Research Paper

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Comparative Evaluation of Conventional Techniques; Mgit And Real Time Pcr For The Diagnosis of Mycobacterium Tuberculosis From Various Clinical Samples

Medical Science

KEYWORDS :

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ABSTRACT

The objective of the study was to Isolate & characterize Mycobacterium tuberculosis from pulmonary and extrapulmonary clinical samples & to standardize Real Time PCR for detection of M.tuberculosis in various clinical samples & to study utility of PCR over conventional methods for the diagnosis of tuberculosis. Various diagnostic modalities were also compared for their sensitivity, specificity & turnaround time.

This study comprised of one hundred samples taken from clinically suspected cases of tuberculosis. Specimens from suspected cases were processed for Ziehl-Neelsen (ZN) stain and culture for M. tuberculosis was performed on Lowenstein-Jensen (LJ) medium as well as the BACTEC MGIT 320 system. All the samples were processed for PCR amplification with primers targeting 123 bp fragment of insertion element IS6110 sequence of M. tuberculosis complex using Taq Man chemistry. These methods were then compared for their rate of isolation. Out of 74 pulmonary samples, 32.75% were positive on ZN, 39.65% on LJ, 72.41% on MGIT and 94.82% on Real time PCR. LJ & MGIT negative and smear, LJ & MGIT negative. PCR was found to be 100% specific. None of the conventional methods combine a reasonable sensitivity with time to detection as real time PCR does. Even though these methodologies might be expensive for developing countries, the cost-benefit of this test must be considered. It is less expensive than a prolonged patient stay in the hospital wards, which is common for tuberculosis patients. The most important feature of Real Time detection chemistry is its high sensitivity for smear-negative specimens.

INTRODUCTION

Tuberculosis is an infection with *Mycobacterium tuberculosis, which can occur in any organ of the body,* but it most commonly occurs in the lungs. It has been a scourge throughout history and may have killed more persons than any other microbial pathogen.[1]

As per 2014 annual TB report, published by WHO, there were 9.0 million new TB cases in 2013 and 1.5 million TB deaths (1.1 million among HIV-negative people and 0.4 million among HIV-positive people).

Diagnosis of tuberculosis is a challenging problem especially in case of paucibacillary and extrapulmonary form of tuberculosis. Microscopy is relatively insensitive as at least 5,000 bacilli per millilitre of sputum are required even in expert hands for direct microscopy to be positive.[2] Smear sensitivity is further reduced in patients with extrapulmonary TB, those with HIV-co-infection, and those with disease due to nontuberculous mycobacteria (NTM). During the last two decades, several methods for achieving early growth of *M. tuberculosis* have been developed.[3] Some of the important automated methods are –MGIT /Bac T Alert systems. These have been widely used for drug susceptibility testing and are currently being used as a comparative standards[4]; Growth of mycobacteria in these systems is detected by non-radioactive detection methods system using (Fluorochromes / Colorimetric) methods.[4] Other automated methods include MB/Bact, Septi-Chek and Reporter phages. Nucleic acid amplification tests represent a major advance in the diagnosis of TB. Advanced molecular methods such as polymerase chain reaction (PCR), a type of nucleic acid amplification system, have shown very promising results for early and rapid diagnosis of the disease due to its detection limit of one to ten bacilli in various clinical samples.[5] Various targets have been used for detecting mycobacterial DNA such as IS6110, 65KD heat shock protein, MBP 64, 38 KD proteins and ribosomal RNA.[6]

IS6110-TaqMan assay is a promising auxiliary tool for the diagnosis of TB when used in conjunction with routine laboratory tests, clinical and epidemiological criteria of the patient, thus increasing the sensitivity and specificity of diagnosis.The better sensitivity of IS6110-TaqMan assay could be attributed to the effect of the multicopy target IS*6110*, which is present at 10 to 25 copies in most genomes of MTBC.[7] Introduction of molecular methods presents a major advance in early diagnosis of tuberculosis. The present study was undertaken to assess the diagnostic utility of Real Time PCR by comparing it with MGIT & other conventional diagnostic modalities.

MATERIALS AND METHODS

The present study was conducted in the Department of Microbiology, Govt. Medical College Amritsar. The study comprised of 74 clinical samples which included sputum and Bronchoalveolar lavage were taken from the patients with suspected Tuberculosis, attending Chest & TB Hospital Amritsar.

