Embase (Elsevier)

ORIGINAL ARTICLE

Molecular Detection of Mycobacterium Tuberculosis Complex from Clinical Sputum Samples in Patients Attending Tertiary Care Centre in Uttar Pradesh Province of India

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Article Information

Received: 15 Nov 2015
Accepted: 10 Dec 2015
Plagiarism software: Turnitin

Keywords:
Tuberculosis,
Polymerase chain reaction,
Non Tuberculous Mycobacteria

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ABSTRACT

Background: In the world, tuberculosis ranks second after HIV of all infectious agents leading cause of mortality and morbidity due to bacterial infections with HIV taking the first spot. India holds the global burden of TB in one fifth with more than 350,000 deaths each year. Though pulmonary TB (PTB) cases, account for the vast majority of the total TB burden, almost 10-15 per cent of total cases are extra-pulmonary infection.
Methods: Mycobacterium Tuberculosis complex were detected in 50 clinical sputum samples by using Polymerase chain Reaction (PCR) and BacT/Alert.
Results: In our study, 50 sputum clinical samples were taken, out of which 11(22%) were smear positive & 39 (78%) were smear negative. Out of 11 smear positive 10 (90%) were MTB (Mycobacterium Tuberculosis) & 01 (10%) was NTM (Non-Tuberculous mycobacteria) and in 39 smear negative,15 (38.47%) were M. tuberculosis & 02 (5.12%) were NTM and 22 (56.41%) samples were negative by using PCR. By BacT/Alert 3D system, out of 50 clinical samples only 15(30%) samples were positive and 35 (70%) samples were negative for M. tuberculosis complex.
Conclusions: It is concluded that result obtained from our study, Mycobacterium tuberculosis complex was detected by PCR from clinical samples has high specificity (99%) and sensitivity (95%) than BacT/Alert 3D system.

INTRODUCTION

In the world, tuberculosis is ranks second after HIV of all infectious agents leading cause of mortality and morbidity due to bacterial infections. In the group of mycobacteria, is the etiological agent of tuberculosis. TB was declared as a global health emergency by the World Health Organization (WHO) in 1993. Approximately 1.7 million lives per annum, statistics have put TB to claim.[1]

The important step for tuberculosis (TB) control programme worldwide for the primary challenges in curtailing the spread of TB is early diagnosis of tuberculosis (TB).[2] *M. tuberculosis* is estimated as one-third of the overall infection in world's population, with around 9 to 10 million new cases reported annually.[3]

Access this article online	
Website: www.actamedicainternational.com	Quick Response code
DOI: 10.5530ami.2016.1.22	

The global burden of TB, India holds one-fifth with more than 350,000 deaths each year.[3] Almost 10-15 per cent of total cases are extra-pulmonary, though pulmonary TB (PTB) cases, account for the vast majority of the total TB burden.[4]

In India, the major public health problem is Tuberculosis. In India TB develop nearly 2 million people in each year, and due to tuberculosis, estimated that around 330,000 Indians die annually.[5] Due to its high risk of person-to-person transmission, *Mycobacterium tuberculosis* remains a serious public health issue, morbidity and mortality.[6,7]

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Though early diagnosis is mandatory in control of TB, especially for pulmonary TB as it is transmissible, it has remained enigmatic. Although, the conventional acid fast bacilli (AFB) microscopy has high positive predictive value for detection of causative agent *Mycobacterium tuberculosis*, it lacks sensitivity. BacT/Alert 3D system liquid culture considered as a gold standard is labor intensive and slower than molecular detection. Thus, detection of *M. tuberculosis* in clinical samples containing small numbers of the organism is still a major challenge.

MTB complex i.e. *M. tuberculosis*, *M. africanum*, *M. bovis* subsp.*bovis*, M. *bovis* subsp. *caprae*, *M. bovis* subsp. BCG, *M. microti*, *M. canettii* & *M. pinnipedii*.

