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Retrospective Analysis of Clinical Isolates of Mycobacterium Tuberculosis in a Tertiary Centre in Kerala: Two Year Study

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Abstract

Tuberculosis is a major public health problem in India which accounts for one fifth of the global burden. It is estimated that about 40% of the Indian population is infected with tubercle bacilli. Automated methods and molecular methods are new developments in the diagnostic field of mycobacteria. But culture is the gold standard for diagnosis. A retrospective analysis of the culture positives obtained by conventional culture method using solid medium Lowenstein Jensen medium for 2 years from January 2015 to December 2016 by processing various clinical specimens collected and sent to the Mycobacteriology laboratory in the Dept. of Microbiology at Govt. Medical College, Thiruvananthapuram, Kerala was done. Among 548 samples received 16 samples yielded Mycobacterium Tuberculosis. Culture positivity was 4.28% smear positive for acid fast bacilli after Ziehl Neelsen staining was 3.83%. Total number of patients and culture positivity were more in males than in females. Since the facility for sensitivity testing is not available all the culture positive cases were referred to RNTCP for treatment and periodic assessment. Mortality rate was 6.25% among the culture positive cases.

Keywords: Mycobacterium tuberculosis, Lowenstein – Jensen medium, Ziehl – Neelsen staining

Introduction

Mycobacterium tuberculosis is an obligate intracellular aerobic bacterium. It is the causative agent for Tuberculosis. Robert Koch (1882) isolated the mammalian tubercle bacillus and proved its causative role in tuberculosis by satisfying Koch's postulates Mycobacteria meaning fungus – like bacteria since its morphology showed branching filaments resembling fungal mycelium. They do not stain readily but once stained resist decolourisation with dilute mineral acids. Mycobacteria are therefore called acid – fast bacilli or AFB.

Materials and Methods

Study design	:	Retrospective study
Study population	:	Clinically suspected cases of pulmonary Tuberculosis and Extra Pulmonary Tuberculosis
Study period	:	2 years (January 2015 to Dec. 2015)
Study setting	:	Dept. of Microbiology
Sample size	:	548 samples
Age group	:	1 year to 90 years.

Collection of samples

Different types of samples received in the Mycobacteriology Laboratory at the dept. of Microbiology, Govt. medical College, Thiruvananthapuram, Kerala.

1) Collection of samples

Sputum was collected in the sterile wide mouthed screw capped plastic container and sent to the laboratory immediately after collection. Early morning urine samples were collected in sterile containers on three consecutive days and were pooled and decontaminated. Then it was subjected to centrifugation and the centrifuged deposit was used for preparation of smear and for culture. Pus was aspirated form abscess after cleaning the site with surgical spirit and aspirated with a sterile syringe and needle. The pus sample was sent to the laboratory in the same syringe itself after covering it with adhesive plaster or the sample may be transferred to a sterile screw capped plastic container and sent to the laboratory. Lymphnode aspirate, biopsy specimens, bone marrow sample, CSF, (collected by lumbar puncture), Ascitic fluid, pleural fluid, peritoneal fluid, pericardial fluid etc. were collected under strict aseptic precautions in sterile leak proof containers and sent to the laboratory immediately after collection. If any delay in processing the sample, it was kept in the refrigerator and processed within 24 hours.

Processing of samples

The samples were processed in class II Biosafety cabinet. Direct smears were prepared from all clinical specimens Ziehl-Neelsen staining was done, for detection of acid fast bacilli in the smear.

All specimens were decontaminated using modified petroffs method. Since CSF is from a sterile site, it was not decontaminated. CSF sample was directly inoculated on to Lowenstein-Jensen medium.

Modified Petroff's method

A fixed volume of the specimen was transferred to a 50ml sterile graduated centrifuge tube. Equal volume of 4% NaOH was added and allowed to stand at room temperature for 15 minutes with intermittent shaking. Then the mixture was centrifuged and 20 ml of normal saline/ Distilled water was added. It was again centrifuged at 3000 rpm for 15 minutes. The supernatant was discarded and the deposit was used to prepare a smear for Ziehl Neelsen staining and for culture.

