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Review:

Diagnosis of Tuberculosis:

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Abstract: Tuberculosis has huge impact in humanity with high mortality rate in developing countries. For diagnosis of tuberculosis sputum smear microscopy has been the primary method in low middle income countries. But sputum microscopy has its some limitation in its performance. But if bacterial load is less then100000/ML of sputum sample the sensitivity is grossly compromised. In 1930 Fluorescent microscopy was introduced for detection of TB, which shows better result in compared to ordinary microscopy/ ZN microscopy. Specificity, sensitivity, cost effectiveness, and cost benefit of fluorescent microscopy have not yet established. International guidance on quality assurance for fluorescent microscopy does not currently exist. Current methods of drug susceptibility testing of Mycobacterium tuberculosis are costly or time consumable task. As the multidrug resistance tuberculosis is increases so the fast, reliable and inexpensive methods are in need of for detection of Tuberculosis. In compared to microscopy sputum culture can detect far lower number of AFB. Moreover, culture make it possible to identify the mycobacterial species as well as perform drug susceptibility testing. Rapid and molecular technique are also established for diagnosis of TB.

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Key words-NTEP, Multi drug resistance, CBNAAT, Line probe assay. Smear microscopy, molecular diagnosis, culture and drug susceptibility testing.

INTRODUCTION:

Tuberculosis is a common disease in India. It is an air born disease. It is caused by bacteria called *Mycobacterium tuberculosis* which bacterium was discovered by Robert Koch in 1882 on 24thmarch. During that time TB killed one out of seven people living in the United States and Europe. [1] A century later, March 24 was designated as World TB Day, a day to educate the common people regarding the disease tuberculosis. So 24th march is being celebrated as "world Tb Day" across the globe.

The *Mycobacterium Tuberculosis* is an acid fast bacilli are rod shaped bacteria measuring 0.2-0.6 μm wide and 1.0-10 μm long. Under the light microscope the bacilli are seen as straight or slightly rod shaped. [2] Taxonomically Mycobacterium is under the kingdom of Bacteria and Phylum is Actinobacteria, order is Actinomycetales, Sub-order is Carynebacterineae, Family is *Mycabacteriun* and species is *tuberculosis*.

Due to coughing and sneezing one person may have infected from an infected person. A Tb infected person may spread the infection up to 10 to 15 normal persons if not treated the infected patient on time [3]

ISSN 2515-8260 Volume 09, Issue 07, 2022

The symptoms of Tuberculosis in a person having cough more than two weeks and fever more than two weeks also significant weight loss and any abnormality in chest radiation also must be seen apart from this people living with HIV and diabetes, cancer. Moreover high-risk population such as prisoners, slum dwellers, health care worker. Miners should be undertaken for TB screening. [4]

Tuberculosis is a communicable disease and one of the leading causes of death worldwide. Before Corona virus pandemic, Tb was the leading cause of death from a single infectious agent even ranking above HIV infection. Tuberculosis is a curable and preventable disease and almost 85% Tb patients can be successfully treated with anti TB drugs regimen. [8]

As per Global tuberculosis report, 2015 an estimated 9.6 million people fell ill with Tuberculosis in 2014. Globally there were 1.5 million Tb deaths. Out of that 5.4 million were men and 3.2 million were female. [9]

. In 2015 an estimated 10.4 million (5.9 million men and 3.5 million woman and 1 million children) people fell ill with TB and the best estimate is that there were 1.4 million death and additionally 0.4 million TB death among HIV Tb cases. In 2015 Tb was the top 10 cause of death globally [10]. In 2016 estimated TB cases were 10.4 million and estimated TB death were 1.3 million and additionally 374000 HIV TB cases. In 2016 Tb was the top 9th cause of death globally. 11] In 2017 an estimated 10 million people fell ill with TB and estimated death were 1.3 million and additionally 3000000 TB HIV patients. In 2017 TB was the top 10 th cause of death globally. [12]. In 2018 estimated TB cases were 10 million and estimated death were 1.2 million and additionally 251000 TB HIV patients. Out of total global Tb burden India bears 27% followed by China bears 9% and Indonesia bears 8%. [13]. In 2019 an estimated 10 million people fell ill with TB and estimated death were 1.4 million. [14] . In 2020 estimated TB cases were 5.8 million and death were 1.3 million and

