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Utility of Microscopic Observation of Drug Susceptibility (MODS) Assay in Diagnosis of Extra Pulmonary Tuberculosis (EPTB)

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Abstract

Objectives: We aimed determine the overall and sample wise sensitivity, specificity and time to culture positivity of MODS assay for diagnosis of EPTB in comparison to culture on Lowenstein Jensen (LJ) medium.

Materials and Methods: The present study was laboratory based at a tertiary care, referral, and teaching hospital in Mumbai from January 2019 to June2020 (18 months). Total 225 specimens including pus, body fluids, lymph node aspirates, tissues and biopsies from clinically suspected cases of EPTB were subjected to Ziehl Neelsen staining, MODS assay and culture on LJ medium.

Statistical analysis: After excluding 26 specimens from the study for the cause of contamination and 199 specimens were analyzed. Statistical analysis was done using Medcalc statistical software.

Results: Sensitivity and specificity of MODS for EPTB was found to be 68.75% and 97.26% respectively using LJ medium as a gold standard. Amongst culture positive cases, sensitivity of MODS assay was 88.8% in smear positive and 42.85% in smear negative cases. Sample wise sensitivity was found to be 100% in samples of

lymph node biopsy, pleural biopsy and pleural fluid. All sample types showed more than 90% specificity except for bone and bone marrow samples where specificity was 66.66%. MODS assay could detect Mycobacterium tuberculosis in 13 days on an average in all types of EPTB samples.

Conclusion: Our results establishes that rapidity and simplicity of MODS assay with a good sensitivity and specificity for lymph node, pleural biopsy and pleural fluid specimens holds promise as a diagnostic tool for EPTB.

Keywords: Extra pulmonary TB, MODS assay

Introduction

Tuberculosis has the dubious distinction of being the most persistent scourge of humankind. Worldwide statistics are staggering: globally, around 10.6 million people fell prey to TB in 2021 equivalent to 134 cases per 100000 population. The South East Asian Region bears an inordinately high share (45%) of the global TB burden. India stands 3rd in ranking of high burden countries.[1]Extrapulmonary TB (EPTB) constitutes about 15 to 20 per cent of all cases of tuberculosis.[2]

In 2021, there were an estimated 1.4 million deaths among HIV-negative people (95% Ul: 1.3–1.5 million) and 187 000 (95% UI, 158 000–218 000) among HIV-positive people, for a combined 1.6 million. Progress made in the years up to 2019 has slowed, stalled or reversed, and global TB targets are off track. [1]

Diagnosis of EPTB is challenging due to myriad of clinical presentations, paucibacillary nature of specimens, difficulty in obtaining specimens from deep-seated organs and inability to get an additional specimen for confirmation.

Smear microscopy lacks sensitivity and cannot distinguish viable from nonviable bacteria and non-tuberculous mycobacteria from Mycobacterium tuberculosis (MTB). Culture methods with solid media are inexpensive but time consuming. Liquid automated commercial systems (MGIT) are rapid but require heavy equipment, expertise and are very costly. CB-NAAT is rapid but expensive and not a good option for follow up. In such a situation, MODS assay has been described as a rapid and sensitive test for detection of TB directly from clinical samples.

MODS assay was approved and recommended by WHO in March 2010 for use in countries with limited resources. It's a liquid culture based assay that detects Mycobacterium tuberculosis (MTB). It is based on two important properties of MTB (i) It grows faster in liquid as compared to solid medium, (ii) In liquid medium, MTB grows in a visually characteristic manner (tangles & cording) which can be observed under a microscope long before the naked eye can visualize colonies on the solid agar.[3] The existence of a toxic glycolipid, trehalose 6-6' dimycolate (cord factor) of M. tuberculosis was known from a long time. In more recent time, the ability of virulent MTB to grow and form cords has been demonstrated by a groups of Moore DA and Yagupsky PV.[4, 5]These groups reported very high sensitivity of the test.

The tuberculosis-working group in Peru first described the assay in the year 2000.[6]The test was further refined by Moore et al. and a standard operating procedure (SOP) of the methodology was made. Based on previous studies it was found that once standardized, it is feasible to perform MODS assay in an existing TB culture laboratory in resource constrained settings. An inverted microscope is used to detect the ropy appearance (cording) that is characteristic of MTB complex in liquid medium. It is less prone to cross contamination, requires

minimal bio safety facilities and minimal training. These characteristics make it potentially an attractive tool for EPTB specimens.

