Section: Microbiology

Original Research Article

EVALUATION OF IS6110 BASED RT- PCR ASSAY AND SMEAR EXAMINATION BY ZIEHL-NEELSEN STAINING IN COMPARISON TO BACTEC MGIT 960 AUTOMATED CULTURE IN DIAGNOSIS OF SUSPECTED PULMONARY TUBERCULOSIS CASES

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Abstract

Background: Tuberculosis (TB) caused by Mycobacterium tuberculosis complex (MTC) is a major cause of death worldwide. According to Global tuberculosis report 2020 by WHO approximately 10.0 million people worldwide suffered from tuberculosis in the year 2019. Several molecular methods are available today which hold advantage of rapid diagnosis. Our study was proposed to compare the role of Ziehl – Neelsen staining method, BACTEC MGIT 960 automated culture method and IS6110 based Real time PCR assay in diagnosis of clinically suspected pulmonary tuberculosis cases. **Material & Methods:** Total 200 suspected cases of pulmonary tuberculosis from different wards and OPDs of Dr. RML Hospital were included in this study. Samples included were Sputum (n=109), bronchoalveolar lavage (BAL) (n=82) and gastric aspirate (GA)(n=9). The samples were subjected to Ziehl–Neelsen staining, BACTEC MGIT 960 automated culture system, and IS6110 based Real Time PCR assay. **Results:** Out of 200 suspected cases, 32 (16.0%) samples were detected positive byMGIT culture.PCR detected MTC in 43 cases (21.5%). 27 (13.5%) cases detected positive by all the three tests performed including ZN stain, MGIT and PCR. MGIT culture positive was considered gold standard of diagnosis of tuberculosis. All samples positive through by ZN staining (27) were positive by MGIT automated culture system and Real time PCR. Real time PCR detected 11 additional cases than MGIT culture. Sensitivity and specificity of IS6110 based real time PCR was 100% and 93.45 % respectively. ZN staining showed sensitivity of 84.38% and specificity of 100%. **Conclusion:** IS6110 based Real time PCR could be a promising diagnostic tool as it has stood the test of time in present era. Patient care will be lot easier by early diagnosis and treatment.

INTRODUCTION

Tuberculosis (TB) is caused by Mycobacterium tuberculosis complex (MTC). It is one of the old diseases known in the human history, which is still a major cause of death worldwide.[1] According to Global tuberculosis report 2020 by WHO approximately 10.0 million people worldwide suffered from tuberculosis in the year 2019.In the same year there were an estimated 1.2 million TB deaths among HIV negative people and an additional 208 000 deaths among HIV-positive people.[2]

Early diagnosis of tuberculosis is important for the proper management of disease and to prevents transmission. Clinical diagnosis is made according to the signs and symptoms supported by radiographic and laboratory evidence. Microbiological diagnosis includes examination of sample by microscopy, culture and molecular methods.[3] Culture remains gold standard for confirmatory laboratory diagnosis. A positive culture of tuberculosis bacilli confirms the disease. Conventional culture media like Löwenstein–Jensen (LJ) medium takes a minimum of 3 to 6 weeks to give positive result. Commercially available broth culture system (e.g., BACTEC Mycobacterium Growth Indicator Tube [MGIT]) allows detection of most mycobacterial growth as early as 4 to 14 days.[4] Molecular methods like polymerase chain reaction (PCR) are gradually taking the lead in the diagnosis for TB in terms of speed and accuracy. Real time polymerase chain reaction (RT-PCR) assay based on IS6110 is one of

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the most commonly used methods available.[5] In this method insertion sequence IS6110 found in DNA of Mycobacterium tuberculosis complex is amplified and then detected.[6] There are very few studies which have compared staining method, culture and PCR assay. Our study proposes to evaluate Ziehl – Neelsen staining method, BACTEC MGIT 960 automated culture method and IS6110 based RT-PCR assay in respiratory samples from clinically suspected pulmonary tuberculosis cases, suspecting that molecular method will prove to be a better alternative diagnostic tool and thus help reduce the morbidity and mortality of the disease.

