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Research Article

COMPARATIVE EVALUATION OF MICROSCOPY, CONVENTIONAL CULTURE AND CARTRIDGE BASED NUCLEIC ACID AMPLIFICATION TEST (CB-NAAT) FOR THE DIAGNOSIS OF *MYCOBACTERIUM TUBERCULOSIS*

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Abstract- Background: Mycobacterium infection results in chronic granulomatous lesion requiring drug treatment for a prolonged period. We compared different methods for diagnosis of Mycobacterium infection in respiratory samples in this study and speciated the positive samples using MALDI-TOF MS. Aim: To evaluate different methods of smear microscopy, comparing Cartridge based nucleic acid amplification test with convention culture methods and speciation of the isolates by MALDI-TOF MS. Settings and Design: A cross sectional study was done in SRMC&RI. Methods & Material: Respiratory samples both before and after concentrations (Modified Petroff's & Bleach method) were stained by Kinyoun's modification. Fluorescent staining performed on unconcerated samples. The smears were interpreted as per National Tuberculosis Elimination Program. Smear positive samples were cultivated in Lowenstein-Jensen slope and incubated at 37oC for 8 weeks. LJ growths were subjected to MALD-TOF MS for speciation. Results: Of the 399 samples 7.3% was positive by Kinyoun's modification without concentration. Modified Petroff's and Bleach concentration methods enhanced the positivity to 8% and 8.3% respectively. Fluorescent staining showed 10.8% sample positivity. Of these 44 positive samples, 41 grew in conventional LJ medium. However, CB-NAAT identified them within 2 hours of inoculation. On speciation of these isolates by MALDI-TOF MS, Mycobacterium tuberculosis complex were predominant followed by *Mycobacterium avium* spp., *Mycobacterium arupense* and *Mycobacterium szulgai*. Statistical analysis showed 100% specificity in all microscopy methods, however, sensitivity varied as follows: Unconcentrated Kinyoun's-53.7%; Modified Petroff's-61.1%; Bleach concentration-59.3%; Fluorescent staining -79.6%. Conclusion: Fluorescent microscopy is a good technique for initial screening as It is imperative to include CBNAAT for early detection and RIF resistance. Species identification is also essential for accurate treatment.

Keywords- *Cartridge Based Nucleic Acid Amplification Test, Matrix-assisted laser desorption ionization - time-of-flight, Fluorescent microscopy, Bleach concentration*

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Introduction

Tuberculosis, a disease more than 100 years old, is still a major concern all over the world and in India [1]. Global TB report 2019 by the World Health Organisation (WHO) estimates that 10 million people fell ill with TB in the world and 27% of the world TB cases were from India in the year 2018 [2]. Early diagnosis and treatment have a critical role in reducing the disease burden and controlling the disease spread [3]. Tuberculosis is a chronic granulomatous disease affecting multiple organ systems of which lungs is mostly affected [4]. Despite of many diagnostic methods available, microscopy is the mainstay of TB diagnosis [5]. The benefit of smear microscopy is its low cost, low technical requirement and in addition it acts as a prognostic indicator to monitor the treatment progress [6]. The positivity of smear microscopy depends on the collection time, processing of sputum and staining method used. Smear microscopy is performed using conventional light microscopic methods such as Ziehl-Neelson staining and Modified Kinyoun's staining. These methods have low sensitivity and the lower limit of detection of AFB in sputum samples is observed to be 104 to 105 bacilli per ml [7]. The sensitivity of these methods can be increased by using decontamination and concentration methods such as Modified Petroff's Method and Bleach Method [6]. In 2009, WHO insisted the use of fluorescent microscopy in the stead of conventional microscopy as it was found to have higher sensitivity than the conventional method [8]. Mycobacterial culture methods done on solid media and liquid media system have a higher sensitivity than smear microscopy and can detect even 102 bacilli per ml. The main drawback of the method is the long incubation required for the growth of bacilli ranging from 6-8 weeks [6].

