Manage TB
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Lecture – 09
Bacteriological Diagnosis of Tuberculosis – Smear and Culture

Hello and welcome to this session on Bacteriological Diagnosis of Tuberculosis. I am Dr. Gomathi, from the national institute for research in tuberculosis Chennai.

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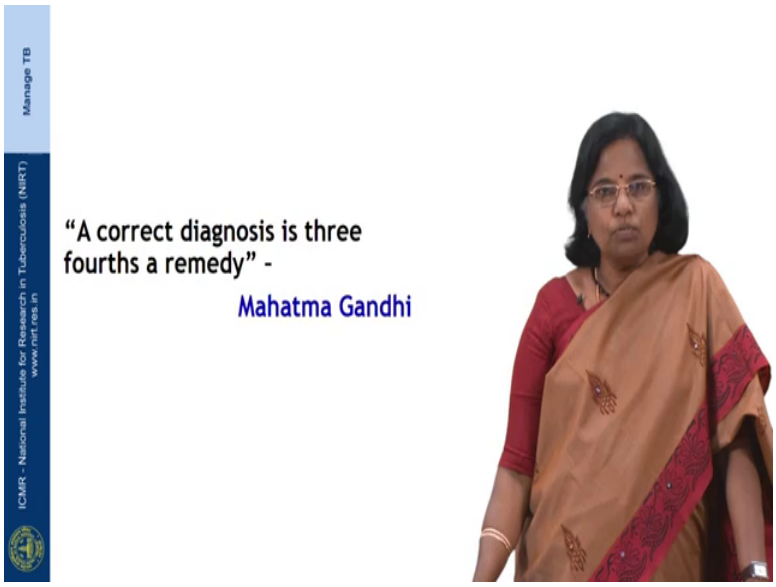

The importance of a correct diagnosis was emphasized by Mahatma Gandhi when he said; the correct diagnosis is three-fourths a remedy.

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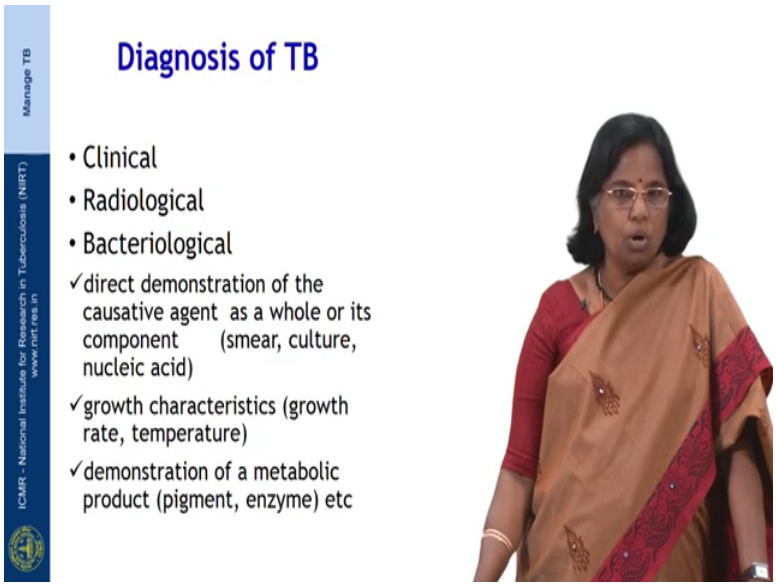

Diagnosis of TB can be done based on clinical findings or radiological findings or bacteriological findings through direct demonstration of the causative agent as a whole or its component for example, the smear, culture or nucleic acid or by means of growth characteristics; example being the growth rate determination or the temperature requirement.

And it could also be by means of demonstration of a metabolic product which could be a pigment an enzyme.

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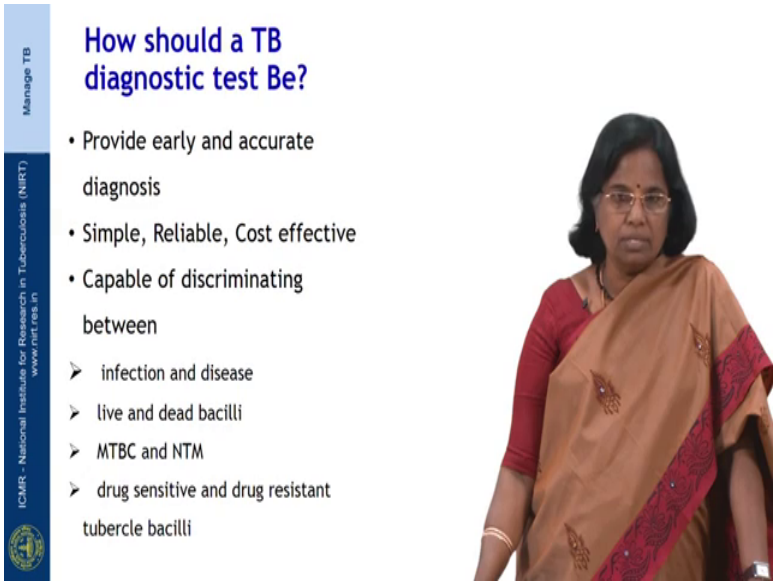

Now, how should a TB diagnostic test be? It should provide early and accurate diagnosis; it should be simple, reliable and cost effective, capable of discriminating between infection and disease live and dead bacilli between the mycobacterium tuberculosis complex and the non-tuberculosis mycobacteria and between drug sensitive and drug resistant tubercle bacilli.

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The good diagnosis is dependent on a good quality specimen; for diagnosis of pulmonary tuberculosis the ideal specimen is sputum and in the case of children who are not able to expectorate sputum gastric aspirate can be collected.

Now, these are some of the types of specimens that are received in the laboratory. First is saliva which is not very useful for smear or culture, second is mucopurulent sputum sample which is very good for all types of test, the third is blood mixed which can be used for smear and culture, but cannot be used for molecular test. The fourth one is a colloidal sputum specimen which may or may not be useful. This picture gives a an ideal sputum sample and an ideally collected gastric aspirate.