1. SELECTION OF CASES

1.1) INCLUSION CRITERIA FOR STUDY POPULATION:

Samples from patients with features suggestive of pulmonary tuberculosis whose samples were received with a request from OPD/IPD to determine the possible presence of *Mycobacterium tuberculosis* from Chest and TB Hospital Amritsar.

Patients of any age and gender were included.

1.2) EXLUSION CRITERIA FOR STUDY POPULATION:

Patients who ATT (Anti Tubercular Treatment).

Patients who have taken ATT in the past.

History of the patient was taken which included age, gender, relevant past clinical history & risk factors. All samples were processed by standard procedures[8] and the organisms were identified by direct AFB smears, culture on BACTEC MGIT 320 & LJ medium and Real time PCR. These methods were then compared for their rate of isolation and time for detection.

2. COLLECTION AND PROCESSING OF SAMPLES

Early morning sputum specimens were obtained on forceful coughing in a wide mouth, sterile, screw capped container. BAL samples were centrifuged at 3000 rpm for 30 mins & deposits were used for staining, cultures & other procedures In case of anticipated delays in processing, they were stored at 4°C. The portion of the sample to be processed for PCR was stored at -20°C.

All sputum and BAL samples were decontaminated by using the NALC-NaOH method as recommended by the CDC's (Centre for Disease Control) Public Health Mycobacteriology: A Guide for the Level III Laboratory.[9]

2-3 drops of processed specimen was inoculated on LJ media slant via hand-operated pipette. The inoculated media was incubated first at 37 °C in horizontal position for 48 hours and then in vertical position for 8 weeks. Typical colonies of *Mycobacterium tuberculosis* were rough, crumbly, waxy, non pigmented (buff coloured).

A lyophilized vial of BBL MGIT PANTA antibiotic mixture was reconstituted with 15ml of BACTEC MGIT Growth Supplement. 0.8ml of MGIT Growth Supplement-MGIT PANTA antibiotic mixture was added aseptically into the 7ml MGIT tube.

After that 0.5ml of the digested, decontaminated and concentrated specimen suspension was added to the MGIT tube. Tubes were entered into the instrument by scanning the bar codes. Positive tube's bar code were scanned to extinguish LED light for that tube's station. After that 0.1ml of aliquot was removed from the bottom of the tube and an acid fast and Gram's stain smears were prepared along with culture on blood agar to rule out contaminating bacteria. Contaminated tubes were reprocessed by decontamination/digestion and inoculation into fresh MGIT tubes. If no microorganisms were present on the smear, the tubes were re-entered into the instrument. Negative cultures were removed as "out of protocol" negatives after 42 days.[10]

For Real time PCR the kit used was procured from Mind Biomeds Kit Contents :

Ref.	Type of Reagent	Presentation (for 25 reactions)
1.	DNA Extraction Buffer	2 X 1.5 ml
2.	TB Reaction Mix	1X950µl
3.	PCR Enzyme Mix	1 X 12µl
4.	Molecular Grade Water	1 X 400µl
5.	Internal Control (IC)	1 X 30µl
6.	TB Positive Control	1 X 30µl

PREPARATION OF MASTER MIX

For single reaction

Eppendorf tube covered with aluminium foil was taken, to this tube was added 35µl Reaction Mix, 0.4µl Enzyme Mix and Internal Control 1µl.

Spin win after removing foil for 10 seconds, reapply foil.

DNA EXTRACTION

DNA extraction buffer was thawed before use.

SPUTUM SAMPLE AND BAL

The decontaminated and concentrated sample in MCT (microcentrifuge tube) stored at 4°C was brought to 37°C. The sample was centrifuged at 13000 rpm for 5 mins for sputum and 2 mins for BAL.

The supernatant was discarded. 1ml of normal saline was added to sputum samples , followed by centrifugation at 13,000 rpm for 5mins, the supernatant was discarded. This process was repeated. 100 µl of DNA extraction buffer was added to the deposit, which was then vortexed for few seconds, followed by centrifugation in spin win for few seconds. Incubated at 100°C for 10 mins followed by Centrifugation at 13,000 rpm for 10 mins. Another tube was taken to which supernatant was added which contained the DNA.

DNA AMPLIFICATION

PCR amplification was done in Roche Light Cycler 480 Real Time PCR system using Taq man chemistry.