Molecular testing by the Gene Xpert MTB/RIF test overcame this drawback by detecting tuberculosis bacilli at a much faster rate with high sensitivity and specificity and provided the resistance status of the organism. The limitations of the test are the short self-life of the cartridges, requirement of uninterrupted power supply and a few temperature and humidity restrictions for its operation [9].

After a diagnosis of pulmonary tuberculosis is made, the process stops, and speciation of culture isolates are seldom performed. The involvement of non-tuberculous mycobacteria (NTM) in causing disease is slowly increasing and it needs to be addressed especially in countries with high incidence of tuberculosis. NTMs run the risk of being diagnosed as multi drug resistant tuberculosis as they are mostly resistant to antibiotics and routinely employed anti tuberculous therapy. This makes it important to speciate the isolated organism as vital step to introduce prompt and accurate treatment [10].

Conventional methods to speciate mycobacterium are easy to perform but require a long incubation period of 12 weeks. The rapid molecular methods are expensive and are most often restricted to reference laboratories. Alternative method for the identification and speciation of mycobacterium is provided by a newer technique involving Matrix-assisted laser desorption ionization - time-of-flight (MALDI-TOF). The use of this method is increasing as identification of mycobacteria is essential for controlling the disease [11].

Hence in our centre we undertook this study to perform a comparative analysis of smear microscopic methods, conventional culture, and cartridge based nucleic acid amplification tests and speciated culture positive isolated by MALDI-TOF MS.

Table-1 *RNTCP Grading of Acid Fast Bacilli for Sputum Smears*

Examination findings	No. of fields examined	Grading	Result
No AFB in 100 oil immersion fields	100	0	Negative
1-9 AFB per 100 oil immersion fields	100	Scanty (Record actual number of bacilli in 100 oil immersion fields)	Positive
10-99 AFB per 100 oil immersion fields	100	1+	Positive
1-10 AFB per oil immersion field	50	2+	Positive
More than 10 AFB per oil immersion field	20	3+	Positive

Table-2 *RNTCP Grading of Acid Fast Bacilli for Sputum Smears with Fluorescent Staining*

200-250x magnification: 1 length = 30 fields = 300 HPF	400x magnification: 1 length = 40 fields = 400 HPF	Grading	Result
No AFB per 1 length	No AFB per 1 length	0	Negative
1-29 AFB per 1 length	1-19 AFB per 1 length	Scanty (Record actual number of bacilli in 100 oil immersion fields)	Positive
30-299 AFB per 1 length	20-199 AFB per 1 length	1+	Positive
10-100 AFB per 1 field on average	5-50 AFB per 1 field On average	2+	Positive
More than 100 AFB per 1field on average	More than 50 AFB per 1 field on average	3+	Positive

Aims and Objectives

To evaluate and determine the sensitivity and specificity of different methods of smear microscopy for the detection of acid-fast bacilli in respiratory samples.

To compare the smear positivity with Cartridge Based Nucleic Acid Amplification Test (CB-NAAT) and conventional culture methods.

To speciate culture positive isolates by MALDI-TOF Mass spectrometry.

Materials and methods

A prospective study was done between 1st April 2019 to 31st September 2019 at a tertiary care Centre in South India. The study was conducted after procuring Institutional Ethics Committee approval (REF: CSP-MED/18/AUG/45/123).

All the respiratory samples *viz*., sputum, pleural fluid, bronchial wash, tracheal secretions sent to Microbiology Central Lab for acid fast staining were included in the study and other samples such as gastric aspiration, body fluids (except pleural fluid), biopsy tissue, pus, blood, urine, and stool were excluded from the study. More than one sample representing the same patient was also excluded.

