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Original Article

Assessment for Performance of Middlebrook 7H9 with Oleic-Albumin-Dextrose-Catalase Culture Media for Isolation of *Mycobacterium tuberculosis* in Ziehl–Neelsen Smear-positive Sputum Samples in Clinically Suspected Cases of Tuberculosis

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Abstract

Introduction: Pulmonary tuberculosis (TB) is a second foremost cause of death from a communicable disease, after the HIV. Being communicable should be diagnosed at the earliest. Smear examination is preliminary step for the confirm diagnosis, but culture is still a gold standard method.

Materials and Methods: 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 staining before and after decontamination. After microscopy, specimens were subjected to culture on LJ and Middlebrook 7H9.

Results: *Mycobacterium tuberculosis* was isolated in 23.33% of samples. 110 (78.57%) were detected by microscopy (ZN and LED), respectively. ZN smear positivity before and after decontamination was maximum in mucopurulent 78% and 76.63% and LED 73.63% and 72.03%. Culture positivity on Middlebrook 7H9 was 100% while 87.85% on LJ media. The rate of contamination was 5% and 7% on Middlebrook 7H9 and LJ media, respectively.

Conclusions: Middlebrook media was superior to the conventional LJ medium in being rapid, easy to use and interpret, and significantly low time-to-growth detection and had lesser contamination rate because the liquid media contains growth supplement oleic-albumin–dextrose-catalase, provides additional nutrition.

Key words: LJ media, Middlebrook media, Pulmonary tuberculosis

INTRODUCTION

For numerous centuries, tuberculosis (TB) has been the very important of the infections in its global incidence. In ancient days, TB remains one of the world's deadliest transmissible diseases. Newer diagnostic facility, modern treatment, and better preventive measures have almost controlled the disease in the past decade. However, after the arrival of HIV, coinfection of HIV and TB created the havoc. On the top of it, the advent of multidrug resistant (MDR) and extensively drug-resistant tuberculosis has agitated the situation further. MDR accounts for 5% of the global TB burden; however, <5% of existing MDR-TB patients are currently being diagnosed as a result of serious laboratory capacity constraints.[1]

Laboratory confirmation and proper follow-up are extremely significant. TB may look like clinically to other respiratory diseases such as pneumonia; similarly,

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common radiological finding in pneumonia and pulmonary aspergillosis has almost same radiological finding. That is why it is very difficult to differentiate, thus confirmation of TB can only be done after microbiology examination. According to Revised National Tuberculosis Control Program (RNTCP), microscopic examination of sputum is, as a rule, the only way by which the diagnosis of pulmonary TB can be confirmed. ZN microscopy is still the most useful among all methods and even done at PHC and CHC level due to its simplicity, speed, low cost, and minimal requirement of equipment and technical skills. Only disadvantage is that it requires 103–104 organisms/ml and acid-fast bacilli (AFB) and each smear examination requires on average 5–10 min, creating considerable workload for laboratories with limited resources. An alternative technique to ZN smear microscopy, LED staining gives better result as compared to ZN. Fluorescent microscopy is little bit expensive and complexity of the microscope, it reduces laboratory workloads.[2]

During the past two decades, several methods for achieving early growth of *Mycobacterium tuberculosis* have been developed. Sputum sample contains normal flora, which may overgrow on culture, and makes the detection of mycobacteria difficult, this emphasizes the importance of a good decontamination technique before culture, chemical agents employed for this purpose should be able to effectively destroy non-tubercular organisms in sputum and release the intracellular tubercle bacilli from the epithelial cells.

In the current study, Middlebrook 7H9 liquid media is used to prevent the contamination or unwanted growth, Middlebrook 7H9 and oleic-albumin-dextrose-catalase (OADC) provide additional nutrition, it offers a more conducive environment, for helping the growth of tubercular bacilli and whereas liquid media contains polymyxin, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (PANTA). A series of antimicrobial agents, namely PANTA, which prevent contamination and LJ medium, was used. In the present study, we tried to compare the Middlebrook 7H9 and LJ for shorter turnaround time and feasibility for use in smaller laboratories.