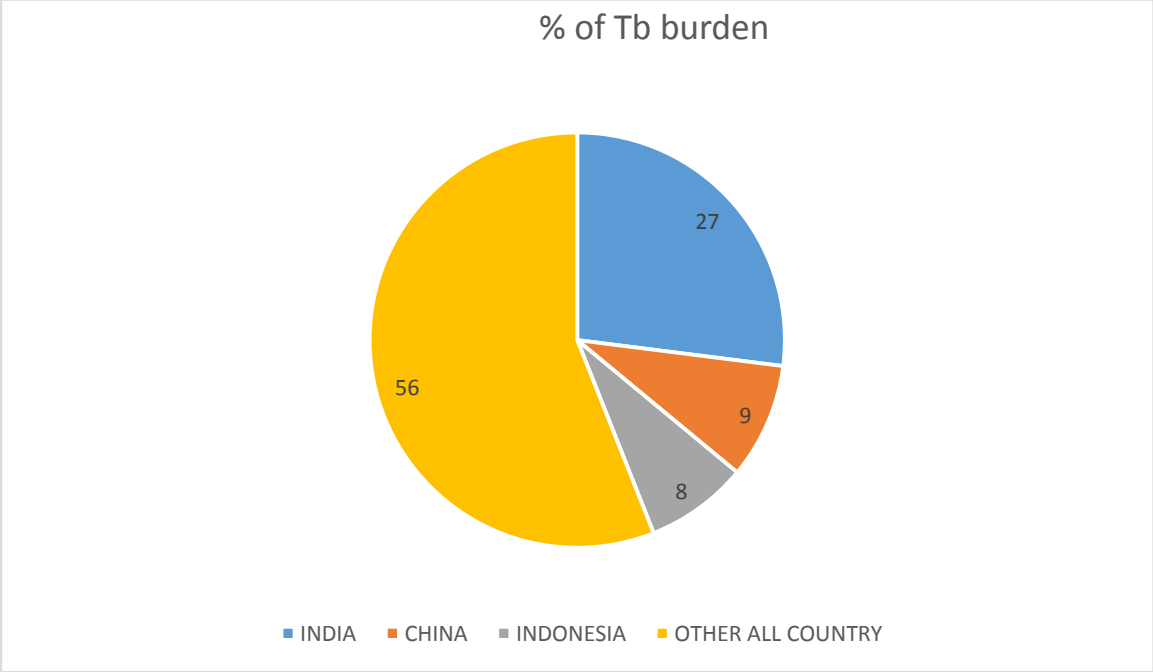

Fig: Pie diagram showing % TB burden of India

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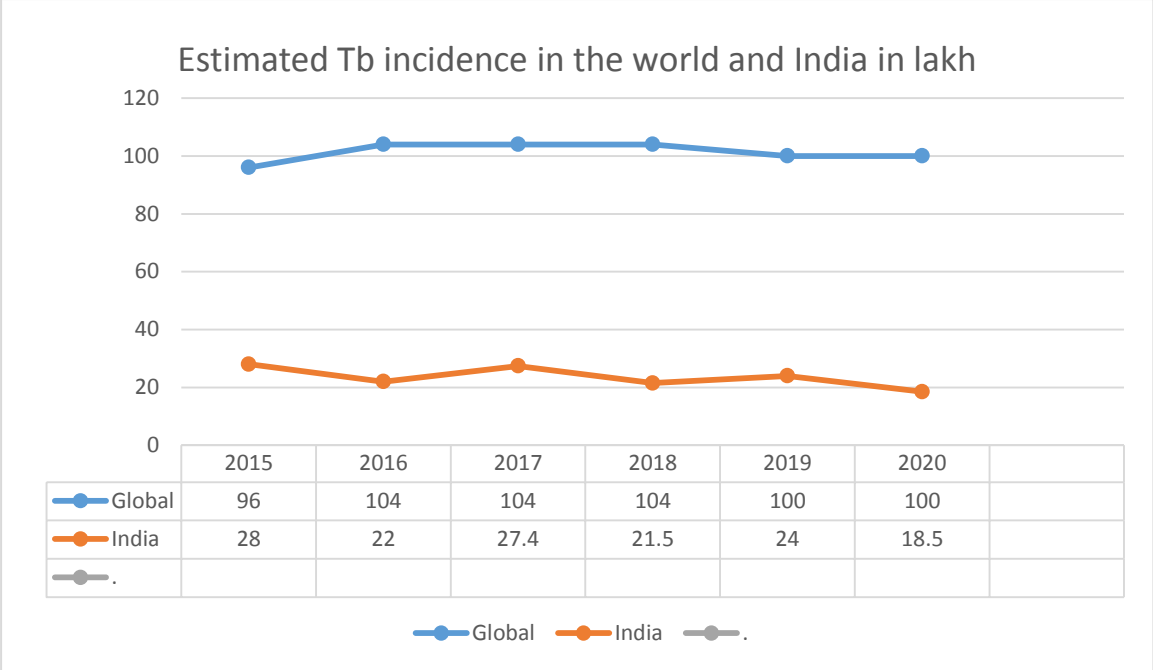
Additionally 2 14000 HIV TB cases. Due to Covid -19 pandemic in the beginning of 2020 an enormous health impact has occurred.- [15]

After China, India is the second populous country across the globe One fourth of global Tb incidence occur in India annually. As per statistics out of estimated global annual TB incidence of 9 million, 2.1 million were estimated to have occurred in our country in 2013. [16]. Estimated 28 lakh TB cases occurred in India in 2015 and 4.8 lakh TB cases were died of TB. Estimated 4.8 Lakh MDR TB cases occurred- in the world and in India this number were 1. 3 lakh.

In 2016 estimated 27.9 lakh TB cases were occurred and 4.23 Lakh cases were died. Out of 104 lakh TB cases of the world India accounts for one fourth of the total global burden [17]. In 2018 total number of notified TB cases were 21.5 lakh. [18]

In 2019 total notified TB patient were 24 lakhs in India with an increase of 12 % as compared to the notified TB cases of 2018. [19]. In 2020 notified TB patients were 18.5 lakh. Due to Covid 19 pandemic there were 25% reduction of notified case in compared to 2018. [20]



India have the highest TB burden in the world. India have the one fourth of the global TB cases.. India bears 26% of the entire global TB burden. [5] Every second an Indian is infected with tuberculosis and one person dies off TB in every second in India. Thus in every year five lakhs peoples died in TB in India. But still tuberculosis is a such type of infectious disease that not getting publicity [6]. Today tuberculosis disease has come out with a new look that is Multidrug resistance TB (MDR/R-R) and creates lots of tough challenges to medical science. Tuberculosis is most opportunistic infection and it infects HIV patients and HIV infection has complicated the TB disease. Almost 3% new TB patients and about 20% of previously treated patients in the world have multidrug resistant strains, mostly resistant to Rifampicin and isoniazid. Nowadays MDR TB has become a public health problem in India and many countries of world and appears as major problems. TB is the 6th leading causes of death in India [7]

