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PERFORMANCE OF LJ MEDIA, MIDDLEBROOK 7H9 MEDIA, ZN MICROSCOPY AND LED FM MICROSCOPY FOR DETECTION OF MYCOBACTERIUM TUBERCULOSIS

Medical Science

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ABSTRACT

Introduction - There are number of techniques for detection of AFB in sputum samples as well as extrapulmonary samples. There are rapid molecular methods as well as culture methods which take few weeks for detection of bacilli as mycobacteria are generally very slow growing organisms. Although culture methods are considered gold standard for diagnosis of MTB but mostly staining and rapid molecular methods are the ones which dictate starting of treatment in most cases.

Material & Method – The present study was carried out in the Department of Microbiology on a total of 600 smear sputum samples from clinically suspected cases of pulmonary TB attending the outpatient and inpatient departments of MMIMSR, Mullana, Ambala, from December 2016 to June 2018. Specimens were subjected to ZN and LED FM staining before and after decontamination. After microscopy, specimens were subjected to culture on LJ and Middlebrook 7H9.

Results – A total of 600 samples were included in the study. Out of these 140 were positive on culture (123 on LJ Medium or 140 on Middle Brook 7H9 Medium), 110 were positive on FM staining and 100 were positive on ZN staining. Higher grading on smear corelated with earlier growth of mycobacteria on both culture mediums. Middlebrook medium showed earlier growth than LJ medium (2nd week vs 3rd week) and CPC treated samples showed better growth.

Conclusion - Our study also showed that CPC treated samples showed improved yield. FM microscopy is better than ZN staining with more sensitivity and specificity for detecting AFB. Among culture methods, Middle Brooke 7H9 broth showed faster growth of mycobacteria and better positivity rate as compared to LJ Medium.

KEYWORDS

Mycobacterial culture, TB Diagnosis, Middlebrook Media

Introduction –

Tuberculosis is a disease with significant morbidity and mortality. It is one of the most important health concerns in India at the present time. Despite implementation of RNTCP and PMDT, the morbidity and mortality from TB is still high in India[1]. As per the Global TB report 2017 the estimated incidence of TB in India was approximately 28,00,000 accounting for about a quarter of the world's TB cases[1]

One of the key steps in TB control is case detection. Although advances in diagnostics are leading to the introduction of new tests, the backbone of TB diagnosis worldwide continues to be smear microscopy. ZN microscopy is popularly used, but Fluorescent staining is yielded better results than the ZN staining and recommended by WHO too in being >10% sensitive than the conventional staining, but it comes with a drawback of being evaluated by LED Microscope which is expensive and requires an expert eye. The World Health Organization in 2011 recommended replacing conventional FM by LED-FM and phase in LED-FM as an alternative to ZN microscopy. LED microscopic examination requires 104 bacilli per milliliter of sputum in order to be detected on smear, hence has best sensitivity than ZN microscopy. But the limitation of LED microscopy is that it may give false positive results because of fabricated detection of dead bacilli as live, mainly in patients who have recently started anti tubercular treatment.

Although culture methods are considered gold standard for diagnosis of MTB but in practice it has been seen that staining and rapid molecular methods are the ones which dictate starting of treatment in most cases. Under RNTCP, any patient having sputum smear positive for acid fast bacilli, is designated as sputum smear positive TB and started on treatment. An important factor that determines the positivity rate of any staining or culture method is the quality of sputum. The ideal sample should be mucopurulent containing less epithelial cell and more pus cells. The processing of sample before culture is also an important factor that can alter the results. Contamination is a major obstacle in the diagnosis of tuberculosis. The effectiveness of culture systems is greatly undermined by contamination with bacteria and fungi. Contamination reduce the proportion of interpretable results, thereby limiting the diagnostic value of culture systems at additional cost to public health systems, which delays or ultimately prevents TB diagnosis. Our study intended to analyse the performance of various staining and culture methods commonly employed for diagnosis of TB and determining the influence of Cetyl-pyridinium Chloride on smear and culture positivity.

Material and Methods-

A total of 600 samples were taken from suspected pulmonary TB patients. An early morning sample on empty stomach was collected in sterile wide-mouthed, screw capped translucent container (at least 35 mm in diameter) so that the patient can expectorate easily inside the container without contaminating the outside and to observe specimen volume and quality without opening the container. Recently collected sputum of at least 4-5 ml was accepted, in some cases even less volume but good quality sputum (mucopurulent) was also accepted. ZN staining, FM Auramine staining was done and used for microscopic examination. For culture methods, sample was split into 3 equal parts, and was processed by digestion, decontamination and concentration by Cetyl-pyridinium Chloride (CPC) and Sodium Chloride as per standard protocol. 500 microlitre of specimen was then inoculated LJ Medium and Middle Brook 7H9 OADC Media was incubated at 37ºC. LJ Medium & Middle Brook 7H9 OADC medium was checked twice weekly for first two weeks and then every week for a maximum of eight weeks.

Results –

A total of 600 samples were included in the study. Out of these 140 were positive on culture (123 on LJ Medium or 140 on Middle Brook

7H9 Medium), 110 were positive on FM staining and 100 were positive on ZN staining. **(Table 1).**

Table 1 - Total number of samples

Total	Middle-Brook 7H9 positive	LJ Medium	FM Stain positive	ZN stain +ve
600	140 (23.3%)	123 (20.5%)	110 (18.3%)	100 (16.7%)

Table 2 – Quality of sputum and positivity rate on staining

Macroscopic appearance	Number (n=600)	ZN positive	FM positive
Salivary	300 (50%)	15 (5%)	20 (6.66%)
Mucopurulent	200 (33.33%)	78 (39%)	81 (40.5%)
Blood Tinged	100 (16.66%)	7 (7%)	9 (9%)
Total	**600**	**100**	**110**

Mucopurulent sputum showed highest positivity rate followed by blood tinged and salivary sputum samples. So, the quality of sputum is an important factor in determining positivity rate of any method. (Table 2)

Cetyl-pyridinium chloride was used as decontamination techniques. CPC is better for detection of mycobacteria because it causes disturbance of cell membrane and seepage of cell contents ultimately cell death of epithelial cells causing release of intracellular mycobacterial bacilli, hence useful as an aid in detection. Our study showed, more positivity rate after specimen decontamination (Table 3).

