Mortality reached 200 to 700 per 100,000 populations each year, accounting for 20 to 30% of all deaths in municipal centers and winning tuberculosis the designation of the “white plague.” Morbidity was many times advanced. The disease has had

major sociologic components, boom with ignorance, paucity, congestion, and poor hygiene, mainly during the social disruptions of war and economic misery. Under these circumstances, the deprived are the major victims, but all sectors of humanity are at risk. Chopin, Paganini, Rousseau, Goethe, Chekhov, Thoreau, Keats, Elizabeth Barrett Browning, and the Brontes, to name but a few, were all lost to tuberculosis in their intellectual prime. With knowledge of the cause and transmission of the disease and the development of effective antimicrobial agents, tuberculosis was increasingly brought under control in developed countries. Unfortunately, mortality and morbidity remain at 19th-century levels in many developing countries despite extensive national and international control programs. Globally, there were an estimated of 9.27 million incident cases of TB in 2007. This is an increase from 9.24 million cases in 2006, 8.3 million cases in 2000 and 6.6 million cases in 1990 as shown in figure 2. The five countries that rank first to fifth in terms of total numbers of cases in 2007 are India (2.0 million), China (1.3 million), Indonesia (0.53 million), Nigeria (0.46 million) and South Africa (0.46 million). Of the 9.27 million incident TB cases in 2007, an estimated 1.37 million (15%) were HIV-positive; 79% of these HIV-positive cases were in the African Region and 11% were in the South-East Asia Region[9]. The great majority of tuberculous infections are contracted by inhalation of droplet nuclei carrying the causative organism. Humans may also be infected through the gastrointestinal tract following the ingestion of milk from tuberculous cows (now uncommon due to pasteurization) or, rarely, through abraded skin. It has been estimated that a single cough can generate as many as 3000 infected droplet nuclei and that less than 10 bacilli may initiate a pulmonary infection in a susceptible individual. The likelihood of acquiring infection thus relates to the numbers of organisms in the sputum of an open case of the disease, the frequency and efficiency of the coughs, the closeness of contact, and the adequacy of ventilation in the contact area.

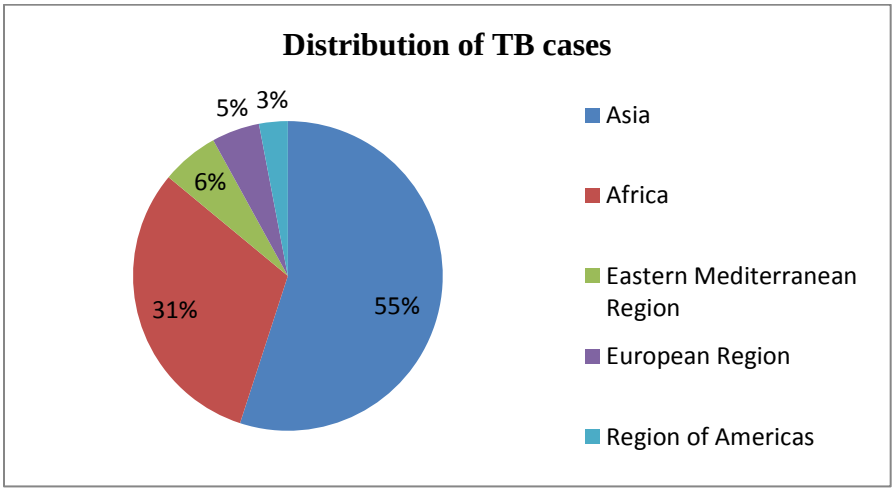

Figure 2: shows distribution of TB cases in different regions of world in 2007

Specimen Collection

Proper sputum collection is extremely critical for best results, and early morning specimens are preferred. The specimen should be expectorated sputum and not saliva that often would not yield correct results. A specimen should be between 2-10 ml in volume. Ideally, from a new patient, three specimens should be collected on consecutive days and should be processed separately. WHO recommends two morning specimens and a third spot specimen when a patient visits the clinic. Pooled specimens are not recommended. The results of tests, as they affect patient diagnosis and treatment, are directly related to the quality of the specimen collected and delivered to the laboratory.