MATERIALS AND METHODS

This cross sectional analytical study was conducted in the Department of Microbiology, ABVIMS and Dr. Ram Manohar Lohia Hospital, New Delhi from November 2017 to March 2019. All the clinically suspected cases of pulmonary tuberculosis attending various departments were included in the study. Already diagnosed cases of pulmonary tuberculosis were excluded from the study. The study was approved by the ethical committee of the institute and written informed consent was taken from of the study subjects or their guardians. Sample like, Sputum, bronchoalveolar lavage(BAL) fluid or gastric aspirate fluid were obtained from the patient after taking proper history. All samples were subjected to gross examination. The selected samples (sputum, BAL and GA) were then processed by - Ziehl-Neelsen staining method, BACTEC MGIT 960 automated culture system and IS6110 based Real Time-PCR assay. Grading of sputum smear was done according to RNTCP guidelines. Culture was done by MGIT (Mycobacteria Growth Indicator Tube) (Becton Dickinson, New Jersey, USA) automated culture system. The intensity of fluorescence is detected which indicates positive culture. Extraction procedure for IS6110 based Real time PCR assay was done on 'NucliSENS easyMAG' Nucleic acid extraction system (Biomerieux, Marcy-l'Étoile, France). Extracted Nucleic acid was subjected to Real time PCR amplification by 'Rotor-Gene Q' instrument. (Qiagen, Hilden, Germany). CareTB PCR assay diagnostic test for detection of mycobacterium tuberculosis (By Qiagen, Hilden, Germany) was used. CT (cycle threshold) value was calculated for the sample as per manufacturer's instructions. CT values determined the presence or absence of insertion sequence IS6110 and analyzed for calculation of positive or negative result.

Stastical Analysis

The results were analyzed using Microsoft excel and SPSS (Stastical P Package for Social sciences) package version 21.0 and Epi-info version 3.0 for relevant statistical comparisons. Chi-square test was used to investigate whether distributions of categorical variables differ from one another.

RESULTS

Total 200 clinically suspected cases of pulmonary tuberculosis were included in this study. Out of total 200 cases,109 samples were sputum (54.5 %) , 82 samples were BAL (41.0 %) and 9 samples were Gastric aspirate (4.5%) .Majority cases were observed in the age group of 41- 60 yrs (43.5%) with a median age of 45 years. Minimum age of the patient was 6 years and maximum was 90 years. In this study most of the cases presented with cough 59.5%, followed by fever contributing 31.0% Other features like weight loss and hemoptysis were found in (7.0%) and (2.5%) respectively.

Twenty-seven cases were detected positive by all 3 test methods. Real time PCR was able to detect additional cases when compared to other two tests [Table 1].

The distribution of test results was compared between Real time PCR, MGIT and ZN staining using the Chi-square test. There was a significant difference in the distribution of test results between PCR, MGIT and ZN staining. (p value < 0.05). MGIT could detect 16% cases, while PCR could detect 21.5% cases and ZN smear could only detect 13.5% cases [Table 2].

In this study, 43 (21.5%) cases were found positive through Real Time PCR and 27 (13.5%) cases were detected positive by ZN staining. There was a significant difference in the distribution of test results between PCR and ZN staining. Positive results were significantly more with PCR [Table 3].

Comparison of Real Time PCR to gold standard (i.e. MGIT) showed a sensitivity of 100 %(95% CI=89.11%-100.00%) specificity of 93.45 %(95% CI=88.59%-96.69%) Positive Predictive Value was found to be 74.42 %(95% CI=62.16%-83.74%) Negative Predictive Value was 100.00 %(95% CI=89.11%-100.00%) accuracy was 94.50 %. (95% CI=90.37%-97.22%) [Table 4].

Comparison of ZN staining to gold standard (i.e. MGIT) showed a sensitivity of 84.38 % (95% CI=67.21%-94.72%) specificity of 100% (95% CI=97.83%-100.00%) Positive Predictive Value to be 100 %(95% CI=97.83%-100.00%) Negative Predictive Value to be 97.11% (95% CI=93.76%-98.69%) and Accuracy to be 97.50 %. (95% CI=94.26%-99.18%) [Table 5].