Microscopy

A total of 399 samples sent for smear microscopy during the study period were processed for direct microscopy by 1. Modified Kinyoun's method [Fig-1] 2. Fluorescent Staining [Fig-2] 3. Modified Kinyoun's Method after concentration by Modified Petroff's Method [Fig-3] and 4. Modified Kinyoun's Method after concentration by Bleach Method [Fig-4]. Smears were interpreted as per the Revised National Tuberculosis Control Programme (Now, National Tuberculosis Elimination Program) 12 guidelines. [Table-1 & Table-2]

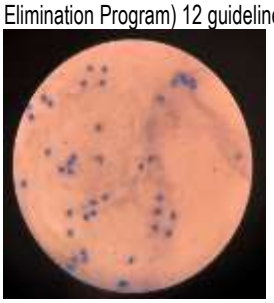

Fig-1 Sputum smear stained with Modified Kinyoun's method showing grade 3 Acid Fast Bacilli/ 100X Oil Immersion Field

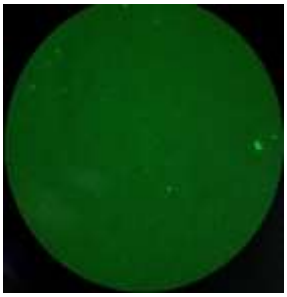

Fig-2 Bronchial Wash smear stained with Fluorescent staining showing Acid Fast Bacilli in 20X

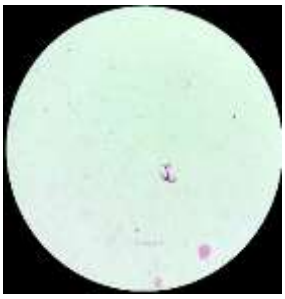

Fig-3 Smear showing AFB after Modified Petroff's concentration

Fig-4 Smear showing AFB after Bleach concentration

Culture

All the samples positive by either of the microscopic methods were inoculated in Lowenstein Jensen medium (L-J Medium) after decontamination using Modified Petroff's method and incubated at 370c for 8 weeks. The culture bottles were examined biweekly for the presence of growth [Fig-5]. Those culture bottles showing absence of visible growth at the end of 8 weeks were reported as culture negative. Culture bottles with visible growth (yellow buffy colonies) were confirmed as acid fast bacilli by performing Modified Kinyoun's method.

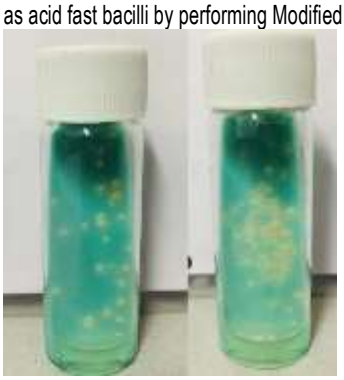

Fig-5 Lowenstein Jensen Medium showing growth of Mycobacteria

Cartridge Based Nucleic Acid Amplification Tests (CB-NAAT)

CB-NAAT results of the study samples were retrospectively obtained from the hospital information system (CB-NAAT results for 168 out of 399 samples were available in hospital database).

Matrix Assisted Laser Desorption Ionisation Time of Flight Mass Spectrometry (MALDI-TOF MS)

Randomly, 22 out of the 44 culture positive isolates were subjected to MALDI-TOF MS identification. Bruker Daltonics GmbH (Bremen, Germany) protocol for the optimised extraction of mycobacteria was followed. Zirconia-silica beads and the database of Mycobacteria Library (v5.0 Bruker Daltonics) with 780 reference spectra of mycobacteria was used. The Biotyper software was used to analyse the spectra of the samples against the spectra in the database. The results of the smear microscopy by different methods were tabulated and analysed for statistical significance (Sensitivity and specificity).

Results

A total of 399 respiratory samples sent for AFB smear examination were included in the study out of which 252 (63.2%) were males and 147 (36.8%) were females with a male to female ratio of 1.7:1. Age group distribution of the study population is shown in the [Table-3]. Among the 399 samples, majority were sputum samples 220 (55.14%). The breakup of clinical samples screened is shown in the [Fig-6].