Marco Tovar et al and Maxine Caws et al have evaluated MODS assay in pleural biopsy and CSF samples respectively.[7, 8] Kirwan De et al assessed it only in lymph node samples.[9] Zadbuke et al had done a study on EPTB samples but the number of samples were ninety.[10] Dang Ha and Sinh Thi Tran have used MODS assay in pediatric specimens.[11, 12]

The present study was therefore undertaken to evaluate the performance of MODS assay in comparison to conventional culture method and to add value to the existing literature.

Materials and Methods

This study aimed to evaluate sensitivity and specificity of MODS in detection of MTB in EPTB samples. In addition, an attempt was made to include all possible varieties of extra pulmonary samples and to assess sample wise sensitivity and specificity. The time to culture positivity of EPTB specimens by both MODS and LJ culture was also determined.

This was a clinical microbiology laboratory based study conducted in the department of Microbiology of a tertiary care, referral and teaching hospital in western India. Waiver of consent was approved by the institutional ethics committee since the department routinely receives specimens from cases of EPTB and this study was conducted on leftover samples received for routine testing. It did not require any additional samples.

All patients above 18 years with clinical signs and symptoms of EPTB were included in the study. Both new and previously treated cases were included.

Patients under 18 years and presenting as only pulmonary TB (PTB) were excluded.

All these patients already presented with signs and symptoms that could raise suspicion of TB. Hence, we did not include Composite reference standard (CRS) as a comparison tool.

A total of 225 consecutive EPTB samples from Jan 2019 to June 2020 were analyzed in the study. Sample size had been calculated considering a previous prevalence rate of 16 % for EPTB in our laboratory.

The EPTB specimens comprised of pus, biopsy, tissues, lymph nodes, urine, CSF, pleural fluid and other body fluids (pericardial, synovial, ascitic fluids). Collection of samples was done by treating clinicians as per standard protocol.

The samples like biopsy and tissue were processed directly after crushing in sterile way. The sterile body fluids were centrifuged and pellet was used as inoculums. Urine samples were decontaminated by NALC-NaOH method. The processed or direct specimens were divided in four parts and used for

1. Making smears- Smears were stained with Ziehl Neelsen method.
2. Were inoculated on LJ medium. Positive cultures were confirmed by secondary smears, culture on PNB, MPT64 test, niacin and nitrate test. [13]
3. Used for MODS assay
4. Used for manual liquid culture

MODS Assay

The MODS assay was performed as described in the standard operating procedure (SOP) given by Singh S, Kumar P.[14] One part of the sediment was resuspended in Middle brook 7H9 broth (Hi MediaM198-500G) with 10% OADC growth supplement (oleic acid-albumin-dextrose-catalase) (HI

Media FD 018) and PANTA supplement (Polymyxin B- Amphotericin B- Nalidixic acid- Trimethoprim- Azlocillin) (PENTA MIX HI Media FD 260). This was used to inoculate 24 well tissue culture plates for MODS assay (Hi Media TPG24-1X100).

The cell culture plates were sealed by cellophane tape, packed in ziplock pouches and incubated at 37^0C. Sample processing and culture manipulation was done in a Biological Safety Cabinet (ClassIIA2). Each plate had two wells as negative control (only MODS medium) and two wells of positive control (M. tuberculosis H37Ra ATCC 25177 strain). Two wells were used to inoculate each of the specimen. The cultures were examined under an inverted light microscope at a magnification of 40X everyday (except holiday) from day 4 to day 15 and on alternate days from day 16 to 21 and twice weekly from day 21 to day 40.

When cording was observed in any of the two wells, the day of positivity was noted. If results were still negative on day 21, the final result was noted as negative. LJ medium cultures were read once every week.

The sensitivity and specificity of MODS assay were compared to TB culture on LJ medium as a reference standard using contingency two-by-two tables. P value < 0.05 were taken as statistically significant. All statistical analyses were performed using MedCalc statistical software version 17.9.6 [15]

Manual liquid culture

Samples inoculated in screw capped transparent glass tubes containing Middle brook 7H9 broth with 10% OADC growth supplement and PANTA supplement. These tubes were incubated at 37^o C and observed macroscopically for bread crumb appearance of MTB colonies.

