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Original research article

COMPARISON OF EFFECTIVENESS OF CONVENTIONAL AND NEWER METHODS IN DETECTING MYCOBACTERIUM TUBERCULOSIS IN SPUTUM

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ABSTRACT

Background: Tuberculosis is emerging as a disease of major public concern causing immense morbidity,mortality and distress to individuals,families and communities all around the world.Early diagnosis plays a vital role in the control of TB. Early and accurate detection of active cases remain an important objective for improved implementation of chemotherapy and for reduction in the spread of the disease.

Aim of the study : To compare effectiveness of conventional and newer methods in detecting Mycobacterium tuberculosis in direct smear negative sputum.

Materials and methods : A Prospective hospital based clinicomicrobiological observational study conducted in the department of Microbiology,at MNR Medical College, and the State TB Centre, Hyderabad from August 2012 to August 2014.100 patients who were clinically suspected to have tuberculosis were selected for the study from among those attending the Pulmonologyand Medical OP .

Results : Out of Direct100 Smear-Negative cases taken up for our study, 7(7%) were positive for Acid Fast bacilli by the ZiehlNeelsen's Stain, 9(9%) were positive for Acid Fast Bacilli by the Auramine- O Stain, 8 (8%) of them showed growth on Lowenstein Jensen's medium (6 on LJ without PNB, and 2 on LJ with PNB), 9 (9%) were positive for Mycobacterium tuberculosis by Line Probe Assay, and 12 (12%) of them showed growth in MiddleBrook7H9 broth base - BACTECMGIT960 SYSTEM,all following concentration technique-NALC-NaOH method

Conclusion: ZeihlNeelsen's Staining Technique:Seven of the 100 smear-negative sputa were positive (7%) for AFB,showing a sensitivity of 58.33% and a specificity of 100%.Auramine-O staining technique:Nine of the 100 smear-negative sputa were positive(9%) for AFB showing a sensitivity of 75% and a specificity of 100%Culture on Lowenstein Jensen's medium:Six(6%) of the 100 smear-negative sputa were positive for M.tuberculosis showing a sensitivityof 66.6% andspecificity of 100%Culture in MiddleBrook7H9 broth base BACTEC MGIT 960 system:Twelve(12%)of them were positive for M.tuberculosis showing a sensitivity of 100% and a
specificity of 75%Line Probe Assay:Nine(9%) of the hundred smear-negative casessputum specimens tested positive for M.tuberculosis showing a sensitivity and specificity of 100% Modern diagnostic techniques are more sensitive and specific compared to conventional methods for detection of Mycobacterium tuberculosis bacteria. This study also emphasize the importance of concentration technique (NALC-NaOH) for direct sputum negative specimen

Key words : ZiehlNeelsen's Stain ,Auramine- O Stain

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INTRODUCTION

Pulmonary tuberculosis is one of the major air borne infectious bacterial diseases known.Despite the discovery of the causative organism,availabilty of effective drugs and vaccine,Tuberculosis still remains a major world wide healthproblem.Tuberculosis or koch's disease,as it is more popularly known,is a necrotizing bacterial infection with protean manifestations and wide distribution.Lungs are more commonly affected, but extra pulmonary lesions may occur also in thekidneys,bones,lymph nodes or meninges or be disseminated throughout the body.The infection may cause clinical disease either shortly after inoculation-primary tuberculosis or after a period of months,or decades of dormancy,often called post primary or reactivated tuberculosis (1)

It is estimated that 1/3rd of the global population is infected with tuberculosisasymptomatically,5-10% of whom will develop clinical disease during their lifetime,and approximately 8.9 to 9.9 million new cases of tuberculosis arise annually.Tuberculosis is thus emerging as a disease of major public concern causing immense morbidity,mortality and distress to individuals,families and communities all around the world.(2)

The diagnostic modalities should have certain desirable features like, sensitivity,specificity, speed, reproducibility, cost effectiveness, safety, simplicity and easy application for wider use.