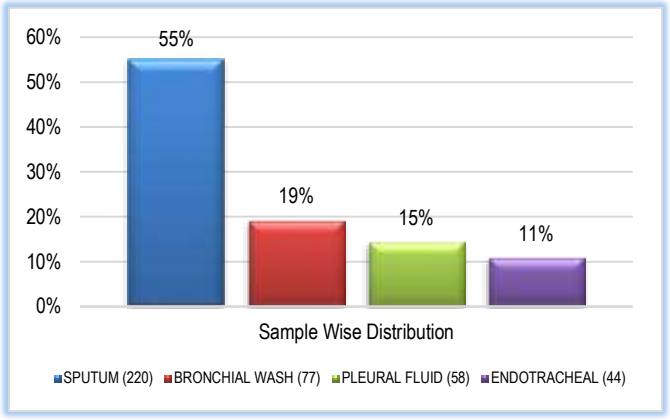

Fig-6 Sample Wise Distribution of the Study Population

Table-3 *Age group distribution of the study population*

Age in years	Male (n=252)	Female (n-147)	Total (n=399)
0-10	5 (2%)	3 (2%)	8 (2%)
Nov-20	9 (3.6%)	6 (4.1%)	15 (3.8%)
21-30	16 (6.4%)	18 (12.2%)	34 (8.5%)
31-40	14 (5.6%)	20 (13.6%)	34 (8.5%)
41-50	39 (15.5%)	28 (19.1%)	67 (16.8%)
51-60	62 (24.6%)	29 (19.7%)	91 (22.8%)
61-70	71 (28.1%)	31 (21.1%)	102 (25.6%)
>71	36 (14.2%)	12 (8.2%)	48 (12%)
Total	252	147	399

Microscopy

A total of 44 samples (11%) (n=399) samples were positive by one or more of the above methods. The age group distribution of the microscopy positive samples is shown in [Table-4]. Among the 44 positive samples, 37 (84%) were males and 7 (16%) were females.

Table-4 *Age group distribution of smear positive samples*

Age in years	Male (n=37)	Female (n=7)	Total (n=44)
0-10	0 (0%)	0 (0%)	0 (0%)
Nov-20	1 (2.3%)	1 (2.3%)	2 (4.6%)
21-30	1 (2.3%)	3 (6.7%)	4 (9%)
31-40	4 (9.1%)	1 (2.3%)	5 (11.4%)
41-50	10 (22.7%)	0 (0%)	10 (22.7%)
51-60	11 (25%)	1 (2.3%)	12 (27.3%)
>60	10 (22.7%)	1 (2.3%)	11 (25%)
Total	37	7	44

Out of the 44 smear positive study samples, 29 (7.3%) of the samples were positive in Kinyoun's method without concentration. Comparison of the results of smear microscopy methods is depicted in [Table-5].

There was an increase in positivity of 2-3% observed in fluorescent staining compared to other three methods. In the 44 smear positive samples, sputum were 27(61.4%) bronchial wash were 12(27.3%), endotracheal secretions were 4(9.1%) and pleural fluid was 1(2.2%). Comparison of smear positivity of the clinical samples by different methods is shown in [Table-6].

Table-5 *Comparison of 4 microscopic methods*

Method	No. of Positives	No. of Negatives	Total
Kinyoun's Method	29 (7.3%)	370	399
Modified Petroff's Method	32 (8%)	367	399
Bleach concentration	33 (8.4%)	366	399
Fluorescent staining	43 (10.8%)	356	399

Culture

Out of the 44 smear positives inoculated in Lowenstein Jensen medium 41 (93%) of them were culture positive. Breakup of the growth by clinical samples and its smear microscopy results is shown in [Table-7].

Table-7 *Comparison of Culture and Microscopy*

Samples	Positive by any or all 4 methods of microscopy	LJ Culture Positives
Sputum	27	26
Bronchial Wash	12	11
Endotracheal Secretions	4	4
Pleural Fluid	1	0

CB-NAAT

Of the 165 samples for which CB-NAAT results were retrieved, 55 samples had been reported MTB complex detected. Comparison of CB-NAAT results with smear microscopy positive is shown in [Table-8].