MATERIALS AND METHODS

This cross-sectional study was conducted in the Department of Microbiology, Maharishi Markandeshwar (Deemed to be University), Mullana, district Ambala. The study was conducted on 600 smears from clinically suspected cases of pulmonary TB after ethical clearance from ethical committee. A detailed clinical history was taken from clinically suspected patients. Sputum samples were 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. Samples were transported to the laboratory as soon as possible after collection. If unavoidable delay, the samples were refrigerated at 4° to inhibit the growth of unwanted microorganism. After processing of samples, growth detection was observed.

Processing of the Sample

The sputum samples, which are at least 2 ml, were selected for the study. The specimen was split approximately into three equal parts and was processed by digestion, decontamination, and concentration by cetylpyridinium chloride (CPC) and sodium chloride as per standard protocol.

Inoculation

About 500 microliters of the samples processed by all three methods will be then inoculated on LJ medium and Middlebrook 7H9 OADC.

Incubation

All inoculated LJ medium and Middlebrook 7H9 OADC media were incubated at 37°C.

Growth Detection

LJ medium and Middlebrook 7H9 OADC medium were checked twice weekly for the first 2 weeks and then every week for a maximum of 8 weeks.

The colony characteristics were noted and confirmed by acid-fast staining from culture.

Inclusion Criteria

Smear-positive sputum samples from clinically suspected cases of pulmonary TB were included in the study.

Exclusion Criteria

Smear-negative sputum samples from clinically suspected cases of pulmonary TB were excluded from the study.

Results were observed and compared statically.

RESULTS

Table 1 demonstrates that out of 600, *M. tuberculosis* was isolated in 23.33% of samples while 76.66% of samples showed invalid results including contamination.

Table 2 illustrates that out of 140 culture isolates, 110 (78.57%) were detected by microscopy (ZN and LED) among the culture-positive cases.

Table 3 shows by direct smear examination, fluorescent (100%) staining was better than ZN microscopy (90.90%). Sensitivity, specificity, positive predictive values (PPVs), and negative predictive values (NPVs) of conventional ZN microscopy and LED microscopy were 90.82%, 98%, 99%, and 98%, respectively.

Table 4 depicts the correlation between nature of sputum and smear positivity. Mucopurulent samples showed maximum correlation, i.e., Zn (39%) as well as LED (40.5%) followed by blood tinged Zn (7%), LED (9%), and least with salivary Zn (15) and LED (6.66%).

Table 5 depicts the correlation, on ZN staining, nature of sputum and smear positivity before and after decontamination in salivary were 15% and 15.8%, mucopurulent 78% and 76.63%, and blood tinged 7% and 7.47%, whereas on LED, smear positivity before and after decontamination was maximum in mucopurulent 73.63% and 72.03% followed by salivary 18.18% and 19.49% and blood tinged 8.1% and 8.4%.

Table 6 shows that the grading of smear by ZN staining before and after decontamination showed decrease in number in scanty and 1+ grade from 15% to 4.67% and 23% to 9.34%, respectively, while in Grade 2+ and Grade 3+ increase in number 25% to 32.7% and 37% to 53.27%, respectively, as well as the grading of smear by LED staining before and after decontamination showed decrease in number in scanty and 1+ grade from 6.36% to 0% and 27% to 11.02%, respectively, while in Grade 2+ and Grade 3+ increase in number 30% to 36.44% and 39.09% to 52.54%, respectively.

Table 7 depicts that out of 140 cases, culture positivity on Middlebrook 7H9 was 100% while 87.85% on LJ media. Sensitivity, specificity, PPV, NPV of culture positivity on LJ media and Middlebrook 7H9 were 97.56%, 96.43%, 85.34%, and 99.35%, respectively.