Smear microscopy using conventional staining techniques like the Zeihl-Neelsen's stain or Kinyoun stain or the modern FluorochromeAuramineO stain for the detection of acid fast bacilli has become a routine practice,and is done free of cost under the Revised National TBControl Programme. Even with the advent of newer methods of diagnosis, culture remains the gold standard for the diagnosis of Tuberculosis.Culture on Lowenstein-Jensen medium is the most common method of isolation of mycobacteria from clinical specimens. Performance of culture will provide mycobacterial isolates, which can be identified by simple phenotypic tests.Such information will help in proper differentiation of mycobacteria into M. tuberculosis and non tuberculous mycobacteria (NTM) (2) .

The Ziehl-Neelsen staining method,is certainly rapid and inexpensive but it can detect bacilli only when there are more than 10,000 bacilli/ml of the sputum (3)

Aims and objectives: To compare effectiveness of conventional and newer methods in detecting Mycobacterium tuberculosis in direct smear negative sputum.

Objectives:

- To detect the presence of Mycobacterium tuberculosis by direct microscopy using Zeihl-Neelsen's technique, in the sputum of patients suspected to have pulmonary tuberculosis,attending the Pulmonology and Medical Outpatient Departments,MNR Hospital,and in the sputa processed in the State TB Centre,Hyderabad
- To study the incidence of Pulmonary tuberculosis among patients suspected to have Tuberculosis,but ZeihlNeelsen’s stain smear negative on Direct Microscopy,by subjecting their sputum specimens to1.Conventional Diagnostic techniques like (a)ZN stain following concentration technique (b)Culture on Lowenstein Jensen’s medium and 2.Modern Diagnostic techniques like (a)Auramine O fluorochrome stain,(b)Culture in MiddleBrook Liquid medium contained in BBL MGIT Mycobacterial (Fluorescent)Growth Indicator Tubes,and incubated in MGIT BACTEC/960 System,and(c)Line Probe Assay ,all following concentertion technique
- To evaluate and compare the sensitivity, specificity, and therefore, the effectiveness and reliability of conventional & modern techniques in the detection of M. tuberculosis in smear-negative cases.
- To detect the prevalence of multidrug resistant tb if any, among smear negative cases of pulmonary tb suspects by line probe assay, using the genotype mtbdrplus ver 2.0.

MATERIALSANDMETHOD

This study was conducted after approval by the Institutional Research & Ethics Committee. Informed consent was obtained from the patients for the sample collection and for enrolment in the study .A Prospectivehospital based clinicomicrobiological observational study conducted in the department of

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Microbiology,at MNR Medical College, and the State TB Centre, Hyderabad from August 2012 to August 2014.100 patients who were clinically suspected to have tuberculosis were selected for the study from among those attending the Pulmonology and Medical OP .

Inclusion criteria

- Age 21 -60 years
- Patients clinically and/or radiologically suspected to have Tuberculosis,but smear negative when stained by ZiehlNeelsen's Direct technique.
- Suspected Drug Resistant-Tuberculosis patients referred for evaluation and management

Exclusion criteria

- Age less than 20 years and more than 60 years
- Patients whose sputum were positive for AFB by Direct ZiehlNeelsen staining .
- Patients who were unwilling to get enrolled in the study.
- Patients who submitted improper specimens-saliva or nasal secretions
- Patients who had used oral antiseptics .

Methodology

IdentificationofTBsuspects : A proforma was filled up for each patient documenting age ,sex, address, clinical information including chief complaints, duration of symptoms, and any history of Anti Tuberculosis Treatment earlier.Apulmonary TB suspect was defined as any personwith coughfor 2 weeks or more with or without other symptoms suggestive of TB.