Table 3 – Effect of Decontamination on smear positivity

Macroscopic examination of sputum	ZN Positivity		FM Positivity	
	Before Decontamination	After Decontamination	Before Decontamination	After Decontamination
Salivary	15(15%)	17(15.8%)	20(18.18%)	23(19.49%)
Mucopurulent	78(78%)	82(76.63%)	81(73.63%)	85(72.03%)
Blood tinged	7(7%)	8(7.47%)	9 (8.1%)	10(8.47%)
Total	100	107	110	118

Table 4 – Co-relation of Microscopy positivity with Culture positivity

CULTURE MEDIA	CULTURE POSITIVE SAMPLES	ZN MICROSCOPY AFTER DECONTAMINATION		LED MICROSCOPY AFTER DECONTAMINATION	
		POSITIVE	NEGATIVE	POSITIVE	NEGATIVE
LJ MEDIUM	123	100 (81.30%)	23 (18.9%)	110 (89.4%)	13 (10.6%)
MIDDLE-BROOK 7H9	140	100 (71.42%)	40 (28.57%)	110 (78.5%)	30 (21.5%)

81.30% smear positive and 18.9% smear negative on ZN microscopy showed mycobacteria growth while 90.24% smear positive and 9.75% smear negative on LED FM microscopy showed mycobacteria growth on LJ media. 71.42% smear positive and 28.57% smear negative on ZN showed mycobacteria growth on Middle Brook media& 79.28% smear positive and 29.71% smear negative on LED Microscopy showed mycobacteria growth on Middle Brook media.

Table 5 – Growth time comparison on LJ and Middle Brook medium

	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Total
LJ Medium			10 (8.1%)	14 (11.38%)	37 (30.08%)	37 (30.08%)	25 (20.32%)		123
Middle Brook 7H9		13 (9.2%)	20 (14.2%)	30 (21.42%)	21 (15%)	42 (30%)	14 (10%)		140

MIDDLE BROOK									
Smear Grading	I st week	II nd week	III rd week	IV th week	V th Week	VI th Week	VII th week	VIII th week	Total
Scanty									0
1+				9	4				13
2+			10	11	10	5	7		43
3+		13	10	10	7	15	7		62
Negative						22			22

Table 6 - Correlation of LED Smear Grading with Total Time of Isolation of Mycobacteria on Lowenstein Jensen and Middle Brook Media

LOWENSTEIN JENSEN MEDIUM									
Smear Grading	I st week	II nd week	III rd week	IV th week	V th Week	VI th Week	VII th week	VIII th week	Total
Scanty									0
1+					5	3	5		13
2+				4	17	10	12		43
3+			10	10	15	24	3		62
Negative							5		5

Middle Brook 7H9 medium showed earlier growth than LJ medium in our study. Middle brook liquid media provided early growth of isolates i.e. 25 % were positive within 3 to 4 weeks while 75 % were positive in 4 to 6 weeks on Middle Brook 7H9 as compared to 20% positivity by 4 weeks and 80% in 5-6 weeks on LJ media.

Discussion –

Although, rapid tests like BACTEC, septic check AFB system and MGIT have become available but are not used commonly in rural setting, because these are expensive methods and are not available in most rural areas. So, microscopy and culture are still the most widely used methods for diagnosis of TB. In this study we compared the usefulness of different commonly used methods for diagnosis of tuberculosis.

The positivity rate of any method is highly dependent on the quality of sputum, both macroscopically and microscopically, the technique of collection of sputum, technique of processing and decontamination of samples, finally the culture method or the staining method being used for detection of AFB. In the Present study, the rate of Contamination of 7% and 5% on LJ and Middle brook respectively.

Proper decontamination of sample is an important step in any mycobacterial culture. Mycobacterium is a slow growing microorganism and its growth will be outnumbered and hampered by other rapid growing gram positive and gram-negative bacteria if the sample is not properly decontaminated. H Hiza et al in their study showed that he CPC treated samples showed higher culture recovery and lower contamination compared to non-CPC samples, with no negative effect on sample viability even when stored at ambient temperature (25 °C) for up to 21 days.[2] In our study we treated the samples with CPC and NaOH and it improved the culture yield and improved AFB yield by staining methods as well.

In our study sputum smear grading directly corelated with the early detection of mycobacteria on cultures. Higher smear positive grade (3+) showed early growth by 3rd week in LJ Medium and by 2nd week by Middle Brooke medium. Among staining methods, FM staining is more sensitive, faster and less time consuming than ZN staining. The most important advantage of the fluorescence technique is that the slides can be examined at a lower magnification, thus allowing the examination of a much larger area per unit of time. In fluorescence microscopy, the same area that needs examination for 10 minutes with a light microscope can be examined in two minutes.[3]

Conclusion -

Our study showed FM microscopy to be better than ZN staining with more sensitivity and specificity for detecting AFB. Among culture methods, Middle Brooke 7H9 broth showed faster growth of mycobacteria as compared to LJ Medium. Treatment of samples with CPC improved yield of mycobacterium on staining and culture.

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