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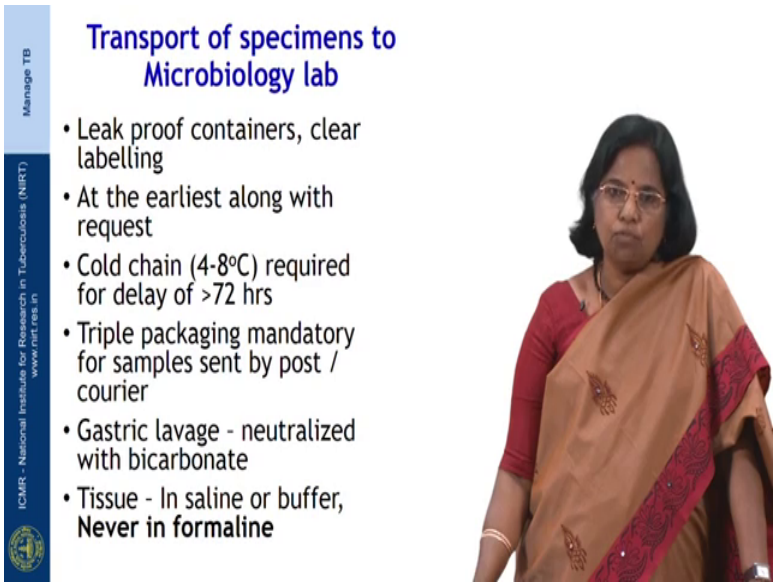

The second important factor to consider for tuberculosis is the transport of specimens to the microbiology laboratory.

First the sample should be collected in leak proof containers with clear labelling and they should be sent to the laboratory at the earliest time point along with request and a cold chain of 4 to 8 degree C is required a whenever a delay of more than 72 hours is expected. A triple packaging system is mandatory for samples if they have to be sent by post or by courier and in the case of gastric lavage the acid content has to be neutralized using bicarbonate solution.

And in the case of tissues or biopsy they should be sent either in saline or in buffer and never in formaline solution.

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We will now have a look at the triple packaging system for transporting infectious material; the material has to be collected in at screw caped tube or a container which is a primary container and after ensuring that the cap is tightly closed it should be sealed using a paraffin film and next it should be pay wrapped with absorbent material like tissue paper and it should be placed in a zip lock cover.

Several such zip lock covers can be placed in a second container and the request forms for all these should be placed separately in a zip lock cover and both these should be placed in a third larger container; this will be the third container which will have the ice packs which will maintain the temperature at 4 to 8 degree C for transportation.

This third container has to be properly sealed labeled with biohazard signs before their transmitter transported by courier or by post.

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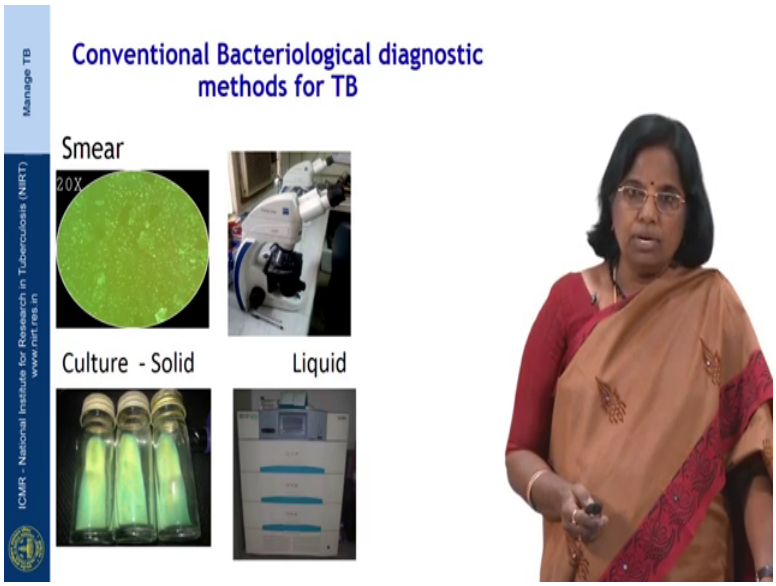

Now, we will come to the conventional bacteriological diagnostic methods for TB; first is a smear which is your usually visualized under the microscope, a second is the culture using solid media or by liquid media.

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Smear microscopy is a century old technique, but its continues to be used as a basic diagnostic tool; it requires minimal infrastructure, it is simple and inexpensive, offers quick results in about 1 hour time, it is suitable for samples like sputum, pus, csf, gastric

aspirate and biopsy and it is also available at all levels of laboratories right from the designated microscopy centers to reference laboratories.

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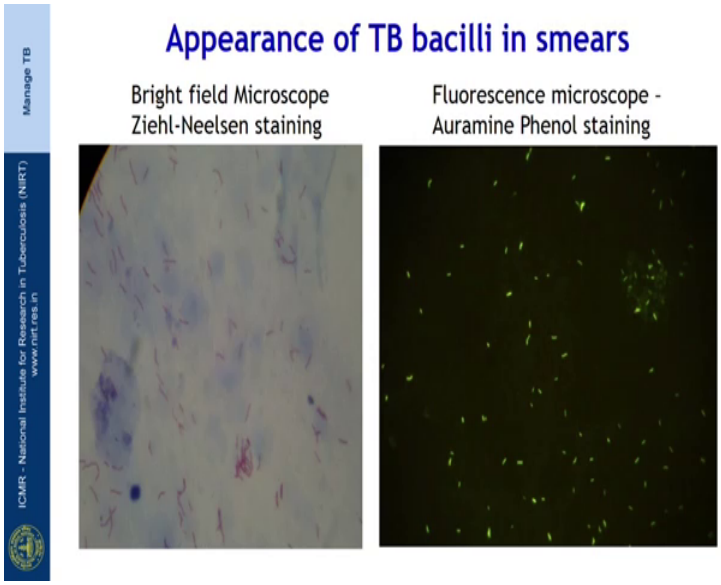

How does the organism appear in the smear under the microscope? When the smear is changed using the Ziehl-Neelsen staining and visualized under bright field microscope; the organism appears as a bright pink slender beaded road against a blue background.