Collection of the Sputum Specimen :

Eachpatientwasinstructedtotakeadeepbreath,holditmomentarily, andthen coughdeeplyandvigorously. The patientwasalsoinstructedtocoverhis/hermouthcarefully andspitinto a sterile leak proof labelled container. Two such sputum specimens, one random sample, and the second, an early morning sample, were collected as per RNTCP guidelines. Both specimens were collected aseptically in order to minimize contamination with other bacteria. Allthesampleswereтransportedtothelaboratoryand processedasearlyaspossible in order to avoid the overgrowth of other microorganisms.Ifany delaywasanticipatedinprocessing,theywerestored at 4˚C.

All 100 sputum were stained by

1.Ziehl- Neelsen's Technique for regular Light microscopy and

2. Auramine O staining technique for Fluorescent microscopy following concentration.

CULTURE :

The recommendations made by the U.S Centre for Disease Control & Prevention (CDC) for laboratories to use the most rapid methods available for diagnostic Mycobacterial testing, include the use of both liquid and solid medium for mycobacterial culture.

Hence, the conventional culture methods, Lowenstein Jensen's medium without p-nitrobenzoic acid (for the cultivation of M. tuberculosis), & Lowenstein Jensen's medium with p-Nitrobenzoic Acid (for the cultivation of Non Tuberculous Mycobacteria, if any), and the modern method - Modified Middlebrook Broth contained in BBL MGIT Mycobacteria Growth Indicator Tubes, used with a BACTEC MGIT 960 System, were chosen for the study.

OBSERVATIONS AND RESULTS

In the present study age distribution varied from 11 years to 90 years. Majority were included among 31-50 years (41%) followed by 51-70 years (25%) ,71-90(3%),10-30 years (31%)

69% were Males, and 31% were Females. 68% were from Rural area, and 32% of them were from Urban area. 98% presented with cough(98), followed by fever (39), loss of appetite (35), loss of weight (16), and haemoptysis (8). Out of Hundred Direct **Smear-negative** patients studied, X-ray Chest was suggestive of Tuberculosis only in six patients (6%), while it was NOT suggestive of TB in theremaining 94% of TB suspects.

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89 (89 %) of the **Smear-Negative** patients included in our study were New cases, while 11 (11%) of them were already on Anti Tuberculous Treatment - Ist Line.

Five (5%) of the **Smear-Negative TB-suspects** randomly chosen for our study were found to be HIV positive, while Ninety five (95%) were HIV negative. HIVSTATUS -:5% positive,95% negative.
Out of Direct 100 **Smear-Negative** cases taken up for our study, 7(7%) were positive for Acid Fast bacilli by the ZiehlNeelsen's Stain following concentration, 9(9%) were positive for Acid Fast Bacilli by the Auramine- O Stain, 8 (8%) of them showed growth on Lowenstein Jensen's medium (6 on LJ without PNB, and 2 on LJ with PNB), 9 (9%) were positive for Mycobacterium tuberculosis by Line Probe Assay, and 12 (12%) of them showed growth in MiddleBrook7H9 broth base - BACTECMGIT960 SYSTEM.
All samples from this system showing an increase in fluorescence after a minimum of 7 days following inoculation, and identified by the BACTEC MGIT 960 instrument as Culture positive, were subcultured. Smears were made, and stained by ZiehlNeelsen's technique, and were confirmed to be Acid Fast Bacilli with morphology resembling M. tuberculosis as per CLSI guidelines.. They were also subjected to standard biochemical tests (like growth or absence of growth on LJ medium with PNB, Niacin test and Nitrate Reduction test), as per CLSI guidelines.
Although 9 culture samples out of the 100 smear-negative cases , were positive for M.TB by both Middlebrook-BACTECMGIT960 SYSTEM, and LPA (Multiplex PCR), an additional 3 samples were found positive by Middlebrook- BACTECMGIT960 SYSTEM. Since LPA was specific for M.TB only, and since all 12 MGIT tubes were positive for AFB, 9 of which were which were Niacin positive, and 3 were Niacin negative, it was confirmed that 9 of the isolates were M.TB, while 3 were Non tuberculous Mycobacteria. Hence,LPA/PCR was taken as Gold Standard for M.TB.only detected by other techniques, while MiddleBrook7H9 broth base - BACTECMGIT960 SYSTEM was taken as Gold Standard for diagnostic techniques detecting the total number of isolates (Mycobacterium tuberculosis and Non Tuberculous Mycobacteria).
7 of the 100 Smear-Negative cases in our study were positive for AFBby ZN Staining following Concentration technique.
Following Concentration Technique, 7 (7%) of the 100 direct **smear negative** sputum specimens were positive for AFB by ZN staining, while 9 (9%)of them were positive for AFB by Auramine O staining. Their Sensitivity and specificity were calculated with LPA as the Gold Standard.