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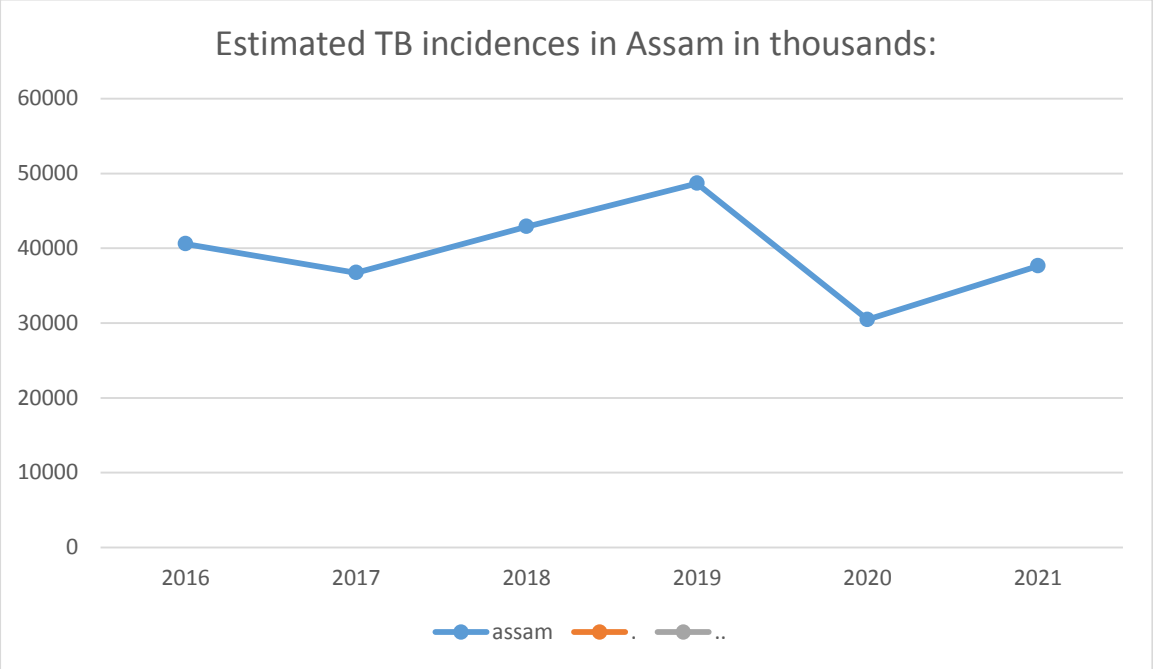
In Assam in 2016 total notified TB cases were 40851, and in 2017 this figure was 36720 and in 2020 this figure was 30456 with death rate of 3.5%. And in 2021 total notified TB cases were 37641.



It has been seen that in slum areas, hard to reach areas, riverine areas, industrial areas like char areas a large number of TB cases is being reported. Kamrup district of Assam has some peculiarities i.e.-having some char areas, slum areas, teagarden, some hard to reach areas. Kamrup district is one of the largest district of Assam having population of 15.18 lakhs and area is 3105 square kilometre [21] . Average Literacy percentage is 75.55% and major language is Assamese and Amri, a local languages taught by Karbi people .Tewa another language taught by 10000 Tewa people. The mighty Brahmaputra River is flowing through the district and the Brahmaputra River divides the district in two half that is north bank and south bank. In south bank there are some brick industry and some tea garden from where TB patients are detecting

Kamrup district has three numbers of tea gardens and some river island, where communication is very bad. The people of those areas have to rush several miles to reach hospital or public health facilities for their health problems. The people from River Island can cross the river by boat and then by paddle they can reach the hospital. Most of the people are illiterate and due to lack of knowledge about TB, and due to social stigma they even cannot disclosed about their disease. In the Tea garden areas, peoples prefer to treat their disease by local doctor called quack doctor.