Results

Out of total 225 samples, 26 samples were excluded from the study. Eleven showed contamination on LJ medium, 12 were contaminated in MODS assay while three samples were contaminated in both the cultures. Hence, 199 samples were analyzed. (Table 1)

Of the 199 samples, 11 samples were culture positive for MTB on both LJ medium and MODS assay, 178 were culture negative on both LJ medium and MODS assay. While five samples were positive on MODS assay but negative on LJ medium and 5 were positive on LJ medium but negative on MODS assay. Thus the overall sensitivity & specificity of MODS assay as compared to LJ culture was 68.75 (CI 41.33-88.98) and 97.26 (CI 93.73-99.10) respectively. (Table 2)

Maximum sensitivity of 100% was observed in samples of pleural fluid, pleural biopsy, LN aspirate & FNAC in our study. Pus samples showed sensitivity of 66.66%. (Table 1)

Pleural biopsy also showed 100% specificity followed by 98.41 (CI 91.47-99.96) specificity in pleural fluids. Specificity of 95.83 (CI 78.88-99.89), 94.44 (CI 72.70-99.85) was seen in pus and lymph node specimens respectively. However, bone and bone marrow aspirate had a specificity of 66.66(CI 9.43-99.16). (Table 1)

Earliest detection of MTB growth MODS assay was detected on 9^{th} day. All the isolates grew by 17^{th} day. Mean time to growth detection by MODS assay was 11.81 days i.e. 12 days.

Mean time to growth detection on LJ medium was 3 weeks i.e. 21 days (CI, 14-28 days).

The difference between the two was statistically significant (p value 0.0001) on applying Mann- Whitney U test.

In the present study out of 199 EPTB specimens analyzed, 23 were (11.5%) ZN smear positive of which 10 were positive by MODS and 176 (88.4%) were smear negative of which six were positive by MODS.

The sensitivity and specificity of MODS assay in comparison to LJ medium in ZN smear positive samples were 88.88% (CI 51.75-99.71) and 85.71% (CI 57.18-98.22) respectively. (Table 3)

The sensitivity and specificity of MODS assay in comparison to LJ medium in ZN smear negative samples were 42.85% (CI 9.89-81.59) and 98.22% (CI 94.9-99.63) respectively. (Table 3)

Discussion

Sensitivity of detecting MTB by MODS assay (Table 4)

In the present study, overall sensitivity of MODS assay for all the EPTB samples was 68.75% when compared to culture on LJ medium. Sample wise sensitivity was seen to be highest 100% each in pleural fluid, pus, pleural biopsy and lymph node biopsy as seen in table no.1.

In literature, sensitivity of detection of MTB by MODS assay in other studies was found to be higher. In a large study using a broad group of patients, Moore et al (2004) reported a sensitivity of 97.8%, for MODS.[4] Higher sensitivity in this study can be explained by inclusion of pulmonary samples and both solid and liquid cultures for comparison which helped in increasing the number of true positives. Sensitivity in a study by Agrawal A et al was 91% and reasons for higher sensitivity was inclusion of both pulmonary and extra pulmonary samples and system of comparison were LJ and MGIT cultures. [16]

In a study of Zadbuke et al sensitivity for EPTB samples was 83.3 % wherein mainly pus (44.4%), FNAC (27.7%) and other body fluids (27.7%) were included.[10]These samples have high bacterial load as compared to other variety of samples as included in our study. This explains higher sensitivity of this study than that of our study. Our overall sensitivity was 68.75%, which was comparable with 65.4% in a study by Kirwan DE et al. [9] Lower sensitivity maybe caused by the number or type of samples, sample storage, grinding and processing of sample, which can significantly reduce the bacillary volume in each inoculum. Modest sensitivity in our study may be because we have included consecutive EPTB samples irrespective of smear findings. A study by Dang Ha et al mentions 42.3 % and that of Sinh Thi Tran showed a sensitivity of 46% of MODS in EPTB. [11, 12] These two studies had included only paediatrics samples like gastric lavage, pleural fluid and CSF which are known to have lesser bacterial load as it's difficult to obtain appropriate and adequate material of sample in pediatric patients. In addition, their standard of comparison was composite reference standard (CRS) and not any identification system.

A study by Kirwan DE et al had reported a sensitivity of 65.4% in lymph node biopsy samples while our study could show 100% sensitivity in LN biopsy and FNAC samples, probably due to less number of samples.[9]Hence, it will not be statistically appropriate.

A study by Tovar et al (2010) which included pleural fluids and pleural biopsy sensitivity was 20% and 81% respectively; as compared to our study, which showed sensitivity of 100% for both types of samples.[7] However in our study, positivity rate for pleural fluid on LJ media was very less and pleural biopsy samples was only three in number.

Another study by Huang Z et al reported a sensitivity of 37.5% for pleural fluid and 20.5 % for CSF. [17]In our study we could not calculate sensitivity for CSF samples because of scarcity of number of CSF samples.