Table 1:

	POSITIVE CASES
All 3 tests (IS6110 based RT -PCR + MGIT + ZN staining)	27
IS6110 based RT -PCR + MGIT	32
IS6110 based RT -PCR	43
MGIT	32

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ZN staining	27

Table 2:

	Real Time PCR		MGIT		ZN STAINING	
	No.	Percent	No.	Percent	No.	Percent
Positive	43	21.5%	32	16.0%	27	13.5%
Negative	157	78.5%	168	84.0%	173	86.5%
Total	200	100.0%	200	100.0%	200	100.0%
Chi-square value = 4.748, p-value = 0.049*						

Table 3:

	Real Time PCR		ZN STAINING	
	No.	Percent	No.	Percent
Negative	157	78.5%	173	86.5%
Positive	43	21.5%	27	13.5%
Total	200	100.0%	200	100.0%
Chi-square value = 4.433, p-value = 0.035*				

Table 4:

	Test result by MGIT (Gold Standard)	
Test result by PCR	Positive	Negative
Positive (43)	32	11
Negative(157)	0	157

Statistic	**Value**	**95% CI**
Sensitivity	100.00%	89.11%-100.00%
Specificity	93.45 %	88.59%-96.69%
Positive Predictive Value	74.42%	62.16%-83.74%
Negative Predictive Value	100.00%	89.11%-100.00%
Accuracy	94.50%	90.37%-97.22%

Table 5:

	Test results by MGIT (Gold Standard)	
Test result by ZN staining	**Present**	**Absent**
Positive (27)	27	0
Negative (173)	5	168
Statistic	**Value**	**95% CI**
Sensitivity	84.38%	67.21%-94.72%
Specificity	100.00 %	97.83%-100.00%
Positive Predictive Value	100.00 %	97.83%-100.00%
Negative Predictive Value	97.11%	93.76%-98.69%
Accuracy	97.50%	94.26%-99.18%

DISCUSSION

Diagnostic methods commonly used in laboratories for detection of Mycobacterial infections are microscopy using Ziehl-Neelsen staining and culture on solid and liquid medium. [7,8]

Microscopic examination is cost effective, rapid and easy to perform and does not require an elaborate setup; but its major drawback is subjectivity and poor sensitivity in paucibacillary cases.[9]

Various types of PCR assays have potential of alternative diagnostic tool for the detection of tuberculosis. [13–22] The sensitivity of PCR is reported to be 90–100% in ZN stain and culture positive samples and approximately 70% in ZN stain and culture negative samples, as compared to that of microscopy which is around 60% sensitive in culture positive samples.[23]

In this study, we compared ZN microscopy and IS6110 based Real time PCR method with automated liquid culture MGIT 960.

Out of the 200 samples, 27 (13.5%) samples were positive by all the three tests. Thirty-two (16.0%) samples were positive by culture as well as PCR. Eleven (5.5%) samples were positive only by Real time PCR. All samples positive by ZN staining were also positive by MGIT automated culture system.

IS6110 is the most widely used target DNA sequence found in repeat copies at different locations in the genome of the microorganisms belonging to the M. tuberculosis complex. [10,11,12] It has been assumed that this restriction resulted due to lack of genetic exchange with other mycobacterial species. A benefit of this exclusivity is that IS6110 has become an important diagnostic sequence in the differentiation of MTBC species from other mycobacteria.

We conducted IS6110 based Real time PCR assay which was additionally able to detect 5.5% cases which were negative by culture as well as microscopy. Result shows 100% sensitivity and 93.45% specificity for PCR assay. A study conducted by Dayal et al,[24] in India conducted a study on both pulmonary and extra pulmonary cases in paediatric population, and showed IS6110 based PCR had sensitivity of 69.01% and 100 % specificity. Siddiqui et al,[25] parameters in extra pulmonary samples with