COMPARATIVE EVALUATION OF LJ MEDIUM, MB-BACTEC MGIT & PCR IN THE DETECTION OF M.TB

6 (6%) out of 100 smear-negative cases showed growth on LJ medium, all Niacin positive, while 9(9%) of the 12 MB-BACTEC MGIT tubes were Niacin positive. All 9 cases (9%) were detected by LPA/PCR. 5 out of the 100 pts. randomly chosen for our study were incidentally found to be HIV positive. 3 of them were culture and PCR positive for M. TB, while 2 were negative.
Out of nine isolates of M. tuberculosis in 100 cases reported smear-Negative, one(1%) was resistant to both Rifampicin and INH, and therefore, Multi Drug Resistant.

Table 1. COMPARISON OF CONVENTIONAL AND NEWER METHODS IN THE DETECTION OF MYCOBACTERIUM TUBERCULOSIS:

Out of one hundred Smear-Negative pts. tested, 7(7%) were positive by Zeihl-Neelsen stain, 9(9%) were positive by Auramine O stain, 6(6%) were Culture positive in LJ medium,12(12%) were Culture positive in MiddleBrook-BACTEC MGIT960, and 9 (9%) were positive by PCR.

Diagnostic technique	No. of patients tested positive	No.of patients tested negative	Total no.of patients tested	Per centage of patients tested positive	Sensitivity	Specificity
Zeihl-Neelsen stain	7	93	100	7%	58.33%	100%
Auramine O	9	91	100	9%	75%	100%

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Stain						
Culture on LJ	6	94	100	6%	66.66%	100%
Culture in Middlebrook	12	88	100	12%	100%	75%
PCR	9	91	100	9%	100%	100%

Table 2:MDR-TB DETECTED IN THE PRESENT STUDY :

Sensitivity Pattern	Rifampicin(Mutation in rpoB gene)	Isoniazid(Mutation in inh gene)	Isoniazid(Mutation In KatG gene)
Sensitive	8	8	9
Resistant	1	1	0
Total LPA positive for MTB	9	9	9

DISCUSSION

Themajorityofpatients in the present study i.e. 23(23%)werefoundtobeintheage groupof21-30 years,followedby 22(22%)patientsamong31-40yrs.Soabout45%wereintheage group 20-40yrs.Thisagegroupis an active part ofthecommunity.Diseaseinthis age group resultsinreductionofmanpowerandeconomic losstothecountry.Ourstudywasalmost similartoShivaramanetal (4)

Outof100 patientsstudied, 69 (69%)weremaleswhile 31 (31%)were females. The Male to femaleratiowas2.2:1. Narang.P etal (5) havereportedthat61.03%oftheirsubjectswere male while 38.97%were female.

Study	Percentage
Shivaraman et al (4)	40.8%
Narang et al (5)	26.30%
Present study	48.5%

Peter Eriki et al (6) and Fandinho FCO et al.(7) reported a male to female ratio of 1.8:1 and 1.6:1 respectively.All these findings are comparable to ours. Residential status-wise, only 32% of our study population were urban. 68% were from the villages, and from poor socioeconomically background.