TYPES OF TUBERCULOSIS:

Tuberculosis is mainly two types, ie- Pulmonary tuberculosis and extra pulmonary tuberculosis. The bacteria responsible for Tuberculosis may attack all the body parts of Human except nails, hairs. If the bacteria attack only human Lung parenchyma then this tuberculosis disease will be designated as pulmonary tuberculosis and except lung if the bacteria attack other parts/organs e g-pleura, lymph nodes, genitourinary track, abdomen,

skin, joints and bones, meninges then this tuberculosis is named as extra pulmonary tuberculosis. In the world incidence of pulmonary tuberculosis is more than extra pulmonary tuberculosis. The pulmonary tuberculosis is classified into two type that is smear negative and smear positive. If the initial sputum samples of a patients is found AFB positive in microscopy then it is called sputum positive pulmonary tuberculosis. If the result of smear microscopy is negative for AFB but the patient is diagnosis based on the sputum culture result is positive for AFB then this type of tuberculosis is called as smear negative pulmonary tuberculosis. The facilities for Diagnosis of pulmonary tuberculosis is easily available and cost effective. Microscope is the prime tools for detection of tuberculosis.

Apart from these some other classification of tuberculosis are

Multi Drug Resistant Tuberculosis-A tb patients whose sputum samples is resistant to both Rifampicin and Isoniazid.

Mono resistant TB- A tb patient whose sputum is resistant to only one drug among Rifampicin, Isoniazid, Pyrazinamide and Ethambutol.

Isoniazid resistant TB- A Tb patient whose sputum is resistant to only Isoniazid (not rifampicin). [22]

HISTORY OF TUBERCULOSIS:

As per Global Tuberculosis Report 2016, *Mycobacterium tuberculosis* has very ancient origin it has survived over 70000 years and it is still remains a major public health problem worldwide and currently infects nearly 2 billion people worldwide with around 10.4 million new cases of TB each year. The bacteria Mycobacterium tuberculosis has an ancient origin. Before 1787+- 230 years, the presence of tuberculosis in fossil of Pleistocene bison in North America were established. This was confirmed by sequencing of DNA which were extracted from bones of bison [23]

Tuberculosis has always been associated with a high mortality rate over the countries and it is estimated that 1.4 million TB death among infectious disease after human immunodeficiency virus (HIV).

AIM:

The aim of the present review was to provide an overview of tuberculosis research mainly on diagnostic part. To eliminate tuberculosis, diagnosis of AFB on time is most important. To reduce diagnostic defaulter's time of sputum examination should be less. In different time to time new methods, diagnostic tools were invented.

Method:

The review is based on available data sources related to tuberculosis control/eradication, treatment, diagnosis. World Health Organization (WHO) is publishing secondary data in their Global Tuberculosis Report. Global tuberculosis report of 2015 to 2020 and Annual Status Reports of 2015 to 2020, published by Revised National Tuberculosis Programme/ National TB elimination Programme and different research paper/journal were reviewed.

DIAGNOSIS OF TUBERCULOSIS:

Among the world's population one third population is infected with mycobacterium tuberculosis. For diagnosis of tuberculosis in human various tools were being used. If any

person having cough for more than two weeks or loss of appetite, weight loss, night sweating, evening rise of temperature then doctor may advise for test of detection of tuberculosis.

To detect the infection with M. tuberculosis the tuberculin test is generally being used. Tuberculin is generally glycerin extract of tubercle bacilli, named as old tuberculin. This name was given by Robert Koch in 1890. Later on, a French physician, Charles Mantoux facilitated the previous work done by Robert Koch. So later on this test was named as Mantoux test. Only 0.1 mL of Tuberculin is injected intradermal and observed after 48 -72 hours. If a person is infected with mycobacterium tuberculosis then the skin reaction is seen by measuring the diameter of induration. This tuberculin test is attractive and have low technology and inexpensive.

Although the tuberculin skin test(TST) is attractive and cost effective but this test is not useful for diagnosis of latent or dormant infection from infection associated with active disease and if person concern have had prior BCG vaccination then this test will misleading with false positive result. [24]

Radiography is another diagnostic tool for detection of tuberculosis. The chest X-Ray, CT scan of thorax and ultrasonography are some tools but further bacteriological examination is required for final diagnosis as tuberculosis infection as proof.