SETTING UP OF PCR REACTION

1. The system was turned on and the software was opened and New Experiment was set up.
2. In this Dual Color Hydrolysis probe was selected for FAM and VIC/HEX.
3. The reaction volume was set to 40µL.
4. Cycle conditions and acquistion of fluoroscent signals:

Program No.	Acquisition Mode	Cycles	Analysis Mode	Target	Hold
1.	None	1	None	37°C	2mins
2.	None	1	None	94°C	2mins
3. 1st part 2nd part	None Single	40 1	Quantification None	93°C 60°C	15secs 60secs
4.	None	1	None	40°C	30 secs

5 Now to the PCR multiwell plate 36µl of the master mix was added and to this was added 4µl of the extracted sample.

6 Along with each run 40µl of the positive control was put.

7 Immediately plates were sealed properly with the self-adhesive sealing foil. Sealing the plate is crucial to eliminate evaporation at high temperatures.

8 Sample information was entered in the software, wells containing the samples and controls were selected.

9 In system absolute quantification was selected and filter was set to 465-510 nm fs

10 The plate was gently rotated clockwise on flat surface before loading it into the instrument.

11 The multiwell plate was now loaded into the Roche Light Cycler 480 Real Time PCR system and the cycle was run.

12 Once the cycle was completed, analysis was done using software.

13 After analysis report was generated and print outs were taken.

RESULTS

Out of 74 patients included in our study, 52 (70.27 %) patients were male and 22 (29.72%) were females. Out of 74 patients 19 (25.67%) were found to be positive by smear microscopy and 55(74.32%) were found to be negative by smear microscopy.

Amongst 19 cases that were positive by ZN staining in sputum samples, 52.63% (10) patients were of sputum grade 3+, 31.57% (6) were of grade 2+ and 17.34%(3) were of grade 1+. Out of 74 samples, 23(31.08%) samples were positive on culture on LJ medium while 51(68.91%) samples were negative on culture on LJ medium. The earliest time to detection was 16 days and the maximum time taken for detection was 28 days.

Out of 74 suspected cases, 42(56.75%) cases were positive in culture on MGIT while 32(43.24%) cases were negative in culture on MGIT. Earliest growth was detected in 4 days and the maximum time taken for detection was 20 days. Out of the 42 positive cultures, 42 (100%) cultures were identified as *Mycobacterium tuberculosis.*

In 74 suspected cases, 55(74.32%) cases were positive with Real Time PCR while 19(25.67%) cases were negative on Real Time PCR.

Out of the 74 suspected cases, 19 were positive on both ZN staining and LJ medium culture. Out of 100 clinically suspected cases of tuberculosis, 19 were positive on both ZN staining and on culture in MGIT. In addition 32/74 cases, which were negative on smear microscopy, were found to be positive on culture in MGIT. 3 cases which were positive for ZN microscopy were negative on BACTEC MGIT320.

Out of 74 suspected cases of Tuberculosis, 22 cases were positive on both ZN staining and Real Time PCR. In addition 48 cases,

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which came negative on, smear microscopy, were found to be positive on Real Time PCR.

Mycobacterial culture was positive in 42 cases by MGIT and in 23 cases by conventional LJ culture method. The rate of isolation on LJ medium as compared with MGIT was 23/42 (54.76%).

Mycobacterial culture was positive in 23 cases by LJ medium and positivity by Real Time PCR was 55. So the rate of isolation on LJ medium as compared with Real Time PCR was 23/55 (41.81%).

Out of the strains isolated by Real Time PCR, 20/55 (36.36%) strains were isolated exclusively in Real Time PCR whereas 1 strains were isolated only on LJ medium.

Mycobacterial culture was positive in 42 cases by MGIT and in 55 cases by Real Time PCR. The rate of isolation of MGIT as compared with PCR was 42/55 (76.36%).

Out of the 55 strains isolated by Real Time PCR, 20/55 (36.36%) strains were isolated exclusively in Real Time PCR whereas 04/55 (7.27%) strains were isolated only on MGIT, these were negative on real time PCR. Real time PCR (94.82%) showed highest sensitivity amongst various diagnostic modalities followed by MGIT (72.41%), LJ media (39.65%) and ZN staining (32.75%) (Table1).