Zaman et al (8), and AbuBakar (9) also reported that most of their study population was rural. It is an established fact that Tuberculosis is associated with poorsocioeconomic conditions, overcrowding, and poor hygiene, public travel, and malnutrition which favour the spread of infection in the community. With all these factors ,there is also a lack of awareness of communicable diseases prevalent, and the health facilities available. (10) However, infection occurs at an earlier age in urban than in rural population.

Place&Study	No.TBpatientexamined	% HIVpositive
TripathiS et al) 11)	400	28.75
Mohanty et al) (12)	3878	26.7
(Lalitafernandes et al) (13)	980	10.91
Samuel NM et al) (14)	112	17
Present study	100	5

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Zeihl-Neelsen'sStaining :

Inthepresentstudy,7outof100 Z.N. stained **Directsmear-Negative**cases(7%)werepositiveforacid fastbacillibythe same ZiehlNeelsen's stained smear made after Decontamination and Concentration of the specimen by the NALC-NaOH method. The sensitivityandspecificityof ZN stain following NALC-NaOHConcentration Technique inourstudywas58.33%and100%respectively.

Study	**Percentage**
Lakshmi et al.(15)	18.6
Jain et al.(16)	32.7
Jena et al.(17)	56.5
SSNegi.(18)	33.79
Our study	7%

Lakshmi et al .(15) reported 18.6%, Jain et al reported 32.7% Jena et al reported 56.5%, a high percentage of AFB positivity in concentrated smears examined in their study, while SS Negi et al reported 33.79%.

The percentage of AFB positivity in the present study seems low, when compared to the above studies precisely because all the hundred persons that constitute our study population were basically **Direct Smear-Negative** cases who were taken up for Zeihl Nielsen's staining by the Concentration technique.

Auramine O Staining ;

Since they were Direct smear-negative, they were probably in the early stages of pulmonary infection, and had a lowbacterial load. So, a majority of patients were of grade 1 (+), which represents the least bacteria among 3 grades.

All the one hundred specimens, after Concentration, were also stained by the Auramine-Ofluorochrome staining technique.9 (9%) out of 100 concentrated smears in the present study were positive by Fluorochrome staining, giving a

	Sensitivity	Specificity
LidyaChaidir et al(19)	75.5%	90%
Forero et al(20)	97.89%	94.67%
Our study	75%	100 %

sensitivity of 75%, and a specificity of 100%. The sensitivity and specificity of Auramine O staining, as well as ZeihlNeelsen's staining with Concentrated specimens were calculated taking LPA as the Gold standard. The sensitivity rate of Auramine O staining technique was found to be 75% in our study, very similar to that reported by LidyaChaidir et al (19) (75.55%)

Result		Min No. of fields to be examined
Negative	0 AFB/1 Length	40
Scanty	1-19 AFB/1 Length	40
1+	20-199 AFB/1 Length	40
2+	5-50 AFB/1 Field	20

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3+	>50 AFB/1 Field	8

All 9 smears that were positive for AFB, were graded as scanty 5.5%,1+ 4.2% , 2+4.2%,3+ 0.5% in accordance with the RNTCP guidelines.

Culture on Lowenstein Jensen medium:

Sl.no.	Study(year)	M.TB -Rateof Isolation(%) MM.TBPercent)
1	J. Jena (1997) (17)	40.7
2	Lathinair(1998)(21)	38.8
3	ABhargava et al (2007)(22))	59
4	SSNegi(2005)(18)	48.9

In our study,rate of isolation of M.TB is 6%in LJ without p-nitrobenzoicacid.All 6 of these isolates were AFBpositive, and Niacin positive.

Isolation of MTB in MIDDLEBROOK 7H9 BROTH/BACTEC MGIT 960:
All hundred Smear-Negative sputum samples were subjected to Decontamination & Concentration before Liquid Culture In a falcon tube.