Microscopy is an age old procedure for detection of tuberculosis and still microscopy is accepting as gold standard tool for diagnosis of tubercular bacilli although so many modern tool and technique are introduced. Sputum microscopy is widely used tool as it is inexpensive rapid, and can be use with minimal human resource involvement. The bacteria that are involved with Tuberculosis is *Mycobacterium Tuberculosis* . This bacteria is gram negative bacilli. After the bacilli are stained by carbol fuchsine(Ziehl- Neelson method), the bacilli retain their red color, hence they are called acid fast bacilli. Sputum smear is graded according to WHO as.

Table . Grading of AFB smears as per NTEP recommendation;

Serial No	Number of bacilli	Grading
1	0/100 immersion fields	Negative
2	(1-9)acid fast bacilli/100 immersion fields	Scanty
3	(10 to 99) acid fast bacilli/ 100 immersion fields	1+
4	(1 to 10) acid fast bacilli/ immersion fields	2+
5	More than 10 acid fast bacilli/immersion field	3+

Ziehl-Neelsen Staining Technique:

. New clean and unscratched slide wiere taken and labeled with the Laboratory serial number on one end of the slide.

. Smear were prepared from the mucoid/ purulent part of the sputum using a wooden stick. Smear were allowed to dry for 15-30 minutes

. The smear were fixed by passing three times through a spirit lamp.

. Filtered carbol fuchsin were poured to cover the entire slide.

.The slide were gently heated to steaming until vapor rise.
.Hot carbol fuchsin were keep on the smear for 5 minutes
. The slide were gently rinsed with tap water until the stain is washed away
.The smear were covered with 20% sulfuric acid for 3 minutes
. Again the slide were gently rinse with tap water until the stain is washed away. Then allow to dry in room atmosphere.
. If the slide is still red then sulfuric acid will be re added for 1 minute
.Methylene blue solution (0 1%) were poured on the slide and allow to dry
. Slide were rinsed gently with tap water and allow to dry
.Then slide were shifted to microscope room and were examined under ZN microscope by using X40 lens to select the areas and one drop of immersion oil on the slide were added before observation.

Sputum smear microscopy is primary tool for diagnosis of pulmonary tuberculosis in low and middle income countries. The sputum microscopy is highly specific in area with a high prevalence of tuberculosis. But in compared to conventional microscopy the sensitivity is 10 % higher with fluorescent microscopy. This fluorescent microscope was introduced in 1930 for sputum microscopy. But as per performance concern sputum microscopy has some significant limitation. With sputum microscopy the sensitivity is compromised when the bacterial load is less than 1000 bacilli/ML sputum sample but to monitor the progress of infection and for giving final result of cure sputum microscopy is being used. [25]

Tb is one of the most infectious disease in the world. So whole world is thinking for end TB .So for that different diagnostic tool are being used. In Ethiopia TB case detection rate remain 50% or below 50%. Current policy of that country emphasizes for sputum microscopy of more case detection. But when TB case come to health center them were ask to come with house hold for screening of TB along with HIV. So due to social stigma people deny for coming to health center for screening of TB [26]

Tuberculosis has huge impact on humanity with more death rate, especially in advanced countries. For detection of tuberculosis sputum smear microscopy is cost effective, less time consumable and high specificity. Different modification has noticed but microscopy will remain the primary diagnostic tools for detection of tuberculosis in resource limited countries. Now a day's fluorescent microscopy has been introduced as alternative of ZN staining. Different relevant studies established that fluorescent microscopy shown 10% more sensitivity than conventional microscope [27]

In low and middle income countries TB cases are more across the world.In that low income countries is widely used for detection of tuberculosis. But in case of sensitivity concern light microscopy is less sensitive in patients with co infection with HIV.In advanced countries fluorocent microscopy is widely using due to its high sensitivity. [61]