Table-8 Smear Positive CB-NAAT positive samples

The samples that were positive for CB-NAAT but negative by Smear Microscopy was seen in high number in pleural fluid (5), followed by bronchial wash (4) and sputum (1).

MALDI TOF Mass Spectrometry

Of the 41 culture positive samples, 22 randomly selected Lowenstein Jensen slopes were subjected to MALDI-TOF MS for speciation of Mycobacteria. The list of Mycobacterium species reported by MALDI-TOF MS are *Mycobacterium tuberculosis* complex (18) and the rest were *Mycobacterium avium* spp. paratuberculosis (2), *Mycobacterium arupense* (1) and *Mycobacterium szulgai* (1).

Statistical analysis

Out of the 54 samples positive by CB-NAAT, 33 were positive by smear microscopy in one or more of the 4 microscopic methods employed for screening. The sensitivity and specificity of the microscopic methods is shown in [Table-9].

Table-9 *Sensitivity and Specificity of 4 different microscopic methods*

Method	Sensitivity	Specificity
Unconcentrated Kinyoun's	53.70%	100%
Modified Petroff's Concentration	59.30%	100%
Bleach Concentration	61.10%	100%
Fluorescent Staining	79.60%	100%

Discussion

Of the 399 samples studied, 252 (63.2%) were males and the rest were females, among this 44 (11%) were positive for the presence of acid fast bacilli (AFB) by different smear microscopy. This is comparable to studies done by Kulkarni *et al*., 201513, Firdaus *et al*., 201714, Gebre-Selassie, 200315 and Uddin *et al*., 201316 with AFB positivity of 13.7%, 12%, 8.5% and 8% respectively.

Available evidence in the literature shows most of the AFB positive patients were from males than females as demonstrated by Rahman *et al*., 197017 (74% males), Bansal *et al*., 201718 (72.9% males), Reed *et al*., 201119 (80.7% males) and Baruwa *et al*., 200820 (75% males). Similarly, our study also has a predominant male positivity percentage of 87 which is slightly higher than the above-mentioned studies.

On analysing the age group distribution of AFB positive patients, most of the patients (n=44) belonged to the age group of 51-60 (27.3%), followed by age group of more than 60 (25%) and age group of 41-50 (22.7%).

Table-6 *Sample wise comparison of smear positives*

Samples	Kinyoun's Method		Modified Petroff's		Bleach concentration		Fluorescent Staining		Total	
	+	-	+	-	+	-	+	-	+	-
Sputum (n = 220)	19 (8.6%)	201	22(10%)	198	26(11.8%)	194	26(11.8%)	194	27(12.2%)	193
Bronchial Wash (n = 77)	7(9.1%)	70	7(9.1%)	70	9(9.1%)	68	12(15.5%)	65	12(15.5%)	65
Endotracheal (n = 44)	3(6.8%)	41	3(6.8%)	41	3(6.8%)	41	4(9.1%)	40	4(9.1%)	40
Pleural Fluid (n = 58)	0	58	0	58	0	58	1(1.7%)	57	1(1.7%)	57

We had least positivity below age group 20 years. This is comparable with study done by Neelu Sree *et al*., 201821 which showed majority 40.3% (n=21) of Tb positivity in the age group of 51-60 years and low positivity between age group of 11-20 years.

On evaluating the various smear microscopy namely Kinyoun's method, Modified Petroff's method, Bleach concentration technique and Fluorescent staining, the positivity rate achieved were 29 (7.26%), 32 (8.02%), 33 (8.26%) and 43 (10.77%) (n=399) respectively. This shows maximum benefit with fluorescent staining. This data is in par with Uddin *et al*., 201316 which showed increase in 1.5% from direct smear to bleach concentration method; Ängeby *et al*., 200422 which showed 7.7% positivity with direct smear and 10.3% positivity with Bleach concentration; Tripathi *et al*., 201423 which showed 2% increase in positivity when compared with Direct smear and Modified Petroff's concentration; Rahman *et al*., 197017 which showed a 3% increase in positivity when using Fluorescent staining; Reed *et al*., 201119 which showed a 6% increase in positivity when using Fluorescent staining; Workineh *et al*., 201724 which showed a 2% increase in positivity when using Fluorescent staining.