	Sensitivity	specificity
Negi et al.(18)	55.86%	100%
C.Rodrigues et al.(23)	97.6%	100%
Chewkk et al.(24)	93%	92%
Our study	100%	100%

Various authors have reported similar findings ranging from 80 to 100% for M960 and from 59.7 to 87.2% for LJ.5-7 In our study, isolation rate by M960 system was 43.44% morethan that by LJ method. There was a **low positivity rate shown by LJ method** in our study in comparison to around 69-87.2% reported in literature6,7.This could be because of the fact that samples that were grossly contaminated on LJ were considered negative, whereasin M960, since the smears were made from all instrument positive MGIT tubes, it was found that there were samples, which had both contaminants, as well as Mycobacteria grown in them. Such tubes were considered positive by M960.

NTM/MOTT/ ATYPICAL MYCOBACTERIA:

Study	NTMisolation(%)
Trived.(25)i et al	4
Pathak et al.(26)	0.5
Saran et al.(27)	2
Present study	2(LJ),3(MB MGIT)

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Trived et al .(25) reported the isolation of 4% NTM cases., Pathak et al.(26) reported 0.5%, and Saran et al (27) reported 2 cases, exactly similar to was found in the present study. They were AFB positive, but Niacin negative. Apart from noting them to be Rapid growers, growing on LJ medium with p-nitrobenzoic acid, AFB positive, and Niacin negative, no attempt was made to speciate them further.

LPA-LINE PROBE ASSAY:

All liquid cultures that were identified by the BACTEC MGIT 960 instrument as positive, were then subjected to Line Probe Assay. The Line Probe Assay is basically, aMolecular Genetic Assay, a DNA strip test that allows simultaneous molecular identification of M.TUB complex, and the most common genetic mutations causing resistance to Rifampicin and Isoniazid. MDR-TB is defined as TB that is resistant atleast to RMP and INH, the two most important first-line anti-TB drugs . The following species are included in the TB-causing M. tuberculosis complex: M. tuberculosis, M. africanum, M. bovis subsp. bovis, M. bovis subsp. caprae, M. bovis BCG, M. microti, M. canettii, and M. pinnipedii

Outof 12cases,9(9%)were LPApositive for TUB complex and3(3%)were LPA negative for TUB complex indicating they are NTM/MOTT.

One of the nine isolates of M.tuberculosis was resistant to both Rifampicin and INH,and therefore Multi Drug Resistant Tuberculosis showing MDR incidence of 11.1%

Conclusion :

All hundred Smear-Negative specimens were subjected to Concentration Technique by the NaLC-NaOH method.ZeihlNeelsen's Staining Technique:Seven of the 100 smear-negative sputa were positive (7%) for AFB,showing a sensitivity of 58.33% and a specificity of 100%Auramine-O staining technique:Nine of the 100 smear-negative sputa were positive(9%) for AFB showing a sensitivity of 75% and a specificity of 100%Culture on Lowenstein Jensen's medium-six(6%) of the 100 smear-negative sputa were positive for M.tuberculosis showing a sensitivityof 66.6% and specificity of 100% and Culture in MiddleBrook7H9 broth base BACTEC MGIT 960 system-twelve of them were positive for M.tuberculosis showing a sensitivity of 100% and a specificity of 75%Line Probe Assay:Nine(9%) of the hundred smear-negative casessputum specimens tested positive for M.tuberculosis showing a sensitivity and specificity of 100% One of the nine isolates of M.tuberculosis was resistant to both Rifampicin and INH,and therefore,Multi Drug Resistant showing MDR incidence of 11.1%Hence Modern diagnostic techniques are more sensitive and specific compared to conventional methods for detection of Mycobacterium tuberculosis bacteria. This study also emphasize the importance of concentration technique (NALC-NaOH) for direct sputum negative specimen.

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