Specimen quality

- Recently discharged material from the bronchial tree, with minimal amounts of oral or nasal material.
- Expectorated sputum generated from a deep productive cough.
- Induced sputum produced with hypertonic saline, if patient is unable to produce sputum on their own.
- Specimens are thick and contain mucoid or mucopurulent materials as shown in figure 2.
- Ideally, 3-5 ml in volume, although smaller quantities are acceptable if the quality is satisfactory.
- Poor quality specimens are thin and watery. Saliva and nasal secretions are unacceptable as indicated in figure 3.
- Laboratory requisition form should indicate when a specimen is induced to avoid the specimen being labeled as “unacceptable” quality[10].
- Specimens should be transported to the laboratory as soon as possible after collection.
- If delay is unavoidable, the specimens should be refrigerated to inhibit the growth of unwanted micro-organisms. If refrigeration is not possible and a delay of more than 2 days is anticipated, a suitable preservative viz., an equal volume of a mixture of 1% cetyl pyridinium chloride (CPC) in 2% sodium chloride solution is recommended.

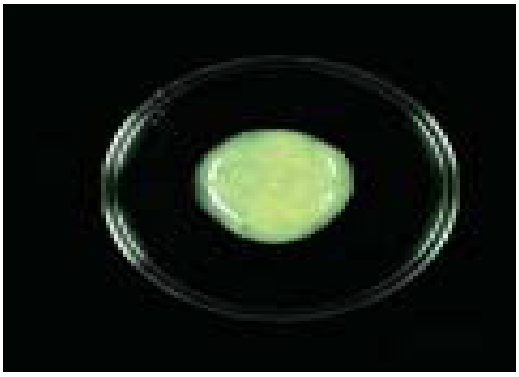

Figure 3: Shows good quality sputum

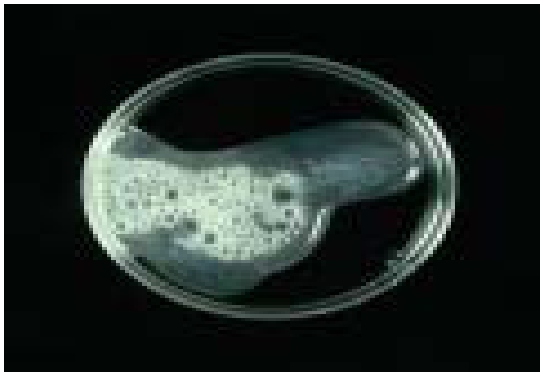

Figure 4: Shows poor quality sputum.

Indications for Sputum Collection

To establish an initial diagnosis of TB

- Collect a series of three sputum specimens, 8-24 hours apart, at least one of which is an early morning specimen.
- Optimally, sputum should be collected before the initiation of drug therapy.

For release from home isolation

- If patient is smearing positive and on treatment: Collect sputum until 3 specimens are negative.

Monitoring of therapy

- Obtain sputum specimens for culture at least monthly until cultures convert to negative[11].

Homogenisation and Decontamination

The bulk of clinical specimens submitted to the tuberculosis culture laboratory are infected to changeable degrees by more rapidly growing normal flora. These would quickly overgrow the entire surface of the medium prior to the tubercle bacilli start to grow. The specimens must, consequently, be subjected to a ruthless digestion and decontamination procedure that liquefies the organic debris and eliminates the unnecessary normal flora. All currently available digesting/decontaminating agents are to some level toxic to tubercle bacilli. Therefore, to ensure the survival of the maximum number of bacilli in the specimen, the digestion/decontamination procedure must be accurately followed. N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) solution is recommended as a gentle but effective digesting and decontaminating agent[12]. Though, at any time a proportion of cultures will be contaminated by other organisms. As a common rule, a contamination rate of 2-3% is satisfactory in laboratories that process fresh specimens. If processing delays are projected, beyond 3 days period, culture fatalities due to contamination may be as high as 5%-10%. It is also significant to note down that a laboratory which experiences no

contamination is most likely using a method that kills a lot of the tubercle bacilli. In such instances the culture negativity in the laboratory increases.

Smear Preparation

Ziehl-Neelsen stain technique is single of the general techniques that are being used to diagnose the TB infection. Smear microscopy with Ziehl-Neelsen technique has been the major way of diagnosing TB patients in budding countries. This is because the method is straightforward, quick, reproducible, low cost and efficient in detecting infectious disease such as TB[13]. Smear must be prepared in convenient batches (maximum of 12 per batch). Labeling of smears should be done at the bench for received specimens using a permanent marker, e.g. a diamond marker. Stay away from touching the surface of the slides. The maximum possibility of finding bacilli is in the solid or moist opaque particles of the sputum and the results of direct smear examination depend to a great extent on the selection of these particles. The recommended size of the sputum smear is 1 cm x 2 cm.