The sensitivity of pus by MODS assay was 72.72% in a study by Zadbuke et al while in our study it was 66.66% that are comparable. [10]

We could not get any positivity in tissue and urine samples; one reason for this was lesser number of samples. Also, it was observed that it was very cumbersome to maintain sterile conditions while grinding of tissues and hence increase in contamination rate. Urine also showed high rate of contamination and hence we had to exclude those samples from already lesser number of samples. Moreover, bacterial load was very low in urine sample. Though we had centrifuged the urine samples, it was not found adequate to increase culture positivity rate. With every addition of steps in the methodology, we introduce additional errors and uncertainties. Also, intricate steps are likely to add more errors.

There were five samples, which were positive by MODS but negative on LJ in our study. Out of these, four samples were positive by Gene Xpert MTB/RIF assay while Gene Xpert was not performed for the fifth sample of bone. Two out of these five were positive on ZN smear. This may point towards MODS being more sensitive than LJ. A study of Moore et al have also reported better sensitivity of MODS compared to LJ or automated mycobacterial liquid culture system.

Specificity of detecting MTB by MODS (Table 4)

In the present study, overall specificity of all EPTB samples was found to be 97.26% as shown in table no.2. Studies by Agarwal A et al and Sinh Thi Tran et al have shown specificity of 98.2% and 99.5% respectively, which is similar to our study.[16, 12]Specificity in a study by Zadbuke et al was 83.3%, which is significantly lesser than that of ours.[10] Attori et al showed that with supervised training detection of cords in liquid media increases and reemphasized that without training and experience serpentine cords can be missed.[18]

Table 1 depicts specificity of MODS for various EPTB samples. Study conducted by Maxine Caws showed a specificity of 100% in CSF samples whereas in our study it was found to be 90.9% for CSF samples.[8] This can be explained by different methods of gold standard used as reference.

Dang Ha had included thirty-two CSF samples but none of them turned out to be positive on culture and hence specificity could not be calculated for CSF samples. The same study showed specificity of 39.7% for pleural fluid and GL samples which is very low. [11] The present study found 98.4% specificity in pleural fluid. The specificity for pus was 95.83% in our study, which was higher than that in study by Zadbuke et al (82.35%).[10]We had found specificity of 94.44% in LN biopsy and FNAC samples. We could not find any other study to compare our findings for these two samples.

Time to detection of MTB by MODS assay (Table 4)

In the present study, time for detection of MTB by LJ and MODS assay were 3 weeks (21 days) and 12 days respectively. As per study of Kirwan DE, time of detection of MTB by MODS assay was 13 days and by LJ culture was 22 days which are comparable to that of our study. In the study of Maxine Caws, time of detection was found by MODS assay and LJ culture were 6 days and 24 days respectively which is lesser as compared other studies.[8] Time for detection by MODS for pleural fluid and CSF were 14 days and 9 days respectively in study of Huang Z. Study of Dang Ha showed time of detection by MODS and MGIT being 8 days and 13 days respectively.[11] Time of detection by MODS assay was 7 days in a study of Sinh Thi Tran.[12] Above mentioned last two studies showed lesser time of

detection as compared to our study as they had included more of PTB samples which has higher load of bacteria. Higher load of mycobacteria give rapid formation of visible ropy colonies.

Number of culture positives by MODS on day 9 were 12.5%; day 10 were 25% and day 17 were 93.75%. The percentage of cultures positive at days 7, 14, 21 were 36.4%, 77.2% and 100% respectively by MODS assay in Agrawal A et al's study. [16]

Contamination rate (Table 4)

Contamination rate of samples by MODS assay and LJ culture in our study has been compared with other studies as shown in the table.

As seen, contamination rate was very low i.e. 0.43% in study of Marco Tovar et al as this study included only CSF sample which is a sterile body fluid.[7] In our study CSF samples showed zero percent of contamination rate. On the other hand, the study of Kirwan DE showed very high contamination rate [9] while study by Zadbuke et al showed contamination rate comparable to that of our study.[10]

Sensitivity of ZN smear

Sensitivity of ZN smear was 56.25% and specificity of 92.35% when compared to growth on LJ medium. This is similar to findings of Bagdia M et al. [19]

In the current study out of 199 specimens analyzed, 23 (11.5%) were ZN smear positive of which 10 were positive by MODS. Of the 176 (88.4%) smear negative,

Six were positive by MODS. Smear positivity and culture negativity could be due to inhibition of growth even when patient is treated with antimicrobials like Amoxiclav or fluoroquinolones for any other infection.