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sensitivity and specificity of IS6110 based PCR as 70% and 100% respectively. Our study showed higher sensitivity of RT-PCR but similar specificity as seen in other Indian studies. There have been several studies globally using IS6110 PCR assay which are in concordance to our results. Aslanzadeha et al,[26] from USA stated the overall sensitivity and specificity of PCR in pulmonary and extra pulmonary cases to be 100% each. Kahla et al,[27] from Tunisia stated sensitivity and specificity of PCR in pulmonary samples to be 93.8% and 98.6%. In Kyoto, Japan, sensitivity and specificity of PCR was 85.7% and 98%, respectively in sputum samples.[28] These values are very close to the present study. Our study revealed 100 % PCR sensitivity in the MGIT culture positive cases. According to Sylvie Armand et al in France IS6110 based real time PCR assay showed sensitivity of 84% in culture positive respiratory samples. This difference in sensitivity could be because our study included a variety of samples which were subjected to MGIT in comparison to their study where both solid culture and BactAlert were used simultaneously.[29] There have been various studies which have targeted multiple genomic sequences of MTBC by PCR and have concluded as having IS6110 better sensitivity and specificity compared to 38kDa, 65kDa and 85B and MPB64.[30,31,32]We conclude that IS6110 based Real time PCR assay is an effective diagnostic test as far as pulmonary samples are concerned.PCR can be considered as an important diagnostic tool in paucibacillary cases and also in those patients who are not able to expectorate a proper sputum sample.

In this study we took MGIT as gold standard for comparing the results of PCR and ZN staining. We cannot do away with the role of ZN staining in the diagnosis of tuberculosis as this is the more commonly used method in all the laboratories and has contributed to the detection of most of the cases. By ZN staining 13.5% cases were detected which were also positive by culture and PCR. We have calculated 84.38% sensitivity and 100% Specificity for ZN staining. These results are comparable to a study from Tunisia.[27] However two studies from India have shown very low sensitivity of ZN staining (9.4% in paediatric population [24] and 5% in extra pulmonary samples,[25] respectively), though the specificity was same as ours.[25] Our study result showed higher sensitivity, may be because two consecutive sputum samples of each patient were subjected to ZN smear and centrifugation of BAL and Gastric Aspirate was done before staining. The sensitivity could also vary due to inclusion of wider age group in our study.

In our study, Real time PCR showed higher sensitivity (100%) compared to ZN staining (84.38%) and specificity of 93.45% compare to 100% in ZN staining. Studies from Tunisia and Nigeria have shown similar results. [27,33] Siddiqui et al,[25] from India also concluded that IS6110 PCR is more sensitive than ZN staining.

Though microscopy and culture still remain the basic useful tools for diagnosis of tuberculosis in resource constrained regions, Real time PCR can serve as a useful alternative tool when used in conjunction with conventional diagnostic methods for rapid and accurate diagnosis of Pulmonary TB.

Tuberculosis has been a great social burden in our country and drug resistance in TB has added even more economic pressure on our health policies. This necessitates continuous monitoring and active surveillance in tuberculosis patient. Early diagnosis can lead to early treatment which reduces morbidity and mortality up to a significant level. As IS6110 based Real time PCR could be a promising diagnostic tool as it has stood the test in present era, in terms of time management and accuracy to detect the disease, so that patient care is lot easier at early diagnosis as well as prevention of transmission of the disease.

CONCLUSION

PCR alone was able to diagnose additional 5.5% cases. This is because of its high sensitivity and specificity. MGIT takes longer time (2-6 weeks) and requires proper lab containment facilities where it is not available in all of the diagnostic centres. The positive result of MGIT cultures is to be further confirmed by examining ZN smear which adds to time while, result of RT-PCR are available on the same day within few hours and does not require any additional test for final result but culture remains gold standard and provides information about drug susceptibility as well. The result of present study could be authenticated by conducting further studies involving even larger number of cases including extra pulmonary samples. The study concludes that intensive clinical evaluation is most important to diagnose pulmonary TB. PCR can be considered alternative diagnostic tool in areas with high prevalence of cases like in India as it complements the conventional bacteriological battery of tests due to its rapidity, high sensitivity and specificity. However further studies involving comparison of battery of tests are the need of the hour.

Conflicts of Interest

The authors have none to declare.

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