Microscopic Examination

The diagnosis of pulmonary tuberculosis in RNTCP is principally based on sputum examination, in agreement with the guidelines of WHO and IUATLD[14,15]. All chest symptomatics are obligatory to get three specimens of sputum examined by worth ZN microscopy for establishing the diagnosis at Microscopy Centres, one for each 100,000 population. The mainly important technique in the diagnosis of tuberculosis is direct microscopic examination of properly stained sputum smear for AFB. Direct microscopy is easy and inexpensive, and detects most of the infectious form of pulmonary tuberculosis. At present, no other diagnostic tool is accessible which could be implemented affordably. Direct microscopy for TB is also performed to assess the response to treatment and to establish cure or failure at the end of treatment. TB diagnosis is usually being done manually by microbiologist through microscopic examination of sputum specimen of TB patients for pulmonary TB diseases. Though, there are a few problems that have been reported with physical screening process, such as time overwhelming and labor-intensive, particularly for screening of the negative slides[16]. When reporting the results of the microscopic examination, the microbiologist should offer the clinician with an opinion of the number of acid-fast bacilli detected. If the smear microscopy is visibly positive, very small examination time is needed to confirm the result. The slide is off the record as TB positive if at least one tubercle bacilli is found in 300 microscopic fields. The case will then be classified into one of four sternness category if the smear on the slide is found to be positive, according to the number of tubercle bacilli found in the

slide. For a well-trained microbiologist, it takes 15-20 minutes to read and confirm one negative slide, with an average of 25 slides can be read per day.

Instructions for sputum examination

- Focus the stained slide under low power objective lens (5X or 10X) and, observe the staining quality and consistency of smear. Choose the field where the smear is lightly distributed and WBCs & mucus can be seen.
- Apply a drop of immersion oil on the stained smear and focus the smear using 100X objective lens. Do not touch the smear with the dropper. Doing so can contaminate the applicator whereby may spread AFB from one slide to the next resulting in false-positive results.
- At all times examine ZN-stained smears with a 100x objective lens under oil immersion.
- Include a well-known positive slide and a known negative slide every day. The positive control ensures the staining potential of the solutions and of the staining method. The negative control confirms that acid-fast contaminants are not there in the stains or other solutions.
- Make a sequence of orderly examination over the length of the smear. After examining a microscopic field, shift the slide longitudinally so that the adjoining field to the right can be examined. Search each field carefully.
- Examine at least of 100 fields before the smear is reported as negative. For expert microbiologist this will take around 5 minutes. In a smear of 2.0 cm x 1.0 cm size, the number of microscopic fields in one length of the slide corresponds to about 100. If the smear is fairly or heavily positive, fewer fields may be examined and a report of "positive" may be made although the entire smear has not been examined.
- Prior to investigative the next slide, clean the immersion lens with a piece of lens tissue.
- Surprising objects may be seen when using the microscope. If these objects move only when the slide is moved, they may be materials rarely found in the specimen or object, precipitated stains, contaminants from the stains or contaminants in the immersion oil.
- Artifacts that move only when the eyepiece is rotated are in the eyepiece or on its lenses. Artifacts may also be caused by material on the condenser, lenses, mirror or light source.
- Maintain all the slides for quality control according to conventional procedures.

Recording and Reporting of Results

The worldwide approved definition of a sputum smear-positive TB case is:

- Two or more preliminary sputum smear examinations positive for AFB.

- One sputum smear examination positive for AFB plus radiographic abnormalities reliable with active pulmonary TB as resolute by a clinician.
- One sputum smear positive for AFB plus sputum culture positive for *Mycobacterium tuberculosis*[17].

The microscopic observation should be established earliest of all and then if there are AFB present in the smear; the 'estimated average number of these bacilli per microscopic field be observed. It is recommended that a standardized pattern of reading be followed, observing up to 100 useful fields. The sputum smears are graded, according to the number of bacilli seen in the slide, as suggested by WHO[18]. A practical microscopic field is regarded as one in which cellular elements of bronchial origin (leucocytes, mucous fibers and ciliated cells) are observed. The fields in which there are no such elements should not be included in the interpretation. Results obtained should be entered or transcribed appropriately into the laboratory register. All results are reported as prior as possible or if possible within 24 hours. Positive results are entered in red. The number of AFB found is a sign of the degree of infectivity of the patient as well as the severity of tuberculosis disease. Results should therefore be quantified. For Ziehl-Neelsen stained smears the following semi-quantitative method of reporting is recommended table 1:

Table 1: Grading of AFB smears as per WHO and IUATLD recommendation

No of acid-fast bacilli (AFB)	Fields	Report
No AFB	In 100 immersion fields	Negative
1-9 AFB	In 100 immersion fields	Positive (scanty)
10-99 AFB	In 100 immersion fields	1+
1-10 AFB	Per filed examine 50 fields	2+
More than 10 AFB	Per field examine 20 fieds	3+

Safety Precautions in Tb Laboratory

Collecting sputum specimens

- Instruct the patient to cover up their mouth as coughing.
- Stand at the back of patient while the patient is coughing.
- Collect samples in open air
- Open the sputum containers containing specimen with care[19].