MATERIALS AND METHODS

Study Design

The study is a 1-year, non-interventional prospective study of 50 suspected patients of tuberculosis had visited the TB chest clinic in TMMC & RC, Moradabad (from December 2014 to November 2015).

Collection of Sputum Specimen

In early morning samples of sputum in a wide-mouth container were collected from the patients.

Z N Staining

Smear was prepared and stained with Ziehl-Neelsen staining and microscopy was done.

Detection of MTB Complex was done using MTBDR plus assay: The Genotype® MTBDR plus assay, a commercially available multiplex PCR DNA strip assay (Hain Lifescience, Nehren, Germany), is designed to simultaneously detect the MTB Complex and using liquid culture media BacT/Alert 3D system (as a gold standard).

Processing of Sample

Procedure for decontamination

The specimens were processed by NALC (N-acetyl-L-cysteine)/NaOH method according to CDC guideline. Two volume of NaOH- NALC solution (0.5%NALC, 4%NaOH, 1.45% Na-Citrate) in sterile test tube the specimen were mixed.

10 volumes of 6.7mM buffer solution of phosphate (PBS-Ph 7.4) added and for 15 min at 30000 Xg the mixture was centrifuged at room temperature.

With PBS it washed twice the pellet and the supernatant was discarded.

The pellet resuspended with 0.5ml of PBS.

DNA (Deoxyribonucleic acid) extraction was directly processed by a 100µl aliquot of the suspension.

DNA Extraction[8]

For DNA extraction, decontaminated clinical specimens were used.

Vortex them & centrifuged for 15 min in 13000 RPM.

Supernatant was discarded by pipette.

Re suspended pellet in 100 µl. molecular water.

Mixed well with vortexing or/& tapping.

Incubated at 95 for 20 min (switched on heat block atleast 1 hr. before).

Incubated in sonicator 15 min. (degas first).

Centrifuged 5 min. in 13000 RPM.

AMPLIFICATION

Amplification mix (45µl) was prepared in a room free from contaminating DNA.

A master mix containing AM-A 10µl and AM-B 35 µl and was added DNA in a separate area.

15min	95^{0}C	1cycle
30sec	95^{0}C	
2min	65^{0}C	20cycle
25sec	95^{0}C	
40sec	50^{0}C	
40sec	70^{0}C	30cycle
8min	70^{0}C	1cycle

Amplification products was stored at -8 to -20C

Hybridization[8]

1. Dispense 20µl of solution of Denaturation (DEN blue) in a corner of each of the wells used.
2. To the solution sample was added of 20 µl of amplified, well mixed the pipette by up and down to and for 5 min it was incubated at room temperature.
3. Carefully added to each well 1ml of pre warmed Hybridization buffer (HYB, green). Gently shake the solution until has a colour of homogenous.
4. In each well a strip was placed.
5. For 30 min at 45^{0}C it was incubated in water bath shaking/Twin cubator by placed.
6. Completely aspirated Hybridization buffer.
7. To each strip added 1ml of Wash Solution of Stringent (SAT, red) and in water bath shaking/Twin cubator for

15 min at 45^{0}C it was incubated.

8. Worked at room temp from this step forwards and completely removed Wash Solution of Stringent.
9. For 1 min it was washed once of each strip with Rinse solution (RIN) of 1 ml on shaking platform/Twin cubator (pour out RIN after incubation).
10. On shaking platform/Twin cubator, to each strip added diluted Conjugate of 1ml and for 30 min it was incubated.
11. In Rinse solution (RIN) of 1ml each strip were washed twice with approx for 1min with and once for 1min and removed the solution. 1ml of distilled water on shaking platform.
12. Diluted substrate of 1ml was added to each strip and it was protected from light without shaking by incubation.
13. Briefly distilled water was rinsed twice, reaction was stop as soon as bands were clearly visible.
14. Between two layers of absorbent paper it made dried by using tweezers removed strip from the try.