PCR showed sensitivity of 100% in smear positive, 95.65% in LJ positive, 92.85% in MGIT positive, 100% in both LJ & MGIT positive and 100% in smear, LJ and MGIT positive. Sensitivity of 100% with Real time PCR was seen in smear negative samples but positive by either LJ/MGIT (Table 2). The specificity of Real Time PCR in smear negative, LJ negative, MGIT negative; both LJ & MGIT negative and smear, LJ & MGIT negative. PCR was found to be 100% specific (Table 3).

Table 1: Overall Sensitivity of various tests

	TOTAL SAMPLES	NEGATIVE	POSITIVE	SENSTIVITY (%)
ZN Smear	74	55	19	32.75
LJ media	74	51	23	39.65
MGIT	74	32	42	72.41
PCR	74	19	55	94.82

Table 2: Comparison of Sensitivity of Real Time PCR test with other tests.

TEST/RESULT	PCR POSITIVE	PCR NEGATIVE	SENSTIVITY OF PCR TESTS
Smear Positive (19)	19	0	100
LJ Positive (23)	22	1	95.65
MGIT Positive (42)	39	3	92.85
LJ& MGIT Positive (19)	19	0	100
Smear, LJ & MGIT Positive (13)	13	0	100
Smear negative samples but positive by either LJ/MGIT (6)	6	0	100

Table 3: Comparison of Specificity of PCR tests with other tests :

TEST/RESULT	PCR POSITIVE	PCR NEGATIVE	SPECIFICITY OF PCR TESTS
Smear Negative (55)	36	19	100
LJ Negative (51)	33	18	100
MGIT Negative (32)	16	16	100
LJ & MGIT Negative (29)	13	16	100
Smear, LJ & MGIT negative (29)	13	16	100

DISCUSSION

The objective of the study was to Isolate & characterize *Mycobacterium tuberculosis* from pulmonary samples & to standardize Real Time PCR for detection of *M.tuberculosis* in various clinical samples & to study utility of PCR over conventional methods for the diagnosis of tuberculosis. Various diagnostic modalities were also compared for their sensitivity, specificity & turnaround time.

Several risk factors are also associated with the development of tuberculosis. In this study, risk factors found to be associated with TB were; H/o close contact 80.55%(29/36) were positive for TB, out of 5 patients who were HIV positive 3/5(60%) had HIV-TB coinfection. Among prisoners 3/3 (100%) patients had tuberculosis and 10/18(55.55%) patients gave personal H/o either Smoking/drug abuse/alcoholism . Mohammed *et al.* reported that smoking was not a risk factor for TB in Ethiopia[11], but several authors have described smoking as an established factor in relation to active TB[12,13].

In this study Out of 74 patients 19 (25.67%) were found to be positive by smear microscopy. This is in accordance with Rishi et al who found positivity rate to be 28%. [14] However, our results are not comparable to study by Bunger et al[138] who concluded that 8.3% cases were found to be AFB positive with ZN Microscopy . This could be attributed to the sample size considered by the author with more number of extra pulmonary samples considered as compared to our study. Factors which determined the results in this study may be time of collection, quality and number of samples taken & nature of samples taken.

In this study, the sensitivity, specificity, PPV & NPV of ZN staining was found to be 32.75%, 100%, 100% & 29.09% respectively. On the other hand sensitivity, specificity, PPV & NPV of LJ medium was found to be 39.65%, 100%, 100%, and 31.37% respectively.

In this study, out of 42 culture positive cases for Mycobacteria on BACTEC MGIT 42/42(100%) were identified as Mycobacterium tuberculosis by MPT64 Ag kit with sensitivity of 100%, specificity of 100%, Positive predictive value (PPV) of 100% & Negative Predictive value (NPV) of 100%. It is in accordance with study by Toihir AOS et al[15] who found that the MPT64 antigen kit had excellent sensitivity of 100% and specificity of 100% compared to that of standard biochemical detection. Therefore they concluded that MPT64 antigen kit can be easily used for the rapid identification of *Mycobacterium tuberculosis* complex cultures. The senstivity, specificity, PPV & NPV of BACTEC MGIT 320 was found to be 72.41%, 100%, 100% & 50.00%.

PCR showed the highest sensitivity 94.82% as compared to other tests as also reported by other workers[16] Significant difference was seen in the sensitivities of different tests, the figures being 94.82% for Real Time PCR test, 72.41% for MGIT, 39.65% for LJ culture and 32.75% for ZN staining ($P<0.05$). However, there was no significant difference ($P>0.05$) as far as specificity of different tests was concerned. The sensitivity of PCR test was compared with three different tests i.e., smear examination, L-J culture and MGIT culture result individually as well as in combination.