The sensitivity and specificity of MODS assay in ZN smear negative samples were 42.85% and 98.22% respectively. A meta-analysis of MODS assay for diagnosis of PTB in HIV infected patients; the sensitivity and specificity were 88.2% and 98.2% in smear negative PTB samples. The higher sensitivity was probably due to higher bacillary count in pulmonary samples as compared to EPTB samples. In a study by Dang HA, the sensitivity of MODS was 25.2% in smear negative specimens from children.[11]

Prevalence of EPTB

Prevalence of MTB in EPTB samples in our study was found to be 8.04% as compared to 7.1% in study of Bankar et al and 18.51% in study of Bag S et al.[20, 21]

Limitations of the study

Bio safety level II combined with individual protection is needed as processing of sample and its inoculation in plate could generate aerosols. However once the plate is sealed with scotch tape and packed in ziplock, it is safe to handle. Only other requirement after that is inverted light microscope.

We have used LJ culture as the gold standard and this has led to some degree of underestimation of test accuracy as some of the liquid culture assay like BACTEC, MGIT etc. have around 10% higher sensitivity.

We could not use automated MGIT because of cost constraint and hence we had to rely on manual liquid culture. The visual check had affected reproducibility of results.

In addition, this study being laboratory based, the clinical and radiological findings could not be obtained. Hence, it was not possible to classify specimens based on case history, age, sex etc. Despite this, our results remain significant since it shares promising diagnostic potential of MODS assay in EPTB.

Conclusion

Sensitivity and specificity of MODS was found to be 68.75% and97.26% using LJ medium as a gold standard. All types of specimens showed more than 90% of specificity except for bone and bone marrow samples (66.66%). MODS assay could detect MTB in 13 days on an average in all types of EPTB samples. Overall contamination rate on LJ medium was 6.22% and on MODS assay was 6.66%. Prevalence of EPTB in total samples which were positive on LJ medium (n=16) was 8.04%.

The MODS assay addresses key gaps in resource-limited settings with a high TB burden like rapid and accurate detection of MTB. Overall, it was found to have modest sensitivity and specificity in extra pulmonary samples. However, its performance varies with the type of EPTB specimen. With the short turnaround time, it helps in rapid detection of newer cases in resource poor settings. MODS are a valuable diagnostic tool in pleural TB and TB lymphadenopathy in which sensitivity of smear is very low. High specificity and rapidity would save patients and society's money by minimizing overtreatment and early detection would interrupt transmission. Nevertheless, it was technically little bit difficult to set up the assay. Prior training and validation needs to be taken into account before the test can be established.

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Legend Figures and Tables

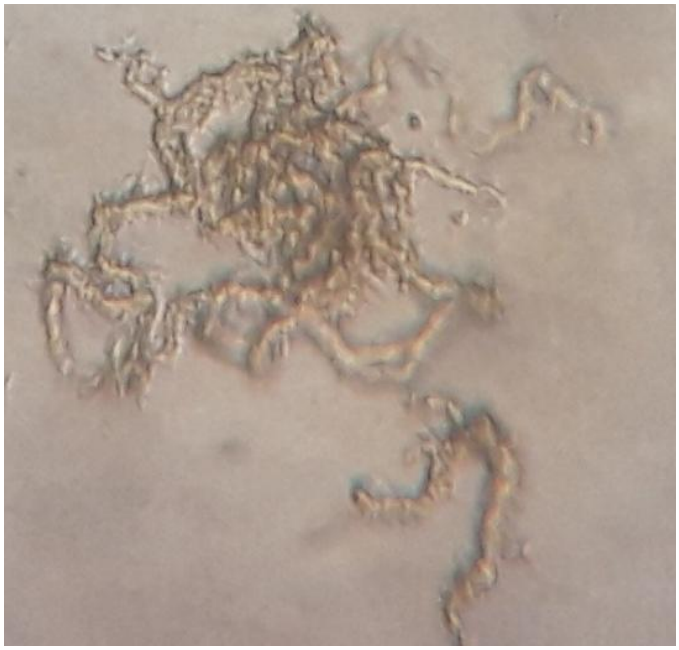

Figure 1: Fully developed stage of cord formation (Ropy appearance of MTB colonies) under inverted microscope in MODS assay on day 9-11 (40X).