In low income countries direct sputum microscopy is mostly preferable tools as it is inexpensive, fast and specific for high TB burden areas. But main drawback of direct sputum microscopy is that it is less sensitive in patients co infection with HIV. So different methods for processing sputum has developed like centrifugation, sedimentation, bleach. Different studies shows that centrifugation with chemical methods (including blench) is more sensitive- [28] Finding of AFB in sputum by smear microscopy is directly

related to the concentration of bacilli present in the sputum. By smear microscopy if bacilli is less than 1000/ML of sputum then only 10 % slide shows the presence of AFB.So in case of Culture of sputum the result of presence of AFB can be declared even presence of 100 bacilli /Ml of sputum .Presently mycobacterial culture can be performed on conventional egg-based solid medium such as Lowenstein –Jensen media and agar based one, like as Middlebrook 7H10 or 7H11 and liquid media like as Kirchner's or Middlebrook 7H 9 broth. But main drawback of culturing Mycobacterium Tuberculosis in conventional media is its slow growth, which takes at least 4 weeks and for drug susceptibility tests it takes another 4-6 weeks. Most of the advanced countries across the globe rely of solid media for culture of Mycobacterium. Combination of solid and liquid media is currently accepted as gold standard for isolation of AFB.

There are three methods for drug susceptibility testing, namely absolute concentration, proportion method, and lastly resistant ration method. Among these proportion method is well accepted method for drug susceptibility test.[29]

Rapid culture method:-In comparison to solid culture method the rapid culture method have more advantages. In rapid culture technique time taken for detection of growth of Mycobacterium is shorter. In Rapid culture method time reduced from 38 days to 18 days.

Bactec system is newer culture system where C14 labeled palmitic acid in 7H12 medium is used. Presence of Mycobacterium is detected by the Bactec system based on their metabolism rather than visual growth. As a result of metabolic reaction C14O2 is produced and measured by the Bactec system instrument and reported as growth index (GI) value. For identification of Mycobacterium tuberculosis bactec system is used. In this system specific inhibitor, para-nitro- alpha-acetylamino-beta-hydrxypropiophenon is used. Drug susceptibility test for antitubercular drugs can also be performed by using Bactec system when sufficient GI is observed. Isolation of positive culture was faster with this system and 87% of positive obtained by 7 days and 96% by 14 days [30] One major advantages of Bactec system is that it can be used for perform susceptibility test for pyrazinamide. [31]

Mycobacterial Growth Indicator Tube (MGIT) is another automated system. In this system growth is detected based on the metabolic O2 utilization.-The growth of Mycobacterium tuberculosis is detected in 15.5 days. The MGIT 960 system is potential rapid, accurate and cost effective method.

Molecular Biological Technique:-Identification of resistance associated mutation of bacteria is a big task. For this following techniques like DNA sequencing, Line probe assay(LPA), DNA microarrays,molecular beacons,single strand conformation polymorphism, fluorescent Resonance Energy Trabsfer probes, other PCR-based techniques or Mycobacteriophages-basedassays like FAST plaque TB and Luciferase receptor phages(LRPs) has been used. World Health Organization with UNITAID and FIND(Foundation for Innovative New Diagnostic) made a new policy endorsing use of Line Probe Assay in low resources countries like India. There are two types-the Geno Type MTB-DR assay and INNO- LPA Rif.TB assay. The advantages of this method is that result can be obtained within 2 days over conventional method. [32]

For rapid detection of Mycobacterium tuberculosis and simultaneous determination of rifampicin resistance profile the INNO-LPA Rif.TB assay is used. This assay is based on amplification of the rpoB gene by Polymerase chain reaction (PCR) using biotinylated

primers followed by hybridisation to oligonucleotide probs immolilised in pawrallel on membrane strips and detection of bacilli is done by addition of streptavidin labelled with alkaline phosphates and a chromogenic substrate resulting in a purple brown precipate. The probes include a- Mycobacterium tuberculosis specific probe, five wild type probes covering the entire amplified region and showing no mutation, as well as four probes representing the most frequently encountered mutation and causing rifampicin resistance, A study based on 75 non respiratory specimens shows 58.8% samples detected Mycobacterium tuberculosis than culture 35.3% when compared with final diagnosis. Sequencing data of the rpoB gene and LPA patterns agreed in 29 of 30 Mycobacterium tuberculosis positive specimen (96.7%) and on the other hand another study revealed that on respiratory specimens found a sensitivity of 78.3% and all results were concordant with those obtained by culture by using Bactec 460 system[33]