BacT/Alert 3D System

Mycobacterium tuberculosis complex was detected from the clinical samples after decontamination, liquid culture (BacT/Alert 3D system) was used as a gold standard for the detection of mycobacterium tuberculosis complex.

The BacT/Alert 3D instrument is the state of the art, automated microbial system. In the presence or absence of micro organism from the clinical samples, BacT/Alert 3D system is used. BacT/Alert 3D system allows to choose the good configuration for laboratory. BacT/Alert 3D bottles are made of unbreakable plastic to protect them from breakage. It also can detect a wide range of micro-organism including bacteria, fungi & yeast.

RESULT AND OBSERVATION

Result for PCR

From research committee after obtaining an institutional ethical clearance the study was conducted with the written consent of the patients in molecular lab of TMMC & RC.

Table 1: Demographic table showing age range and their frequency of distribution

Age range	Frequency	Sex wise distribution of frequency	Percentage
<25	6	Male=4 Female=2	12
26-50	16	Male=10 Female=6	32
51-75	26	Male=22 Female=4	52
>75	2	Male=0 Female=2	4

During the study period, 50 sputum samples were taken. Out of which 11(22%) were smear positive & 39(78%) were smear negative. Out of 11 smear positive, 10(90%) were MTB (Mycobacterium Tuberculosis) & 01(10%) was NTM (Non-Tuberculous Mycobacteria). Out of 39 smear negative, 15(38.47%) were MTB & 02(5.12%) were NTM.

Rests of the 22(56.41%) samples were found negative for M. tuberculosis complex.

Result for BacT/Alert 3D System

Mycobacterium tuberculosis complex was detected from the clinical samples after decontamination, liquid culture (BacT/Alert 3D system) was used as a gold standard.

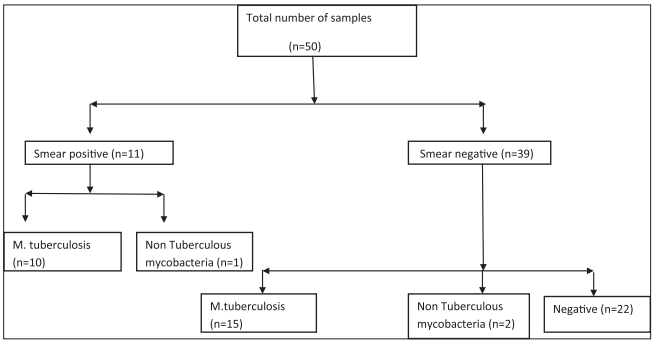

Figure 1: Flowchart to determine Mycobacterium Tuberculosis & Non-Tuberculous Mycobacteria in TMMC & RC

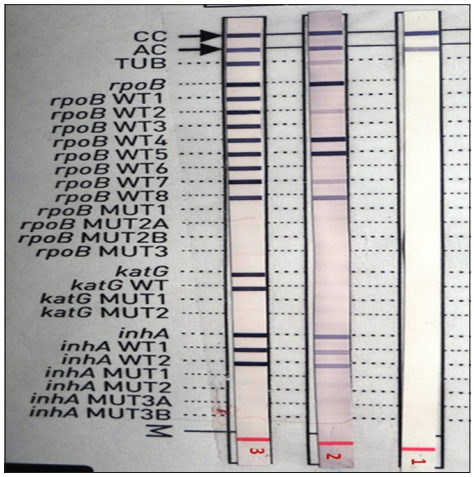

Figure 2: Shows PCR Hybridization strip for detection of Mycobacterium tuberculosis complex from clinical samples. Conjugate control (CC) - CC documents the efficiency of conjugate binding and substrate reaction.Amplification control (AC)- Development of this band excludes mistakes during setup and performance during amplification reaction. M.tuberculosis complex (TUB) - Positive band means member of MTB complex present. Negative TUB Band and Negative Resistant Pattern suggest tested bacterium does not belong to MTB complex, whereas in case of negative TUB band and positive resistant pattern then test should be repeated. Locus controls (rpoB, katG and inhA) - These zone detect a gene region specific for respective locus. Wild type Probes (WT) - When all wild type of a gene probe stain positive then strain tested is sensitive for respected antibiotic. Mutation Probes (MUT) - These probes detect some of the most common resistance mediating mutations.