When the smear is stained using a fluorescent staining such as auramine phenol and visualized under the fluorescence microscope; the organism appears as a bright yellow rod against black background.

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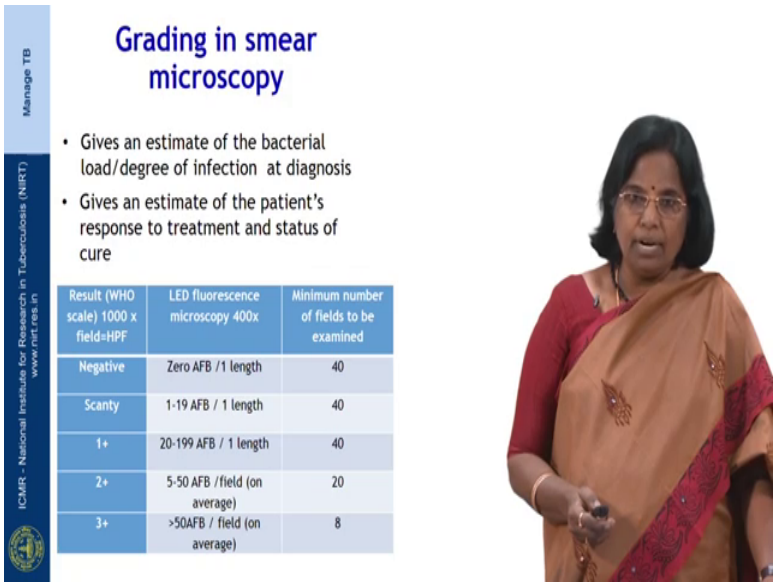

The smear is always reported using grading; grading gives an estimate of the bacteriological load or the degree of infection at the time of diagnosis, it also gives an estimate of the patients response to treatment during follow up on the status of cure.

This is the chart that is used for interpreting think the year results in the form of graded results. 1 length of the smear is equal to 2 centimeter of the smear which roughly has about 40 to 50 fields and if no organisms are seen in 1 length of the smear it was reported as negative and if 1 to 19 organisms are seen in 1 length that is 40 fields; it is reported as scanty and if 20 to 199 organisms are seen in 1 length then it is reported as 1 plus.

And if 5 to 50 organisms are seen per field after visualizing about 20 fields; that it is reported as 2 plus and if more than 50 organisms are seen per field after viewing about 8 fields smear is reported as 3 plus.

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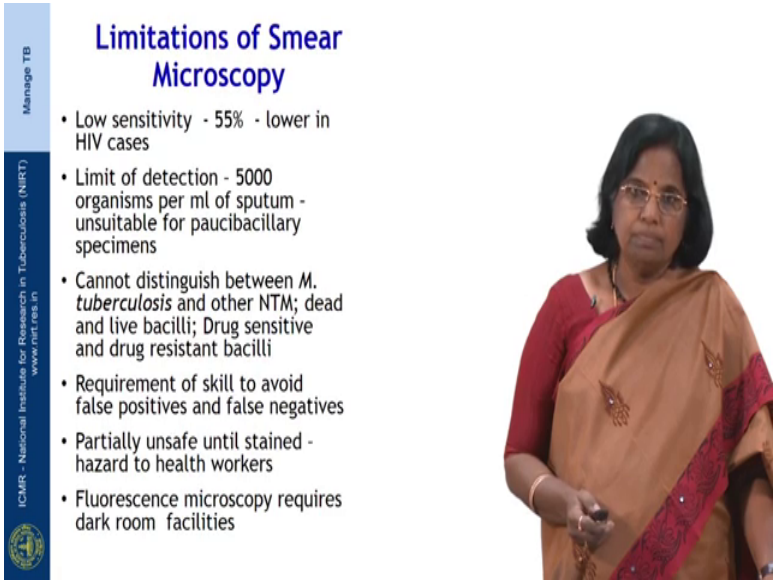

What are the limitations of smear microscopy? It has low sensitivity which is about 55 percent; which could be lower in HIV and pediatric cases. The limit of detection ranges between 5000 to 10000 organisms per ml of sputum hence may be unsuitable for paucibacillary specimens.

Smear cannot distinguish between M tuberculosis and other nontuberculous mycobacteria, nor between dead and live bacilli, not between drug sensitive and drug resistant bacilli. There is a significant amount of skill that is required to avoid false positive and false negative results. The smear is partially unsafe until they are stained and can pose a hazard to healthcare workers and in addition fluorescence microscopy requires dark room facilities.

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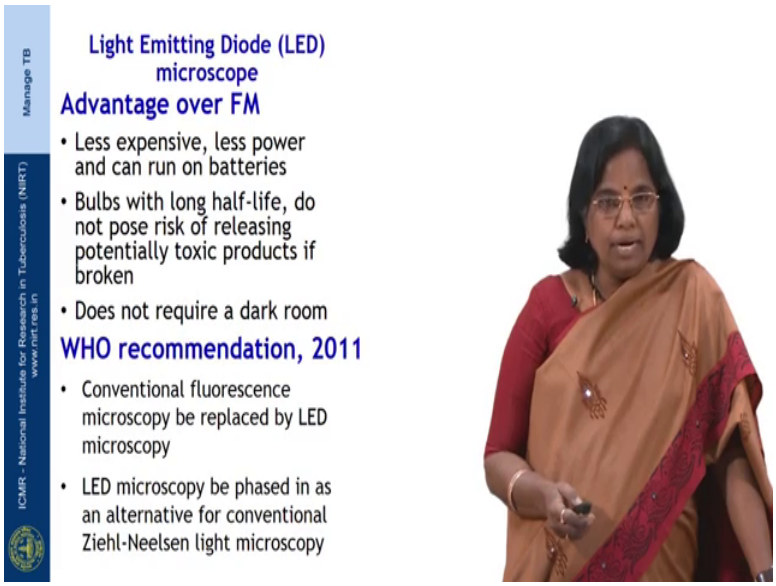

Recent innovation has been the introduction of light emitting diode microscope which has; a few important advantages over the regular fluorescence microscopy. The microscope is less expensive and uses less power and can also run on batteries.