All the positive samples (n=44) were inoculated in Egg based Lowenstein Jensen medium out of which 41(93%) of them grew acid fast bacilli. A total of 3 (7%) samples failed to grow in conventional cultivation methods, maybe due to non-viability of the bacilli. The results are similar with that of a study done by Rahman *et al*., 197017 which also showed 7% cases positive by smear microscopy to be negative by culture.

Of these 399 study samples, Cartridge Based Nucleic Acid Test (CB-NAAT) done by Cepheid GeneXpert® MIB/RIF Ultra assay were available for 168 samples which showed 54 (32.14%) positivity. Among these 54 CB-NAAT positive samples, 44 (82%) were positive by smear microscopy. A total of 10 samples (18%) were reported positive by CB-NAAT but negative by smear microscopy. All these samples were reported as low detected, very low detected or trace detected by CB-NAAT. This data substantiates the requirement of minimum number of bacilli 10000/ml for smear microscopy positive. Similarly, studies done by Chinedum *et al*., 201725 (14.4%) and Moraa Orina *et al*., 201726 (12%) also showed smear negative CB-NAAT Positivity.

Of the four methods evaluated in this study, Fluorescent Staining had the highest sensitivity of 79.63%, followed by Bleach concentration 61.11%, Modified Petroff's concentration 59.26% and Kinyoun's method had lowest sensitivity of 53.70%. This is in par with studies done by Ängeby *et al*., 200422 which showed a sensitivity 57% for direct smear and 65% for Bleach concentration. Similarly, Bruchfeld, 200027 had 54.2% sensitivity for direct smear and 63.1% sensitivity for bleach concentration. Steingart *et al*., 200628 in his review article had reported the sensitivity of conventional microscopy as ranging from 32% to 94% whereas fluorescence microscopy sensitivity ranged between 52% and 97%. Fluorescence microscopy was on average 10% more sensitive than conventional microscopy. Another review article by Cattamanchi *et al*., 201029 had stated 9 studies involving Bleach centrifugation to have sensitivity of 59-71% and specificity of 93-98%, when compared with direct smear microscopy having a sensitivity and specificity of 49-63% and 98% respectively; 5 studies involving Modified Petroff's method with sensitivity range from 61-89% and specificity of 98-99% when compared with direct smear microscopy with sensitivity of 52-75% and specificity of 98-99%. Out of the 44 samples which grew in LJ medium, 22 of them were identified at species level by MALDI-TOF MS. Among this 22 samples, 18 (82%) of them were identified as *Mycobacterium tuberculosis* complex and the rest 4 (18%) as non-tuberculous mycobacteria namely *Mycobacterium avium* spp. paratuberculosis (2), *Mycobacterium arupense* (1) and *Mycobacterium szulgai* (1) but the MALDI TOF Scores for all the 4 were low and hence genus level identification was only reliable.

Conclusion

Our study clearly shows Fluorescent microscopy is a good microscopic method for initial screening as it is 79.63% sensitive and 100% specific when compared to conventional methods routinely followed by most of the diagnostic laboratories. Fluorescent microscopy also reduces the time for detection however; it needs an additional facility like Fluorescent microscope. It is imperative to include CBNAAT assay as well for early detection of Mycobacterial infections as CB-NAAT detected a significant a greater number of our study samples.

Application of research: CBNAAT assay along with smear microscopy methods play a vital role in making the diagnosis of pulmonary tuberculosis, whereas speciation after the culture stands mandatory as non-Mycobacterium tuberculosis complexes are also frequently being isolated.

Research Category: Medical Microbiology

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University: Department of Microbiology, Sriramachandra Institute of Higher Education & Research, Chennai, 600116, Tamil Nadu, India
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Study area / Sample Collection:

Strain name: *Mycobacterium tuberculosis*

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