Table 8a shows comparison of the isolation of mycobacteria on LJ media isolated mycobacterial strains from 81.30% ZN smear positive to 18.9% ZN smear negative and 90.24%

Table 1: Distribution of culture-positive cases among clinically suspected cases of pulmonary tuberculosis

Total cases studied	Culture positive (%)	Culture negative (%)
600	140 (23.33)	460 (76.66)

Table 2: Correlation of culture with microscopy

Total culture positive	Positive by microscopy (%)
140	110 (78.57)

Table 3: Outcome from conventional ZN microscopy (100%) and from LED microscope (110%) from the 110 smear-positive samples

ZN microscopy (*n*=100)	LED microscopy (*n*=110)		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
	+ve	−ve				
+ve	99	1	90.82	98	99	98
−ve	10	490				

Table 4: Correlation of macroscopic appearance of sputum with direct ZN positivity and LED positivity in clinically suspected cases of pulmonary tuberculosis

Macroscopic examination of sputum	*n*=600 (%)	ZN positivity (%)	LED positivity (%)
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

Table 5: Rate of detection of mycobacteria by ZN and LED stains before and after decontamination in relation to gross examination

Macroscopic examination of sputum	ZN positivity (%)		LED 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

LED smear positive to 9.75% LED smear-negative samples as well as on Middlebrook media isolated mycobacterial strains from out of 71.42% ZN smear positive and 28.57% ZN smear negative and 79.28% LED smear positive and 29.71% LED smear-negative samples.

Table 8b shows that sensitivity, specificity, PPV, and NPV on LJ Media in relation with ZN and LED smear for the recovery of mycobacteria were 81.30%, 99.37%, 97.08%, and 95.37%, respectively.

Table 8c shows that sensitivity, specificity, PPV, and NPV on Middlebrook media in relation with ZN and LED smear for the recovery of mycobacteria were 71.42%, 98.91%, 95.23%, and 91.91%, respectively.

DISCUSSION

Concentration and decontamination techniques play an important and critical role in the detection and isolation

Table 6: Detection of smear grading with direct and after decontamination by ZN staining and fluorescent staining

Grades	ZN direct (%)	Staining after decontamination (%)	LED direct (%)	Staining after decontamination (%)
3+	37 (37)	57 (53.27)	43 (39.09)	62 (52.54)
2+	25 (25)	35 (32.7)	33 (30)	43 (36.44)
1+	23 (23)	10 (9.34)	27 (24.54)	13 (11.02)
Scanty	15 (15)	5 (4.67)	7 (6.36)	0 (0)
Total	100	107	110	118

Table 7: The statistical analysis on 100% was culture positive on LJ media, whereas 87.85% were culture positive for Middlebrook 7H9

Culture media	Middlebrook 7H9		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
LJ media						
+ve	120	17	97.56	96.43	85.34	99.35
−ve	3	460				

Table 8a: Correlation of culture with ZN microscopy and decontamination technique

Culture media	Culture-positive samples	ZN microscopy (%)		LED microscopy (%)	
		After decontamination		After decontamination	
		Positive	Negative	Positive	Negative
LJ	123	100 (81.30)	23 (18.9)	111 (90.24)	12 (9.75)
Middlebrook 7H9	140	100 (71.42)	40 (28.57)	111 (79.28)	29 (29.71)
Chi-square (*P* value)		3.503 (0.031)		5.975 (0.007)	

Table 8b: The statistical analysis on LJ media in relation with ZN and LED smear for the recovery of mycobacteria

On LJ media	LED after CPC		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
ZN after CPC	+ve	−ve				
+ve	100	3	81.30	99.37	97.08	95.37
−ve	23	474				

CPC: Cetylpyridinium chloride, PPV: Positive predictive value, NPV: Negative predictive value