Table 1: Nature wise distribution of samples, their contamination rate, positivity on culture methods, sensitivity, specificity and accuracy of MODS assay in comparison to LJ method. (N=225)

Sn..	Nature of sample	Total number (Number of contaminated samples)	Contam-ination rate (%ge)	Positive on LJ	Positive on MODS	Positive on both MODS & LJ	Sensitivity (in %ge)	Specificity (in %ge)	Accuracy
1.	Pleural fluid	75 (11)	14.66	0	1	1	100 (CI 2.5-100)	98.41 (CI 91.47-99.96)	98.43 (CI 91.59-99.96)
2.	Pus*	43 (10)	23.25	3	1	6	66.66 (CI 29.93-92.51)	95.83 (CI 78.88-99.89)	87.87 (CI 71.79-96.59)
3.	Ascitic fluid	15 (1)	6.66	0	0	0	-	-	-
4.	CSF	13 (0)	0	2	1	0	00 (CI 00-84.18)	90.90 (CI 58.72-99.77)	76.92 (CI 46.18-94.96)
5.	Tissue[#]	12 (1)	8.33	0	0	0	-	-	-
6.	LN Biopsy & FNAC	21 (2)	9.5	0	1	1	100 (CI 2.5-100)	94.44 (CI 72.70-99.85)	94.73 (CI 73.97-99.86)
7.	Urine	5 (1)	20	0	0	0	-	-	-
8.	Pleural biopsy	3 (0)	0	0	0	1	100 (CI 2.5-100)	100 (CI 15.81-100)	100 (CI 29.24-100)
9.	Bone & bone	3 (0)	0	0	1	0	-	66.66 (CI 9.43-	-

	marrow							99.16)	
10.	Other body fluids[+]	4 (0)	0	0	0	0	-	-	-
11.	Miscell-aneous[$]	5 (0)	0	0	0	0	-	-	-

*Pus also includes aspirates from liver, kidney and breast abscesses.

[#]Variety of tissues like endometrial and synovial tissue are included.

[+]Other body fluids include pericardial and synovial fluids.

[$]Miscellaneous samples included the samples whose sites are not clearly mentioned.

Table 2: Comparison of MODS assay with LJ medium for all samples (N=199)

	LJ positive	LJ negative	Total
MODS positive	11	5	16
MODS negative	5	178	183
Total	16	183	199

Sensitivity of MODS = 68.75 (CI 41.33-88.98)

Specificity of MODS = 97.26 (CI 93.73-99.10)

Accuracy = 94.97 (CI 90.95-97.56)

Table 3:Comparison of MODS assay with LJ medium among ZN smear positive samples (n=23) and among ZN smear negative samples. (n=176)

	ZN smear positive samples (n=23)			ZN smear negative samples (n=176)		
Findings	LJ positive	LJ negative	Total	LJ positive	LJ negative	Total
MODS positive	8	2	10	3	3	6
MODS negative	1	12	13	4	166	170
Total	9	14	23	7	169	176

Table 4: Comparison of findings of our study with those of other studies.

Name of Study (Year of publication)	Samples	Reference Method	Sensitivity %	Specificity %	Time to detection by MODS (in days)	Rate of contamin-ation on MODS	Rate of contamination on Gold standard culture method
Kirwan DE 2016 [9]	Lymph nodes (144)	LJ	65.4	-	13	26.6%	29.4% (LJ)
Marco Tovar 2010 [7]	Pleural fluid & Pleural biopsy (70)	LJ	20 81	-	11	2.9%	11%(LJ)
Maxine Caws 2007 [8]	CSF (61)	CRS*	64.9	100	6	0.43%	Not mentioned
Huang Z 2014 [17]	Pleural fluid (112) CSF (61) (Total 173)	LJ	37.5 20.5		14 9	Not mentioned	Not mentioned
Zadbuke Sonali 2017 [10]	Pus, urine, FNAC, body fluids,	LJ	83.3	83.3	10	7%	6.6% (LJ)

Agarwal A 2019 [16]	PTB(890) EPTB (173)	LJ/ MGIT	91.3	98.2	10.3	7%	5.2% (LJ)
Dang Ha 2009 [11]	GL (50), CSF (32), Pleural Fluid (3)	CRS	42.3	39.7	8	Not mentioned	Not mentioned
SinhThi Tran 2013 [12]	Sputum, GL, CSF, pleural & tracheal fluid	CRS	46	99.5	7	Not mentioned	Not mentioned
Present study 2019	EPTB samples	LJ	64.28	97.29	12	6.66%	6.22% (LJ)

*CRS Composite reference standard