In 50 sputum samples only 15 (30%) were positive by BacT/Alert 3D system. BacT/Alert 3D system is not differentiating between mycobacterium tuberculosis (TB) and non Tuberculous mycobacteria (NTM). It can only determine the M. tuberculosis complex.

Total samples	BacT/Alert 3D	PCR
N=50	15	28
Tuberculosis	00	25
NTM	00	03

Statistical Analysis

For completeness and consistency checked data were coded into the computer by using Microsoft excel. Then the data were entered and analyzed into the computer.

According to the types of tool used it was expressed in terms of Percentage and presented, descriptive statistics were used to describe the data and results by using tables. No formal statistical hypothesis was tested.

DISCUSSION

Pulmonary Tuberculosis was found more in patients of age group 51-75 years and patients belonging to rural areas rather than from urban areas. In the susceptible individuals, the result of cumulative effects of smoking is the respiratory problems in the association of ageing in the environmental exposure. Our study also found that patients from rural areas were chronic smokers; this might be the reason behind the higher number of patients of Pulmonary T.B. Moradabad area was found to be the thrust area for tuberculosis, because the population which lived in the villages had to come to the cities to earn their livelihood.

Acid-fast staining & microscopy are the main technique for the identification of human tuberculosis in laboratory. On one hand this technique is inexpensive while in other hand this technology has a low sensitivity, & provides no information on the species of mycobacteria causing disease. Mycobacterium tuberculosis complex (MTC) members can be differentiated by conventional laboratory culture & by chemical testing procedures; but it is time-consuming & not practical for surveillance purpose.[9]

Differentiation of MTC members is important in surveillance of public health & appropriation of case management of mycobacterial disease for accurate diagnosis. In most countries, due to MTC specific members accurately & rapidly MTC members can be detected by using PCR method and the total incidence or prevalence are only limited data of mycobacterial disease.[10,11]

MTC members can be detected accurately & rapidly by using PCR method. The PCR method is significantly simpler, faster and gives result within 6 to 8 hours. Whereas in BacT/Alert results were ready in a median time of 11 days (range 7.1-26 days).

In our study, 50 sputum samples were taken. Out of which 11(22%) were smear positive & 39(78%) were smear negative. Out of 11 smear positive, 10(90%) were MTB (Mycobacterium Tuberculosis) & 01(10%) were NTM (Non-Tuberculous Mycobacteria). Out of 39 smear negative, 15 (38.47%) were MTB & 02(5.12%) were NTM. Rests of the 22(56.41%) samples were found negative for M. tuberculosis complex by using PCR.

In BacT/Alert 3D system, out of 50 sputum samples, only 15(30%) were positive by BacT/Alert 3D system. BacT/Alert 3D system is not differentiating between mycobacterium tuberculosis (TB) and non tuberculous mycobacteria (NTM). It can only determine the M. tuberculosis complex.

CONCLUSION

It is concluded that Mycobacterium tuberculosis complex was detected by PCR from clinical samples. On the basis of this study, molecular detection of Mycobacterium tuberculosis complex in the diagnosis of tuberculosis which patients came to the first visit of TB/chest clinic and then early diagnosis was done and also control the spread of the disease. Molecular detection has high specificity (99%) and sensitivity (95%) than BacT/Alert 3D system.

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How to cite this article: Kharibam S, Farooq U, Nudrat S. Molecular detection of Mycobacterium Tuberculosis complex from clinical sputum samples in patients attending tertiary care centre in Uttar Pradesh province of India. Acta Medica International. 2016;3(1):102-106.

Source of Support: Nil, **Conflict of Interest:** None declared.