Table 8c: The statistical analysis on Middlebrook media in relation with ZN and LED smear for the recovery of mycobacteria

On Middlebrook media	LED after CPC		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
	+ve	−ve				
ZN after CPC						
+ve	100	5	71.42	98.91	95.23	91.91
−ve	40	455				

CPC: Cetylpyridinium chloride, PPV: Positive predictive value, NPV: Negative predictive value

of mycobacteria. In samples such as sputum mycobacteria and normal flora are habitually present but are confined within the mucus. Liquefaction by mucolytics and vortex of the specimen disrupts the mucus and releases the mycobacteria. Thus, increasing the rate of detection. Likewise, in the current study showed that *Mycobacterium TB* was isolated in 140 (23.33%) from 600 specimens of clinically suspected patients of pulmonary TB [Table 1] which was in accordance with studies conducted by Shinu *et al.*,[3] in 2013, who observed a isolation rate of 23.07% and 19.88%, respectively. Morcillo *et al.*[4] (2008) also supported this study. The lower the percentage in this study may be because it was conducted in Haryana which is among the rich states of India.

The objective of the present study was to compare the two different types of staining and examination LED microscopy and ZN microscopy which is standard diagnostic procedure in urban, semi-urban, and rural settings, 78.57% smear positivity were detected with two methods, i.e., light microscopy by staining the smears with ZN staining and LED microscopy after staining with auramine stains among the culture-positive cases. [Table 2] in accordance with Kelamane *et al.*, who has reported ZN positive cases were 17.6% and on LED microscopy 82%. when both methods of microscopy were combined. Overall, culture positivity from cases of pulmonary TB shows wide variation and the present study excluded all the patients already on ATT, so the recovery on culture remained on a higher side. Furthermore, culture considers as an the gold standard in the diagnosis of TB and yields higher positivity when compared to smears, findings of the present study were in accordance to Dhingra *et al.,*[5] who in their study also revealed that the culture yielded more positivity 32.7% as compared to microscopy (20.2%).

In existing study by direct smear examination, LED microscopy (100%) showed better results than Zn microscopy (90.90%) and sensitivity is 90.82%, specificity is 98%, PPV is 99%, and NPV is 98% of conventional ZN microscopy and LED microscopy [Table 3] supported by Kelamane *et al.*[6] (2016) showed that sensitivity and specificity of Zn microscopy when compared to culture were 98.50% and 62.70%, respectively.

In the current study, the maximum samples processed were grossly salivary (50%) followed by mucopurulent (33.33%) and blood tinged (16.66%). On evaluation of smears by direct ZN staining, mucopurulent samples yielded the maximum result, i.e., 39% followed by salivary (5%) and minimum results were seen with blood tinged (7%) samples as well as in fluorescent microscopy. Mucopurulent samples yielded the maximum result, i.e., 40.5% followed by salivary (6.66%) and minimum results were seen with blood tinged (9%) samples [Table 4]. This outcome is in concordance with results of Yoon *et al.,*[7] who also found minimum results with salivary and maximum with mucopurulent samples. The reason behind it may be due to the fact that purulence is due to infection which consists of excessive pus cells where the mycobacteria intracellularly yielding more smear positivity, i.e., why RNTCP considers mucopurulent sputum as an ideal sample to be processed for yielding higher number of mycobacteria.