For confirmation of tuberculosis disease in case of presumptive cases, different tools are being used. Microscopy is the cheapest and widely used tools for detection of TB infection. But due to its limitation several techniques has been made for better diagnosis of tuberculosis. As a part of the development of modern technique automated real time sputum processing molecular beacon assay, Xpert MTB/RIF assay are also established for detection of tuberculosis. But due to relatively high operating cost it is being used within limitation [34]The Xpert MTB/RIF was found to be highly sensitive (98%) and specific (98%) in detecting tuberculosis and to know the rifampicin resistance status within 2 hours. It is very easy to perform.

Detection of antibiotic resistance pattern of the bacterial isolates using nucleic acid amplification test (NAAT)

After detection of acid fast bacilli in a particular sputum sample the another two sputum samples to be collected in a sterile falcon tubes especially designed for carrying samples to CBNAAT site for further detection of resistance pattern especially rifampicin resistance. All falcon tubes or sputum cup is labelled indicating date of collection of sputum samples along with patients details.

Fresh sputum samples is need to be transported to the CBNAAT site in cold chain within 72 hours. In this study NTEP approved model will be followed for transportation of sputum.

NTEP aproved model-

Total capacity up to four Falcon tubes are be used and packed with two ice gel pak. Size of the thermocol box for transportation of sample is 18.5 x12.5 x 13 cm^3. Thickness of the thermocol boxes are 2.5 cm. Before carrying the samples, the gel packs are stored in freeze to maintain a temperature between 12-20 degree Celsius. The sputum containing falcon tubes are tightened by parafilm tapes and samples number and name of patients with date are written on the falcon tubes with a help of permanent marker pen. The box is closed with its flap and block with a help of brown tap. As per national guidelines for Biomedical waste management the container used for transporting sputum samples is labelled with a Bio-hazard sticker. [35]

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Working principle of Cartridge based nucleic acid amplification test (CBNAAT)

Cartridge based nucleic acid amplification test (CBNAAT) is conducted in GeneXpert MTB/RIF machine for simultaneous rapid tuberculosis diagnosis and rapid antibiotic sensitivity test.The Xpert MTB/RIF detects DNA sequences specific for *Mycobacterium tuberculosis* and rifampacin resistance by polymerase chain reaction. It is based on the Cepheid GeneXpert system, a rapid, simple-to-use nucleic acid amplification test (NAAT). The Xpert® MTB/RIF purifies and concentrates *Mycobacterium tuberculosis* bacilli from sputum samples, isolates genomic material from the captured bacteria by sonication and subsequently amplifies the genomic DNA by PCR. The process identifies most of the clinically relevant Rifampacin resistance inducing mutations in the RNA polymerase beta (rpoB) gene in the Mycobacterium tuberculosis genome in a real time format using fluorescent probes called molecular beacons. Results are obtained from unprocessed sputum samples in about 2 hrs, with minimal biohazard and very little technical training required to operate. This test was developed as an on-demand near patient technology which could be performed even in a doctor's office if necessary.

Conclusion: For diagnosis of pulmonary tuberculosis sputum microscopy is time tested process. For developing countries or resources limited countries smear microscopy will be the long lasting diagnostic tools for diagnosis of tuberculosis. Now a day's fluorescent microscopy has been established as ZN microscopy has some limitations. But in developed countries Drug susceptibility test and GENE Expert system has adopted. Hope for such type of tools which will be less time consuming with 100% accuracy and required less human effort and infrastructure for screening of tuberculosis will come out for betterment of people.

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