The bulbs that are used in this microscope have a longer half life and do not pose the risk of releasing potentially toxic products if broken; it does not require a dark room. So, WHO in 2011 came out the recommendation that conventional fluorescence microscopy be replaced by LED microscopy and LED microscopy be phase ten has an alternative for conventional Ziehl-Neelsen light microscopy.

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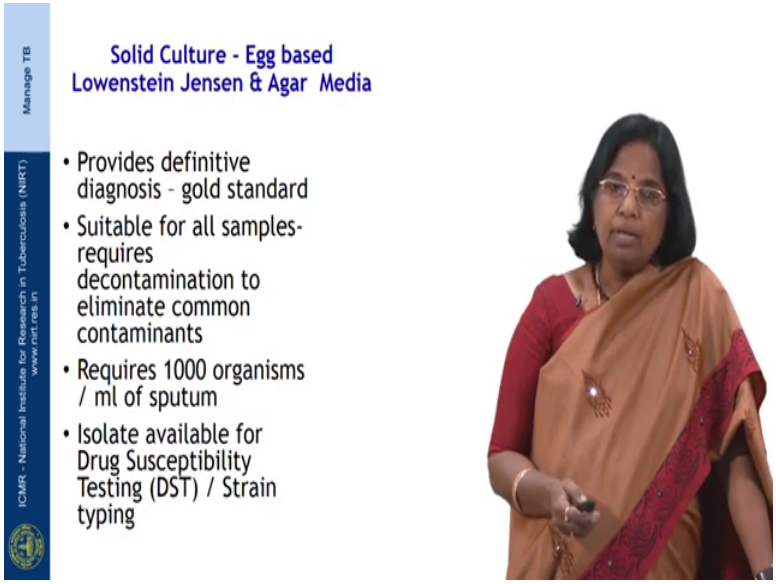

Next we move on to the solid culture or using egg based lowenstein jensen or agar based 7 H 10 or 7 H 11 media.

Culture provides definitive diagnosis and hence is still considered as the gold standards, it is suitable for all samples, but requires decontamination of the specimens to eliminate the common contaminants. Solid culture requires about 1000 organisms per ml of sputum and clinical isolate is available for drug susceptibility testing or for strain typing and other molecular epidemiology studies.

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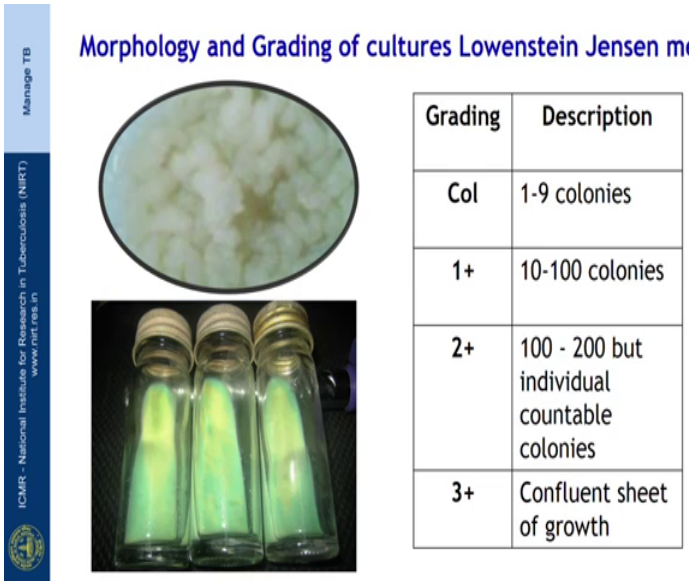

And if you have a look at the morphology and the grading of cultures using Lowenstein Jensen media, classically the morphology is described as; rough, tough and buff. That is it is rough in appearance and buff colored which is semi of white or whatever it is a kind of dirty white color and it is tough to emulsify and this is how it looks on the solid LG medium.

When we when we look at the grading that have to be used for reporting the culture results; when 1 to 9 colonies are observed the actual number of colonies have to be reported, then 10 to 100 colonies are seen if the culture is reported as 1 plus, then 100 to 200 colonies are seen the culture is reported as 2 plus and when a confluent sheet of growth is seen the culture is reported as 3 plus.

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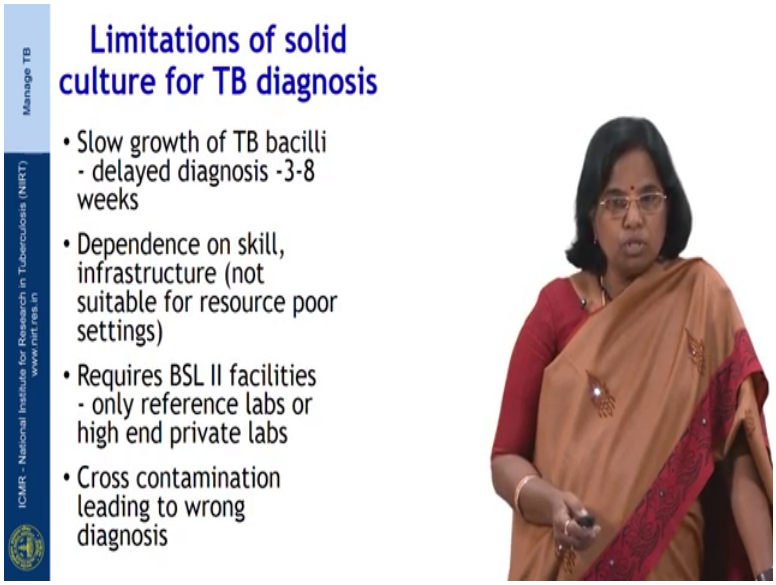

Limitations of solid culture for TB diagnosis; tubercle bacilli has a doubling time of between 18 to 22 hours, which results in slow growth of the organism and hence delayed diagnosis which could range between 3 to 8 weeks.