Ziehl – Neelsen staining

Smear was prepared from the clinical specimen heat fixed and stained with concentrated carbolfuchsin and heated. Heating was continued until steam rises and the stain should not be allowed to boil. Heat intermittently for 10 minutes. Heat being applied at intervals to keep the stain warm. The stain must not be allowed to evaporate or dry. More cone, carbolfuchsin can be poured over the smear to cover it, if required. After 10 minutes, the smear was washed with water. Then the slide was covered with 20% Sulphuric acid and kept for 1 minute. Then it was washed with water. More acid was added and again washed with water. This process was repeated several times until the film becomes colourless or faintly pink which indicates that the decolourisation was finished. Then the slide was washed with water and counterstained with Loeffler's methylene blue and kept for 3 minutes. Then the stain was washed with water and dried using blotting paper. The smear was examined under oil immersion objective of the microscope for presence of acid – fast organisms. Acid fast organisms appear pink and other organisms, pus cells, tissue cells and debris stain blue. This is a differential staining method used for the rapid detection of mycobacteria.

Culture for mycobacterium tuberculosis

Each sample was inoculated into two Lowenstein – Jensen medium slopes prepared in McCartney bottles and incubated at 37^0C. The inoculated slopes were observed every week for 6-8 weeks. The typical rough and tough, buff coloured colonies which appear 4-6 wks after incubation on the LJ medium slopes, suggest Mycobacterium Tuberculosis, a smear may be prepared

from a single colony and stained by Ziehl – Neelsen staining technique. Subculture is done on two LJ medium slopes once again from a single colony for confirmation and incubated at 37^0C and 44^0C respectively. For routine purposes, a slow growing nonpigmented niacin positive acid fast bacillus is taken as mycobacterium tuberculosis.

Direct and concentration smears of sputum are examined. Sputum microscopy is the most reliable single method in the diagnosis and control of tuberculosis. Atleast 10,000 acid fast bacilli should be present perml of sputum for them to be readily demonstrable in direct smear. A negative report should not be given till atleast 300 fields have been examined taking about 10 minutes. A positive report can be given only if two or more typical bacilli have been seen.

Several methods have been described for the homogenisation and concentration of sputum and other specimens. The method we follow is modified Petroff's method. This method does not kill the bacilli and the sample can be used for culture and animal inoculation.

Culture is a very sensitive diagnostic technique for tubercle bacilli, detecting as few as 10 to 100 bacilli per ml. We use the solid medium – Lowenstein – Jensen medium for culture. The use of liquid media with radiometric growth detection such as Bactec 460 and the identification of isolates by nucleic acid probes have simplified culture methods greatly and results obtained in 2 weeks. But these are available only in advanced laboratories.

Special tests

1) Catalase tests

A mixture of equal volumes of 30 vol H_2O_2 and 0.2% catechol in distilled water is added to 5 ml of the test culture and allowed to stand for few minutes. Efferverscence indicates catalase production and browning indicates peroxidase activity. This test helps to differentiate tubercle bacilli from atypical mycobacteria and provide an indication of the sensitivity of the strain to isoniazid. Catalase and peroxidase acitivities are lost when tubercle bacilli become INH resistant.

2) Niacin test

Human tubercle bacilli form niacin when grown on egg medium. When 10% cyanogen bromide and 4% aniline in 96% ethanol are added to a suspension of culture a canany yellow colour indicates positive reaction. This test is useful to differentiate human strains from bovine strains of tubercle bacilli.

3) Nitrate reduction test

This is positive with mycobacterium tuberculosis and negative with M.bovis.

Results

A total number of 548 samples were sent for AFB culture from various clinical department of Govt. Medical College Hospital, Thiruvananthapuram to the Dept. of Microbiology.

Table 1: Gender distribution

Gender	No. of patients & Percentage
Male	305 (55.66%)
Female	243 (44.34%)
Total	548 (100%)

Table 2: Culture positivity

Total No.	Culture Positive	Culture negative
548	24 (4.38%)	524 (95.62%)

Table 3: Smear positivity

Total No.	Smear Positive	Smear negative
548	21 (3.83%)	527 (96.16%)

Table 4: Sample Analysis

Sl. No.	Sample received	No. of percentage
1	Pus from abscesses	136 (24.82%)
2	Urine	97 (17.70%)
3	Sputum	66 (12.04%)
4	CSF	49 (8.94%)
5	Ascitic fluid	43 (7.85%)
6	Pleural fluid	29 (5.29%)
7	Lymphnode biopsy	27 (4.92%)
8	Bone marrow	20 (3.65%)
9	Knee joint aspirate	19 (3.47%)
10	Skin biopsy material	12 (2.19%)
11	Necrotic tissue	10 (1.82%)
12	Peritoneal fluid	20 (3.65%)
13	Gastric aspirate	4 (0.73%)
14	Pericardial fluid	3(0.55%)
15	Bronchial washings	2(0.36%)
16	ET aspirate	2 (0.36%)
17	Bone curetings	2(0.36%)
18	Maxillary sinus aspirate	2(0.36%)
19	Tube-ovarian maks	1(0.18%)
20	Endometrial aspirate	2(0.36%)
21	Thoracoscopy biopsy	1(0.18%)
22	Defected material from mastoid cavity	1 (0.18%)
	Total	548 (100%)