Working with bacteriological loops

- Sufficient cooling of the loop after sterilization and before introducing into the specimen.
- Eliminate the sputum sticking to wire loops in a sand-alcohol jar before flaming.

Smear preparation and heat fixation

- Moderate movement of hands while smearing.

- Absolute air drying of smear previous to heat fixing[19].

Laboratory Hygiene

- Curb the entry to the laboratory only to the approved personnel.
- Ban eating, drinking, smoking or applying make-up inside the Laboratory.
- Do not allow pasting labels or sucking pencils.
- Wash hands with an appropriate soap upon entering the laboratory, after managing potentially contaminated specimen containers, after any bacteriological method and before parting the laboratory.
- Observe all surfaces and equipment within the laboratory as potentially infectious and cleaned frequently before starting and after concluding the work using 5% phenol or 70% alcohol.
- Floors ought to not be swept but should be mopped with a disinfectant to limit dust formation[19].

Disinfectants

Disinfectant	Surfaces	Spills	Prepare
Phenol 5%	✓	✓	Every 2 days
Alcohol 70% v/v	✓	X	Weekly
Hypochlorite 5%	X	✓	Every 2 days

Personal Protective Equipments

Masks

Use of masks is not compulsory. Only extraordinary masks such as the N95 gas mask with a perfect fit are truly effective[20].

Gloves

Gloves are not actually needed either, since infection through the skin is unusual. If used, they should be distorted frequently; if not they will only serve to spread contamination roughly.

Apron

Apron should be worn every time a technician performs work inside the laboratory. The same apron should not be worn outside the laboratory[20].

Laboratory Accidents

Spills inside biological safety cabinet

- Make sure absorbent materials (gauze/cotton), 5% phenol and 70% alcohol is kept inside the cabinet.
- Wear double pair of gloves during decontamination procedure.

- Spread 5% phenol waterlogged gauze/cotton instantly to cover the spillage, while the biological safety cabinet continues to function. Wait for 15-20 minutes.
- Using gauze/ cotton, clean up the spill from the edges to the center.
- Wipe the area once more with a gauze/ cotton soaked in 70% alcohol.
- Discard the worn gauze/cotton and gloves in a biohazard bag to be autoclaved.
- Wash hands by soap after the disinfection[20].

Waste Disposal

No infected objects should leave the laboratory except it is appropriately packed for transport. All specimens, contaminated containers, applicator sticks, pipettes and other laboratory supplies should be sterilized by autoclaving at 121^0C at 15 lbs for 20 minutes before discarding or re-use[21].

Treatment

The most important goals for the treatment of TB disease are: to cure the individual patient; reduce risk of death and disability and diminish transmission of *M. tuberculosis* to other persons. To make sure that these aims are met, TB disease should be treated for at least 6 months and in some cases even longer. The majority of the bacteria are killed during the first 8 weeks of treatment; still, there are persistent organisms that need longer treatment. If treatment is not sustained for a lengthy sufficient duration, the surviving bacteria may cause the patient to become ill and infectious once more, potentially with drug-resistant disease. There are a number of options for daily and irregular therapy, but the goal of treatment for TB disease ought to be to provide the safest and most efficient therapy in the shortest phase of time. Given ample treatment, almost all patients will recuperate and be cured. *M. tuberculosis* is prone to a number of effective antimicrobials. First line drugs which are given to the patient are isoniazid, ethambutol, rifampin, pyrazinamide, rifabutin and rifapentine. INH, RIF, PZA, and EMB form the central part of early treatment regimen. Rifabutin may be used as a replacement for RIF in the treatment of all forms of TB caused by organisms that are known or assumed to be susceptible to this agent. Rifapentine may be used one time weekly with INH in the continuation phase of treatment for HIV-negative patients with noncavitary, drug-susceptible pulmonary TB who have negative sputum smears at conclusion of the initial phase of treatment. The second line treatment includes streptomycin, cycloserine, capreomycin, ρ-Aminosalicylic acid, levofloxacin, moxifloxacin, gatifloxacin, amikacin/kanamycin and ethionamide. Streptomycin was previously considered to be a first-line drug and in some instances, is still used in initial treatment. Increasing incidence of resistance to streptomycin in several parts of the world has decreased its overall usefulness. The residual drugs

are kept for special situations such as drug intolerance or resistance and combinations of these agents represent the primary drugs of choice for treatment of tuberculosis. All of these, except ethambutol, are bactericidal. Isoniazid and rifampin are active against both intra and extracellular organisms, and pyrazinamide, a nicotinamide analog, acts at the acidic pH found within cells. Streptomycin does not penetrate into cells and is thus active only against extracellular organisms.