There is a significant dependence on skill and training and infrastructure hence may not be suitable for resource poor settings. Solid culture requires by safety level 2 facilities and hence may be available only in reference laboratories or high end prevent private laboratories. Cross contamination of sample is possible leading to wrong diagnosis.

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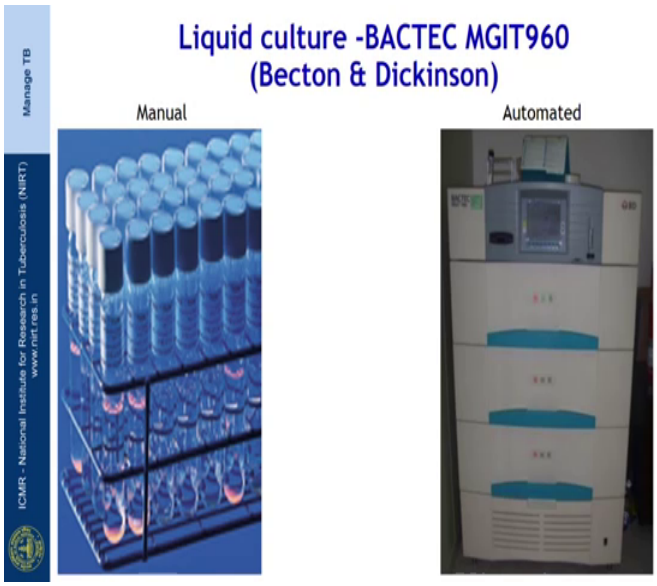

Next we come to the liquid culture based commercial system; the most widely used system which is the BACTEC MGIT960 from Becton and Dickinson. Two versions are available one is the manual version and the automated version which is the actually the MGIT960 system.

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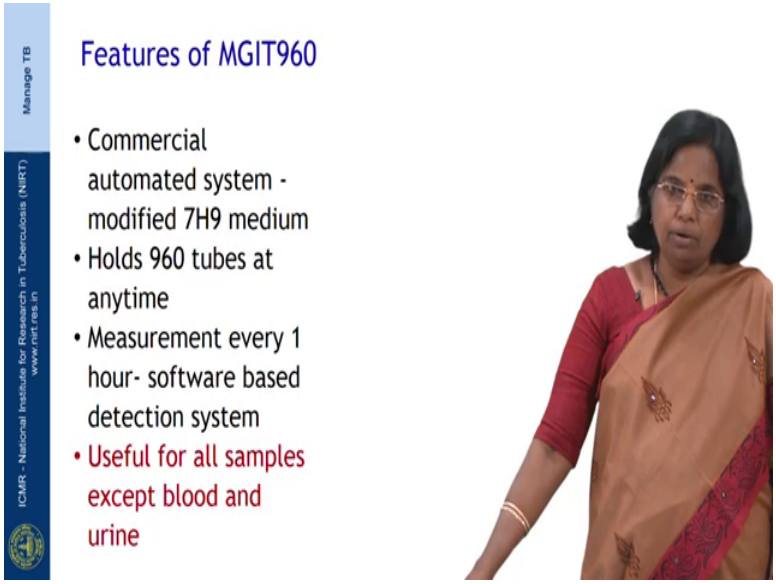

The features of my MGIT960 include that it is a completely commercial autofully automated system using modified 7 H 9 medium. It holds about 960 tubes at any time point and measurement of every tube is done every one hour and a software based

detection system is available. This system is useful for all samples except blood and urine.

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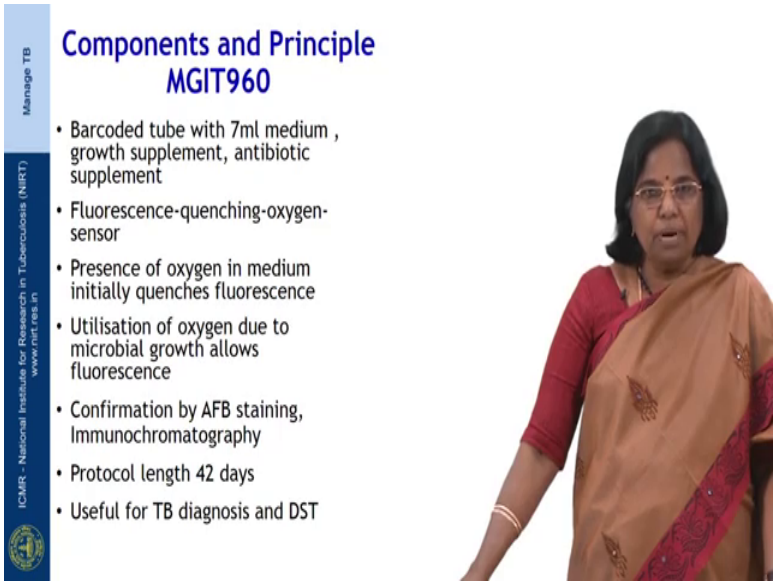

So, MGIT960 makes use of barcoded tube with 7 ml medium to which growth supplement and antibiotic supplements are added. Every tube has a fluorescence quenching oxygen sensor at the bottom, in presence of oxygen in the medium initially quenches fluorescence and utilization of oxygen due to microbial growth allows the fluorescence to be detected.

And when a cutoff is reached the tube is flag the positive by the instrument, but any tube flagged positive by the instrument has to be confirmed by means of confirmatory test such as AFB staining or immunochromatography.

The protocol length is 42 days for diagnosis of tuberculosis; this system is useful for TB diagnosis as well as for drug susceptibility testing.