Table 5: Age group distribution

Age group	No. of cases of percentage
<1 year	1 (0.8%)
1-10	15 (2.73%)
11-20	30 (5.47%)
21-30	66 (12.04%)
31-40	81 (14.78%)
41-50	116 (21.17%)
51-60	122 (22.26%)
61-70	80 (14.2)
71-80	27(4.93%)
81-90	10 (1.82)
Total	548 (100%)

Table – 6 Positive sample analysis

Sl. No.	Sample	Total No.	Smear +ve	Culture positive
1	Sputum	12	11 (91.6%)	8 (16.7%)
2	Pus	9	3 (33.3%)	6 (66.6%)
3	Tissue	2	1 (50%)	1 (50%)
4	Urine	1	-	1 (100%)
	Total	24	15 (62.5%)	16 (66.66)

Discussion

Tuberculosis is one of the leading causes of death in developing countries. The incidence is quite high among people living with AIDS worldwide. WHO reported 1.5 million deaths in 2014. Every year 9 million new cases are reported worldwide. 12 million cases were reported to WHO in 2010 and 14 million in 2012 of which 95% cases were from developing countries. The disease occurs in children and adults. Since it is an easily spread airborne disease and diagnosis should be made easy to interrupt the transmission.

In the present study, a total number of 548 samples were received from Dept. of Medicine, Surgery, Gynaecology, Dermatology, Pulmonary Medicine, Orthopaedics, Neurosurgery etc. Different types of clinical specimens were sent by the clinicians for AFB culture, whenever there was suspicion of Tuberculosis Table 4 shows the details of samples received from clinically suspected cases of Tuberculosis. Out of the 548 samples received majority of the samples were pus aspirated from abscesses (136) accounting for 24.82% followed by urine (17.7%), sputum (12.04%) CSF (8.94%), ascitic fluid (7.85%), pleural fluid (5.29%) and Lymph node biopsy (5%) other specimens constitute <5% each. Overall culture positivity was 4.38% and was more with sputum samples (50%) followed by pus samples (37.5%).

Most of the samples were received from males accounting for 55.66%. Females accounted for 44.34%. Culture positivity was also more in males (75%) than females (25%) Many studies have already proved that the prevalence rate is high among males.

The predominant age group of cases in his study was between 51-60 yrs (22.26%) followed by 41-50 (21.17%). The lowest age at which sample was collected was CSF from a 7 months old baby and the highest age was 90 years. Fifteen samples were collected from children below 10 years of age (2.7%). The samples include gastric aspirate (2) synovial fluid (4), syringe pus from cervical abscess (2), pus from axillary lymph node (4) and pleural fluid (3), which accounted for 3% of the total samples. One 2 year old was infected with HIV and on ART. Thick pus was aspirated from cervical abscess. Smear prepared from the sample was negative for acid-fast bacilli: and the culture on LJ medium was also negative.

In the present analysis, culture was found to be more sensitive than direct smear examination Ziehl- Neelsen technique detects acid fast bacilli in the smear when there is 10,000 bacilli/ml of sputum. But culture will be positive even if 10-100 bacilli are present in the specimen.

Data collected from four Govt. medical Colleges in Kerala in 2011 showed 8.5% culture positivity in GMC, Kozhikode and 8.2% at GMC, Thiruvananthapuram 5% from Govt. TDMC, Alappuzha. The maximum number of cultures (1399) have been done at GMC, Thrissur. But the culture positivity was only 2%. Smear positivity after

ZiehlNeelsen staining was 3.4% at Kozhikode. 3.5% at Thiruvananthapuram, 2% at Thrissur. No smear positivity was reported from Govt. TDMC, Alappuzha. All the four centres followed conventional culture method using lowerstem- Jensen medium.

Line Probe Assay was available only at govt. medical college, Kozhikode at the time of data collection. Therefore sensitivity testing was performed in that centre alone. Most of the strains (94.2%) were sensitive to rifampicin, 82.3% were sensitive to ethambutol and 76.5% were sensitive to isoniazid.