In the current study, CPC was used as decontamination techniques. CPC is better for the 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. During infection, due to immune response, there is chemotaxis of pus cells, thus purulent sample comprises excessive pus cells; as mycobacteria are intracellular organism, such specimens yield maximum smear positivity. During decontamination, there is dissolution of mucous by mucolytics and release of intracellular AFB from the pus cells. In the current study, the effect of CPC method on positive sputum samples was better, this may be because overnight treatment of specimens with CPC causes digestion of all cells and debris, which must have cleared during staining process, hence, aiding in better viewing of the bacilli against a clear background under ZN microscopy and LED microscopy. LED smear positivity and culture goes hand in hand, especially in multibacillary cases as auramine O stain can detect bacilli up to 104/ml of sputum. By this method in the present study, maximum samples processed were grossly mucopurulent (78%) followed by salivary (15%) and blood tinged (7%) before contamination. However, after decontamination, mucopurulent (76.63%) followed by salivary (15.8%) and blood tinged (7.47%). On evaluation of smears by LED microscopy, before decontamination, mucopurulent (73.63%) followed by salivary (18.18%) and blood tinged (8.1%) and after decontamination by CPC mucopurulent (72.03%) followed by salivary (19.49%) and blood tinged (8.47%). Mucopurulent yielded maximum result, respectively. Minimum results were seen with blood tinged [Table 5]. This conclusion was in concordance with Yoon *et al.,*[7] who also found that mucopurulent (33.3%) yielded maximum followed (24%) blood stained and least in salivary (2.4%). Decontamination and concentration procedures break down the cell releasing the intracellular bacilli outside, thereby increasing the positivity rate on microscopy as revealed by Hooja *et al.*[8] in their study that the sensitivity increased by 6.67% for ZN microscopy after decontamination

In ZN microscopy, the present study found a decrease in the number of scanty samples from 15% on direct

microscopy to 5% after CPC and while in LED microscopy, number of scanty samples decreased from 6.36% on direct microscopy to 0%. As well as in in 1+ grade found 23% on direct microscopy to 10% after CPC and while in LED microscopy decrease from 27% to 11.02% respectively. There was a significant increase in rest of the grades after decontamination [Table 6]. According to grading of smears. Scanty samples were 15% and 6.36% on ZN and LED microscopy, respectively, and as the load increases to Grade 3+, the rate of detection in LED increases as compared to ZN. After CPC, scanty samples were 5 (4.67%) and 0% on Zn and LED microscopy. It depends on various factors such as time of collection, number of samples taken, nature of sample, antitubercular treatment, and observer's competency. The present study according to the grades of direct ZN microscopy showed highest result for 3+37 (37%) and 43 (39.09%) and lowest for scanty 15 (15%) by direct examination after ZN staining [Table 6] which is in accordance with the study conducted by Hellen *et al.,*[9] who observed that 58.8% were 3+ and 8.8% were scanty. Scanty samples were only 10% and 7% on ZN and LED microscopy, respectively, and as the mycobacterial load increases to 3+, the rate of detection on smears increases.

In this current study, LED microscopic and ZN microscopic result after decontamination, compared with culture on LJ and Middlebrook 7H9 for diagnosis of TB. Culture is the ultimate diagnostic tool for TB. LJ is the internationally accepted media used as the gold standard. In the present study, 100% and 87.85% showed growth on the LJ and Middlebrook; sensitivity, specificity, PPV, and NPV of biphasic media for the recovery of mycobacteria were 97.56%, 96.43%, 85.34%, and 99.35%, respectively [Table 7]. A study by Pawar *et al.*[10] in culture positive on Middlebrook and LJ showed isolation rates of 23% and 62.9%, respectively, as well the study of Naveen and Peerapur[11] culture positive on Middlebrook and LJ showed isolation rates of 34.74% and 26.27% correspondingly. Compared to LJ medium, Middlebrook 7H9 is a liquid medium, OADC provides additional nutrition, it offers a more conducive environment, for helping the growth of tubercule bacilli and whereas biphasic media contains PANTA which has antibacterial and antifungal activity which prevents contamination. In this study, few patients were on antitubercular treatment for variable time periods so that are why some drugs target the cell wall of mycobacteria, as a result dead bacilli take uneven stain, therefore, give beaded appearance and can be differentiated from uniformly stained live bacilli. However, in patients on recently started ATT, it may be difficult to differentiate live and dead bacilli on microscopy. The dead bacilli do not grow on culture media but may show its presence in microscopy, hence, giving false-positive results. This also reveals that microscopy does not always give accurate results for the diagnosis of TB, and all positive smears should be confirmed by culture.