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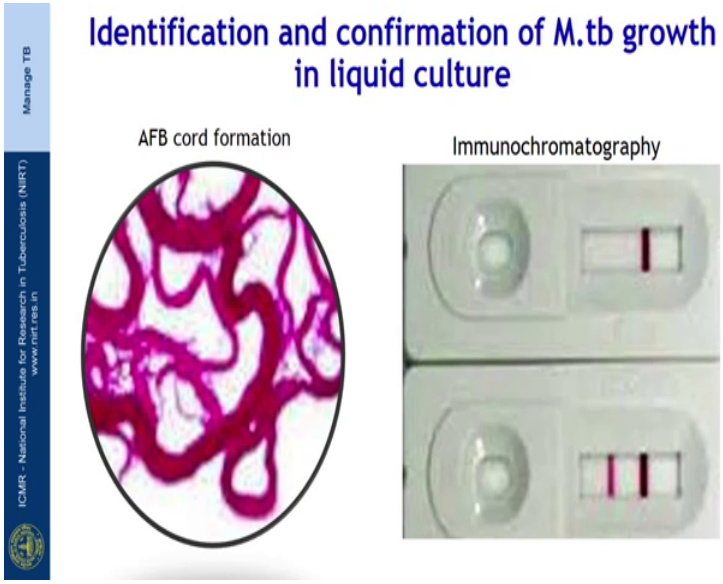

How is the identification or confirmation of MTB growth in liquid culture done? First a smear from the culture is taken and it is stained by ziehl-neelsen method and we look for characteristic cord formation and when conformation is seen the growth is suspected to be of MTB.

Second is the immunochromatography test; here we make use of the gold coated antibodies of the MPT 64 antigen of M tuberculosis, here when the culture is added to the device due to capillary action the antigen flows through the device and comes into contact with a gold coated antibody and results in the formation of a pink complex and the device is it is has an inherent control positive control and the control has to be positive for the test to be declared valid. Now, both these tests are positive for mycobacterium tuberculosis complex.

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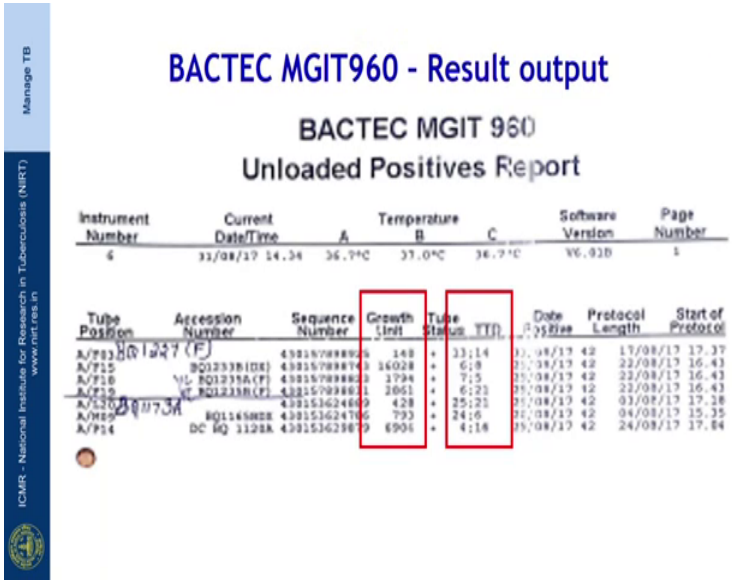

Now, this is an example of the result output from MGIT960; while it gives a lot of information the most important to look for are the growth units which is an a measure of the fluorescence that is detected in each tube and it does not reflect the biomass that is available in the culture.

The second is the TTD which is the time to detection which is the duration of from the time when the tube was a in incubated in the instrument and to the time it was declared positive; it is measured in days and hours.

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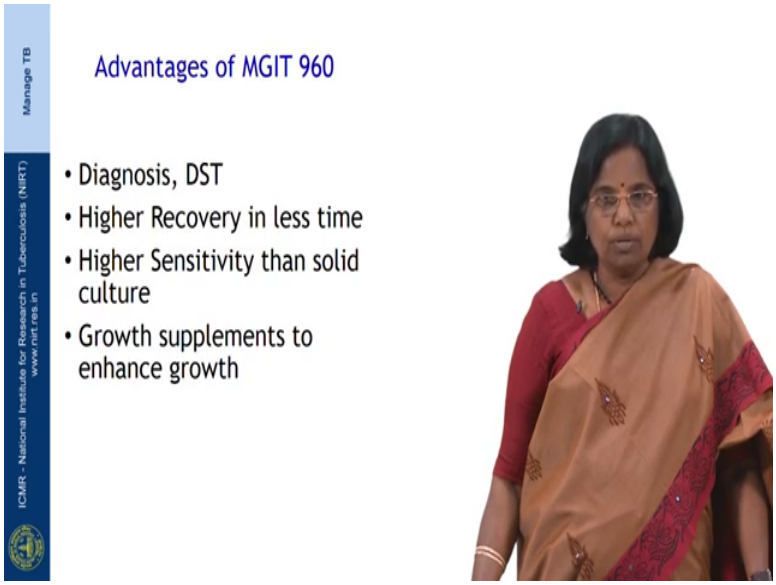

Advantages of MGIT960 the system as I said earlier can be used for diagnosis as well as for drug susceptibility testing; it ensures higher recovery in shorter time, it has higher sensitivity then the solid culture and growth supplements can be added to the medium to enhance growth.

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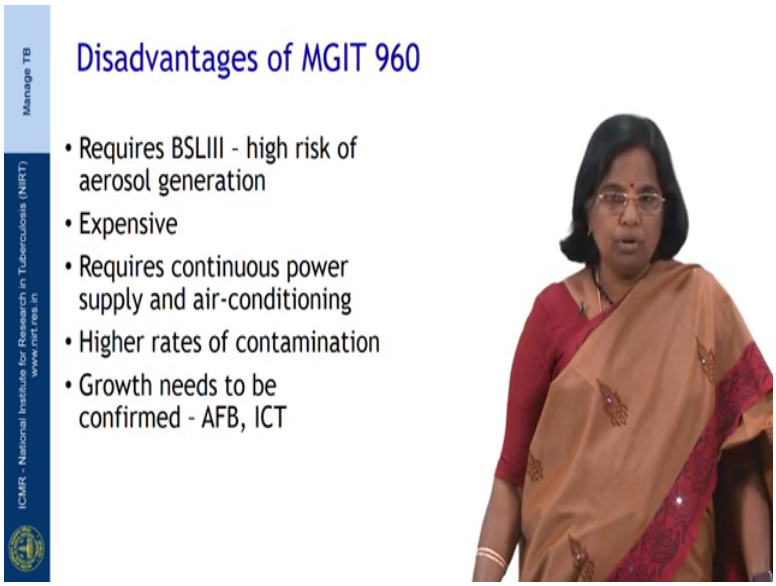

What are the disadvantages of MGIT960? It requires bio-safety level 3 facilities due to the high risk of aerosol generation. It is expensive and requires continuous power supply and air conditioning and the it is also highly prone for contamination and growth needs to be confirmed by means of confirmatory test like AFB staining and immunochromatography test.