As is evident, the PCR test was found to be much more sensitive than smear examination, LJ culture or MGIT culture ($p<0.05$). The sensitivity of detection of *M.tuberculosis* in AFB smear positive samples by PCR approached 100%. The sensitivity of PCR tests approached 100% in smear negative samples, with specificity of 100%. On comparing ZN staining with Real Time PCR chi square statistic is 12.0879, the result is significant $p<0.05$.

In this study there was 1 sample, which was positive on LJ medium but was negative on Real Time PCR. However Real Time PCR was able to detect additional 33 cases, which were negative on LJ medium. The sensitivity of Real Time PCR was found to be 95.65% in LJ positive cases. On comparing LJ medium with Real Time PCR Chi square statistic is 10.7302, P value is 0.001054, and result is significant $p <0.05$

Amongst MGIT Positive cases Real Time PCR was negative in 4 cases. However PCR was able to detect additional 23 cases,

which were negative on MGIT. The sensitivity of Real Time PCR in MGIT positive samples approached 92.15% & specificity was 100% in MGIT negative cases. When analyzed with Chi-square, statistics was 24.3316, P value was IE-06, and result was significant p <0.05

There were 4 samples which were AFB smear & PCR negative but were positive on culture this could be due to presence of PCR inhibiting substances in the samples or some operational errors as supported by earlier studies.[17]

Amongst smear negative combined MGIT & Real Time PCR was able to detect 66.6% cases i.e. when both diagnostic modalities are combined the detection rate increased by additional 5%, which may have significant impact on the treatment of these patients.

The high sensitivity for smear-negative specimens seems to be a great improvement for the new system, since smear-negative samples normally represent a major portion (>90%) of clinical specimens sent to the routine laboratory for initial diagnosis or follow-up of mycobacterial infections.

Among smear positive cases the detection rate of Real Time PCR was found to be 100%. This shows that Real Time PCR was able to detect additional 18% cases amongst smear positive cases when compared with BACTEC MGIT. The Chi square statistic is 4.4, the P value is 0.035939; this result was significant at p, 0.05. Sekar et al and Negi et al reported 100% positivity of PCR in the smear positive cases.[18]

In addition it was seen that the sensitivity of Real Time PCR in combined smear, LJ &MGIT positive samples approached 100%. In our study, the overall sensitivity, specificity, PPV & NPV values were 94.82%, 100%, 100% & 84.21% respectively. Yuan et al reported sensitivity, specificity, PPV & NPV of 91.5%, 98.7%, 91.5% & 98.7% respectively. Chandran & Kenneth compared the performance of TaqMan assay by Real Time PCR & found sensitivity, specificity, PPV & NPV for this assay to be 97.2%, 100%, 100% & 97.2%. [19]

Negi et al conducted a study to compare the diagnostic modalities, for diagnosis of tuberculosis of different tests, they found the sensitivity of PCR to be 74.4% & 48.9% for LJ culture.[18] In our study the sensitivity were 94.82% for Real Time PCR test, 39.65% for LJ culture (P<0.05). Thus it can be concluded that by using Real Time PCR the sensitivity increased to 94.82% as compared to 74.4% by conventional PCR. And in our study the difference between sensitivities of Real Time PCR & LJ medium was 55.17% as compared to 25.5% with Conventional PCR & LJ medium.

CONCLUSION

The conventional tests for detection of *M. tuberculosis*, culture, and microscopy are undoubtedly much cheaper than the PCR systems. However, none of these conventional methods combine a reasonable sensitivity with time to detection as real time PCR does. In addition, PCR has the potential to detect MTBC at an earlier phase of growth. Taqman assays are useful for rapid and accurate diagnosis in cases highly suspected of meningitis TB and also for the assessment of antitubercular treatment response in spite of negative results obtained on conventional methods.

In conclusion, the Real Time PCR approach represents an important diagnostic tool for the rapid identification of MTB infection directly on clinical specimens, which might be useful also for the clinical monitoring of antitubercular treatment.

This technology is a significant breakthrough in PCR amplification and amplicon detection compared to conventional detection methods. We suggest that Real Time detection for *Mycobacterium Tuberculosis* is very quick, sensitive and specific method, which can be introduced in clinical microbiology laboratory of tertiary care hospital.

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