In the present study shows comparison of isolation of mycobacteria on LJ media and Middlebrook with LED microscopy after decontamination and Zn microscopy and concentration techniques. On LJ media, of total 140 isolates, 81.30% and 18.9% were Zn positive and Zn negative after CPC, whereas in LED microscopy, 90.24% and 9.75% were LED positive and LED negative after CPC. Similarly, of 123 isolates on Middlebrook, 71.42% and 28.57% were Zn positive and Zn negative after CPC, whereas in LED microscopy, 79.28% and 29.71% were LED positive and LED negative after CPC. Statistical analysis on LJ media, sensitivity 81.30%, specificity 99.37%, PPV 97.08%, NPV 95.33% and on middle brook Sensitivity 71.42%, Specificity 98.91%, PPV 95.23%, NPV 91.91% [Table 8], whereas 1 (14.28%) and 1 (25%) samples showed no growth on LJ and Middlebrook correspondingly. As far our knowledge is concern, none of the researcher had done similar research.

In existing study, Middlebrook liquid media provided early growth of isolates, i.e., within 3–4 weeks, 25% were positive on Middlebrook and rest of 75% were positive on 4–6 weeks as compared to 20% were positive on LJ by 4 weeks and rest 80% were on 5–6 weeks on LJ. This is attuned with Pawar *et al.*[10] in their study. All 41 samples growth was obtained on the 5th week of incubation on Middlebrook, whereas only 13 cultures were positive on LJ by the 5th week for rest 21 it took 6 weeks for the bacteria to grow on LJ medium. Middlebrook liquid medium could be adapted for early recovery of *Mycobacterium* with better of performance and reliability. Due to high growth supplements in Middlebrook 7H9, it detects more number of mycobacteria other LJ media.

CONCLUSIONS

RNTCP considers staining as an effective method for the preliminary diagnosis of TB. ZN staining is still a popularly used method, especially in resource-limited laboratories. However, LED microscopy after fluorescent staining yields better results than the conventional ZN staining and is recommended by the WHO. In the present study examination, fluorescent LED (18.33%) staining was better than Zn microscopy (16.66%). Gross examination has played an important role in diagnosis of TB. Mucopurulent specimens of sputum yielded the best results and were more relevant and beneficial in diagnosis as the mycobacteria are concentrated in the thick part of the sputum and it is the specimen suggested by RNTCP as ideal.

Decontamination and concentration are a key step, thus it is important to choose an efficient decontamination method. In the present study, CPC method was used, the overnight treatment of specimens with CPC causes digestion of all cells and debris gives better result. In the present study in ZN smear positivity, before and after decontamination was maximum in mucopurulent 78% and 76.63% and LED smear positivity, before and after decontamination was maximum in mucopurulent 73.63% and 72.03%.

Culture is still considered as the gold standard. Solid and liquid culture media has been used nowadays for isolation. Though LJ is internationally accepted media. Liquid culture has become routine microbiology practice and its introduction has improved the sensitivity for detection and reduced the time to result by more than a week in comparison to conventional culture on LJ medium.

The Middlebrook 7H9 medium could be well adapted for early recovery of *M. tuberculosis* with ease of performance and reliability. It does not require gas supplies or radioactive tracers and enable recovery of the mycobacteria without special equipment in small and peripheral laboratories. Whereas Middlebrook media had lesser contamination rate, because the liquid media contains growth supplement OADC, provides additional nutrition, it offers a more conducive environment, for helping the growth of tubercule bacilli and antibiotic mixture contains PANTA which has antibacterial and antifungal activity which prevents contamination.

It is not only comparable with the conventional LJ medium but significantly better for recover and growth of *M. tuberculosis*. It is safer and self-contained and can be used easily in rural laboratories.

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