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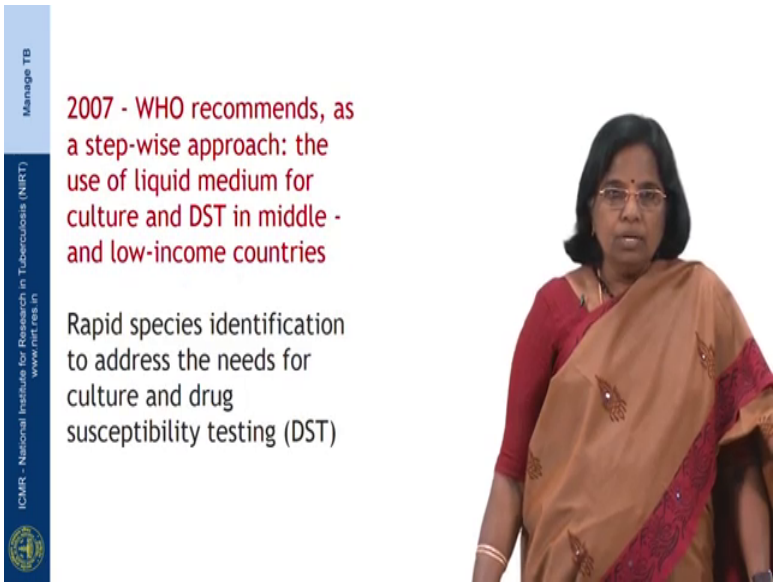

In 2007, WHO came out with recommendation as a stepwise approach; the use of liquid medium for culture and DST in middle and low income countries and rapid species identification to address the needs for culture and drug susceptibility testing.

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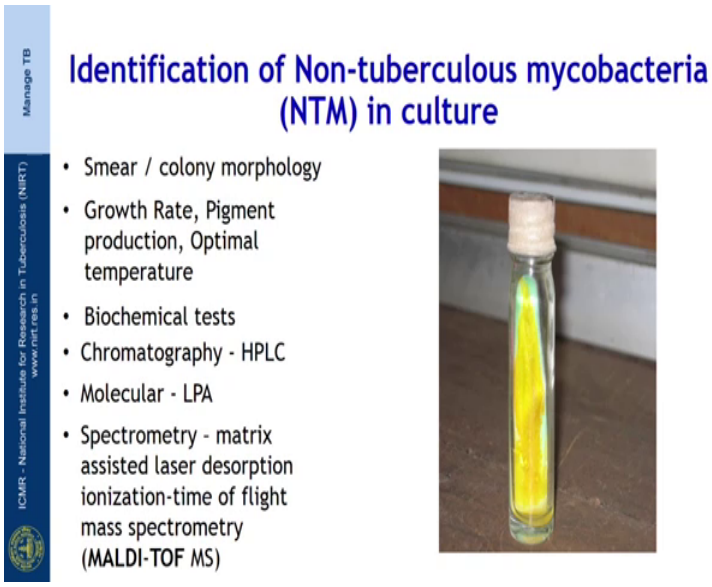

Now, we come to the nontuberculous mycobacteria in culture. The primary identification of NTM in culture is done by smear and colony morphology, growth rate, pigment production and optimal temperature requirements. This is an example of M (Refer Time: 14:48); which has pigment produced and it appears yellow in color. The species level

identification of NTM can be done by means of using a battery of biochemical test or by means of chromatography or by means of molecular test such as line probe assay or by means of spectrometry using MALDI-TOF mass spectrometry.

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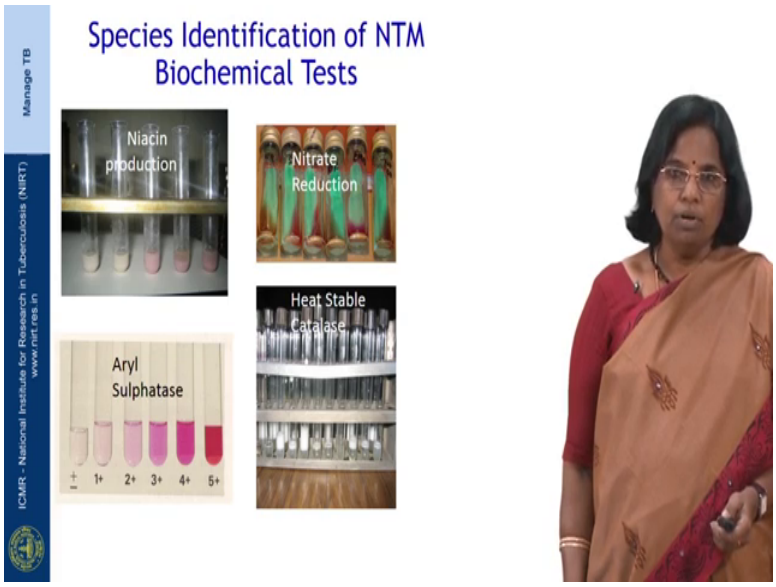

Species identification of NTM can be done by using a battery of biochemical test, here we give a few examples; first is the niacin production which is positive form mycobacterium tuberculosis complex, but negative for MTBC negative for NTM.