CONCLUSION

The diagnosis of pulmonary tuberculosis under RNTCP is mainly based on sputum examination, according to the guidelines of World Health Organization. So, all symptomatic patients are required to get 3 specimens of sputum examined by quality ZN microscopy for establishing the diagnosis at various examination centers across the world.

REFERENCES

1. Grange JM, Collier L, Balows A, and Sussman M. Microbiology and Microbial Infections, 1998; 391-413.
2. Raviglione MC, Snider DE, and Kochi A**.** Global epidemiology of tuberculosis: morbidity and mortality of a worldwide epidemic. JAMA 1995; 273:220-226.
3. Global tuberculosis control: surveillance, planning, financing, Geneva. World Health Organization 2006; 1-242.
4. Dye C, Watt CJ, Bleed DM, Hosseini SM, Raviglione MC. Evolution of tuberculosis control and prospects for reducing tuberculosis incidence, prevalence and deaths globally. JAMA 2005; 293: 2767-75.
5. Behr MA, Warren SA, Salaman H, Hopewell PC, Poncedeleon A. Transmission of *Mycobacterium tuberculosis* from patients smear negative for acid fast bacilli. Lancet 1999; 353: 444- 449.
6. World Health Organisation, Treatment of Tuberculosis: Guidelines for national programmes. 2nd edition. WHO/TB. 1997; 97.220.
7. Enarson DA, Rieder HL, Arnadottir T, Trebueq A. Tuberculosis Guide for Low Income Countries. 4th edition. Paris, 1996; International Union against Tuberculosis and Lung Diseas.
8. Beenhouwer H De, Lhiang Z, Jannes G, Mijs W, Machtelinckx L, Rossau R, Traore H, Portaels F. Rapid detection of Rifampicin resistance in sputum and biopsy specimens from tuberculosis patients by PCR and line probe assa., Tuber Lung Dis. 1995; 76:425-430.
9. WHO Report 2009, *Global tuberculosis control Epidemiology*, *Strategy*, *Financing*, WHO Press, World Health Organization, ISBN 978 92 4 156380 2, Geneva, Siwtzerland.

10. CLSI. Laboratory Detection & Identification of Mycobacteria; Approved Guidelines. CLSI document M48-A. Wayne, PA: Clinical & Laboratory Standards Institute; 2008.
11. Centers for Disease Control and Prevention, Guidelines for Preventing the Transmission of *Mycobacterium tuberculosis* in Health-Care Settings. MMWR. 2005; 54(No. RR-17).
12. Kent PT and Kubica GP. Public health mycobacteriology: a guide for the level III laboratory. USDHHS. Centers for Disease Control. 1985; Atlanta.
13. Luna JAC. A Tuberculosis Guide for Specialist Physicians, International Union against Tuberculosis and Lung Disease. 2004; Paris.
14. World Health Organisation. Treatment of Tuberculosis: Guidelines for national programmes, 2nd edition. WHO/TB/ Geneva. 1997; 97-220.
15. Enarson DA, Rieder HL, Arnadottir T, Trebueq A. Tuberculosis Guide for Low Income Countries, 4th edition. Paris, International Union against Tuberculosis and Lung Disease. 1996.
16. Veropoulos K, Campbell C, & Learmonth G. Image processing and neural computing used in the diagnosis of tuberculosis, Proc. IEE Colloquium on Intelligent Methods in Healthcare and Medical Applications (Digest No. 1998/514), 8/1 - 8/4, York, UK.
17. World Health Organization, Frame work for effeciteve tuberculosis controle. World Health Organization Document. 1994; WHO/TB/94.179; 1-7.
18. World Health Organization, Treatment of Tuberculosis: Guidelines for national programmes, 2nd ed. WHO/TB/ 97.220, Geneva, 1997.
19. Centers for Disease Control and Prevention. National Institutes of Health. Biosafety in microbiological and biomedical laboratories. HHS Publication No. (CDC) 99-8395. US, Government Printing Office. Washington, 1999.
20. Kent PT, Kubica GP. Public Health Microbiology, a Guide for the Level III Laboratory. Centers for Disease Control, Division of Laboratory Training and Consultation. Atlanta, GA, US Department of Health and Human Services, US Government Printing Office, 1985.
21. Isenberg HD. Clinical Microbiology Procedure Handbook, Vol.1. American Society for Microbiology (Publisher), Washington, D.C.