The Automated culture system MGIT 960 technique was compared with lower stein Jensen medium culture method for laboratory diagnosis of active tuberculosis in study conducted in the secondary and tertiary Health care units in Brazil a randomized trail from 2008 to 2011 as. In that study, MGIT technique yielded 10.1% culture positives and LJ method yielded only 3.8%.

In Russia a study was conducted in 2001 to test the efficiency of the automated liquid culture system BACTEC MGIT 960 and MB / BacT with LJ medium (standard dense medium. The isolation rate of mycobacterium tuberculosis was 87.2% in BACTEC MGIT 960 80.9% MB/BacT was 80.9% and 76.6% in LJ medium. The duration required for culture was an average of 10.7 days in Bactec MGTI 960, 18.7 days in MB/BacT and 33.2 days in LJ medium. A 2 year study on comparison of four culture media for isolation of mycobacterium tuberculosis was conducted by RS Martin et al. The culture media used (1975) were LJ medium, Middle brook broth, Petragnani and ribonucleic acid medium. Overall, ribonucleic acid medium performed best but the differences among four media for isolation of mycobacterium tuberculosis bacilli reported 82% isolation in LJ medium, 79% in ATS (American Trudean Society) and 56% in middle brook 7 HIO medium. Culture on LJ medium were found to have the highest isolation rates for each smear category and 7H10 had the lowest isolation rate. Comparing the media from the aspect of number of colonies produced, LJ and ATS had the highest average colony counts followed by 7H10. These findings were relatively constant over the 6 year period of study. One possible reason for the low positive rate of 7H10 was the lack of CO_2 enrichment.

Culture remains the gold standard for the laboratory diagnosis of Tuberculosis (Tenover et al. 1993) and Regeade et al (2014). Direct detection of acid fast bacilli from respiratory specimens showed 89% positivity in culture. But the culture rate was high (98%) in smear positives and low 72%) in smear negatives (Elizabeth M, Marlowe et al).

In cases of abdominal tuberculosis culture positivity was 76% in BACTEC 460 TB automated culture system and 64% in Lowenstein Jensen medium. (Sudeep P Shah et al. 2010). Traditionally solid medium for culture (Lowenstein Jensen medium) was kept upto 8 weeks before issuing a negative report. Eventhough it is well documented that liquid medium detects mycobacteria much earlier, most incubation protocols still require a maximum of 6 weeks.

The automated culture system for detection of mycobacterium tuberculosis in most of the laboratories utilising the BACTEC MGIT 960 can safely issue positive culture reports within 2 weeks. Each MGIT tube contains 7 ml of middlebrook broth. A fluroscent compound is embedded in silicon on the bottom of the tube. This florescent compound is sensitive to the presence of oxygen dissolved in the broth. Initially, the large amount of dissolved oxygen quenches emission from the compound and little fluorescence can be detected.

Later actively respiring microorganisms consume the oxygen and allow the florescence to be detected. All types of clinical specimens both pulmonary and extrapulmonary

can be processed for primary isolation in MGIT (Mycobacteria growth Indicator Tube) and the tubes are incubated at 37^0C and monitored every 60 minutes for increasing florescence. A positive tube contains 105-106 CFU/ml of bacteria.

Dramatic reduction in culture time of mycobacterium may be achieved step by step in near future. A first step is the optimize the culture system. Second step is the strict control of oxygen tension. Third step is the early detection of growth. Fourth step is the use of MALDI-TOF-MS (Matrix Assisted Laser Desorption/ ionization Time of Flight Mass spectrometry) for identification. In the best case, primary culture and rifampicin susceptibility testing can be achieved in 72 hrs. when specimens are inoculated directly on the medium supplemented by antibiotic in the beginning of the culture.

Conclusion

The laboratory diagnosis of tuberculosis usually relies on culture based isolation of the causative agent mycobacterium tuberculosis bacterium. Mycobacteria remain a global public health problem causing on average 170 deaths every hour worldwide. Despite significant advances in the molecular diagnosis of tuberculosis over the part 2 decades, culture is still the universal gold standard for the laboratory diagnosis of tuberculosis enabling complete postcultural antimicrobial susceptibility testing and genotyping. The World Health Organisation still recommends inoculating specimens in parallel into a liquid medium for accelerated diagnosis of high titer specimens and onto a solid culture medium to increase the sensitivity of laboratory diagnosis of tuberculosis. The present study suggests that early, efficient and inexpensive laboratory methods are required for the diagnosis of pulmonary and extrapulmonary tuberculosis for effective patient management and to interrupt transmission of the disease prevalent in all the developing countries.

Conflicts of interest

There are no conflicts of interest.

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