Second is nitrate production again which is positive for MTBC, but negative for NTM and this is aryl sulphatase test and the next is heat stable catalase both these tests are negative for mycobacterium tuberculosis complex, but positive for non-tuberculosis mycobacteria.

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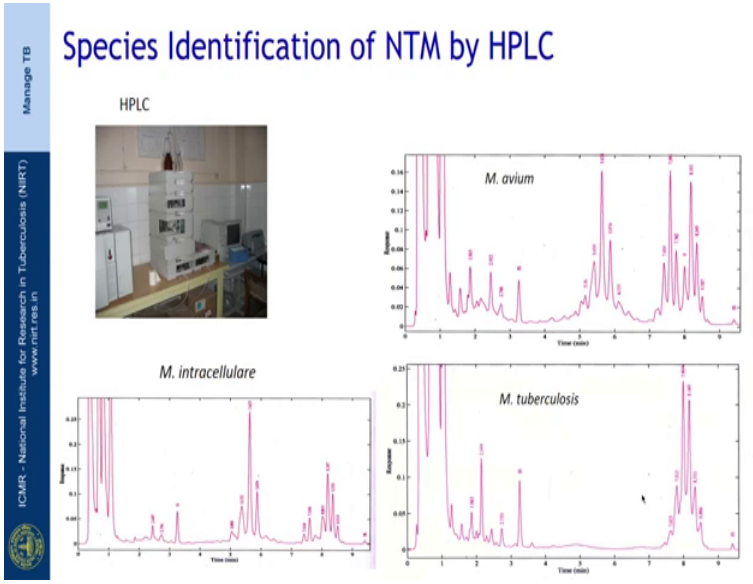

Second species identification of NTM can be done by high performance liquid chromatography; where the mycolic acid of the culture mycolic acid is extracted from the culture and subjected to HPLC and the peak formation is made use of for interpreting the results which will be characteristic for every species; here we give examples for M avium, M intracellulare and M tuberculosis.

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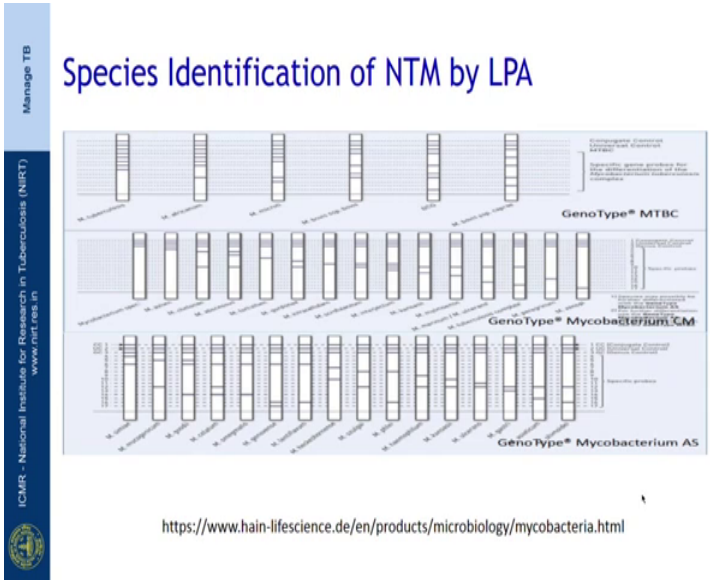

Next we come to the line probe assay; which is the molecular methodology used for species identification of NTM. There are 3 kits that are available and this is

manufactured by Hains life sciences and we have taken the slide from the literature available and there are 3 kits available first is a genotype MTBC kit; which can be used for species identification within the MTBC complex.

The second which is the genotype mycobacterium, CM and genotype mycobacterium AS kits can be used for identifying up to 30 pathogenic non-tuberculosis mycobacteria species.

Now, here in this methodologies the DNA is extracted from the culture subjected to amplification and hybridization with specific probes and specific or characteristic band formation is made use of in interpreting the results or for species identification.

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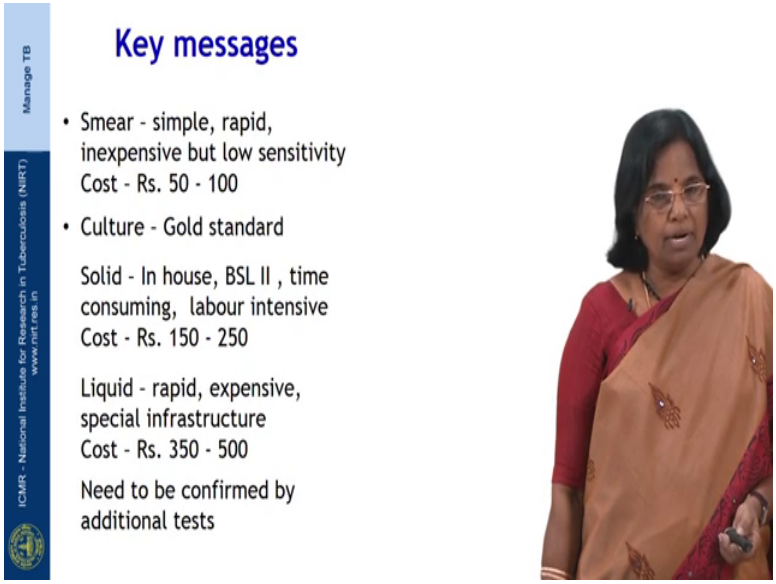

So, the key messages from this session are that smear is simple rapid inexpensive, but has low sensitivity and it may cause anything between 50 to 100 per sample. The culture is the gold standard and solid media can be prepared in house, but solid culture requires by safety level 2 facilities, it is time consuming and labor intensive and the cost may range anything between 150 to 250 rupees per sample.

Liquid media or systems basic using liquid media are more rapid, but they are expensive and require special infrastructure and the cost may range between 350 to 500 rupees per sample.

Now, all these culture whether it is from solid media or from liquid media need to be confirmed by additional test. With this we come to the end of the session on bacteriological diagnosis of tuberculosis.

Thank you for your attention.