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Comparative Study of Fast Plaque Assay and Real Time PCR for Detection of *Mycobacterium tuberculosis* in Pulmonary Samples

Priya Thapliyal[1], Pallavi Dheer[2], Satish Chandra Nautiyal[3], Rajesh Rayal[4], Rakesh Rai[5] and Indra Rautela[6]*

[1]*Department of Biochemistry, H.N.B. Garhwal (A Central) University, Srinagar-246174, Uttarakhand, India.*
[2]*Department of Biotechnology, School of Basic and Applied Sciences, Shri Guru Ram Rai University, Patel Nagar, Dehradun, Uttarakhand, India.*
[3]*Central Molecular Research Laboratory, Department of Biochemistry, Shri Guru Ram Rai University, Patel Nagar, Dehradun, Uttarakhand, India.*
[4]*Department of Zoology, School of Basic and Applied Sciences, Shri Guru Ram Rai University, Dehradun, Uttarakhand, India.*
[5]*Department of Zoology, Buddh Snatkottar Mahavidyalay, Kushi Nagar, Uttar Pradesh, India.*
[6]*Department of Biotechnology, School of Applied and Life Sciences, Uttaranchal University, Dehradun, Uttarakhand, India.*
**Corresponding author: rautela.indra7@gmail.com*

ABSTRACT

Tuberculosis has become the cause of major diseases burden in developing countries such as India. Its etiological agent is a bacterium called *Mycobacterium tuberculosis* (MTB). This calls for the need for a rapid and accurate method of diagnosis as TB detection if not diagnosed timely can be proved to be fatal. In the present study, 90 pulmonary samples were taken into the study and a comparative study on the conventional method of Fast Plaque Assay (FPA) and Real-Time PCR (RT-PCR) was performed to find out a more accurate and reliable method of diagnosis. Of all the samples 18 samples were only diagnosed by RT-PCR but failed to be diagnosed by FPA which proves RT-PCR to be a more sensitive, rapid, reliable, and accurate method of diagnosis. Moreover, the percentage positivity rate of total samples again revealed that RT-PCR (44.4%) is more efficient than FPA (13.33%).

Keywords: Tuberculosis, *Mycobacterium tuberculosis*, Mycobacteriophage, Fast Plaque Assay, Real-time PCR

INTRODUCTION

Tuberculosis (TB) is a serious public health problem and it is estimated by the World Health Organization (WHO) that approximately one-third of the world's population is infected by *Mycobacterium tuberculosis* (MBT) (Marei et al., 2003) because of the interaction between TB and HIV/AIDS epidemic, the TB cases are increasing substantially worldwide. Tuberculosis leads to more kills in adults in India than any other infectious disease thus compounding to the economic loss of the country. Nearly, 9 million people suffer from this disease every year and approximately 2 million die as a result (Barman, et al., 2007). TB causes death in more than 1,000 people a day or one in every minute in our country due to the high burden and the least prospects of favorable time trend of tuberculosis (TB) as of now, a problem that is further compounded by limited resources (Katiyar, et al., 2009). Moreover, studies have shown that during surgery of spinal TB for prevalence, pulmonary TB has come up with major concern and risk factor (Wang, et al., 2020). For the direct detection of *Mycobacterium tuberculosis* complex (MTC) in clinical specimens, various nucleic acid amplification (NAA)

tests have been used in favor of a more rapid pathogenic diagnosis (Chen, et al., 2012). The rapid sensitive and specific identification of the causative agent is necessary for the control of TB and the development of rapid methods (high-performance liquid chromatography, thin-layer chromatography, RNA sequencing and polymerase chain reaction (PCR), nucleic acid sequence-based amplification assay (NASBA), Transcription mediated assay (TMA) and Ligase chain reaction (LCR) have made faster diagnosis and treatment possible (Wattal, 2002).

Mycobacteriophage is used in the Fast Plaque technique which infects and replicates in slow-growing pathogenic strains e.g M.tb, *M. ulcerance* and also in some of the growing strains as *M. smegmatis*. In this technique, viable target bacilli are infected by adding phages in the decontaminated sputum samples. The addition of potent virucide leads to the destruction of bacteriophages outside the target cells and they do not harm phages inside the bacilli. The phages inside the tubercular bacilli replicate inside and cause bacterial cell lysis in order to release the progeny bacteriophage (Barman, et al., 2007).

The use of RT-PCR for TB diagnosis has increased due to its high sensitivity and specificity towards the direct (from clinical samples) and indirect (from culture isolates) detection of the Mycobacterium tuberculosis complex (MTBC) (Broccolo, et al., 2003; Armand, et al., 2011; Lira, et al., 2013). Many mycobacterial genome regions, such as IS6110, 16S rRNA, hsp65, rpoB, sdaA, devR and mpt64 (Lachnik, et al., 2002; Kim, et al., 2004; Chakravorty, et al., 2005; Negi, et al., 2007; Hwang, et al., 2013) have been used in molecular diagnosis of TB. The genome of MTBC species contains a single copy of mpt64, which is mostly used for the diagnosis of both pulmonary and extrapulmonary TB (Martins, et al., 2000; Bhanu, et al., 2005; Takahashi and Nakayama, 2006; Kusum, et al., 2012). Real-time PCR is a closed-tube, fluorescence-based, online and end-point detection technique that uses automated thermocyclers and fluorimeters and hence is a rapid technique having low rates of contamination. There are two common methods of detection in RT-PCR which are either based on detection of non-specific fluorescent dyes which intercalate any dsDNA or detection of sequence-specific DNA probes which consist of oligonucleotides labeled with a fluorescent reporter (Khadka and Kharel, 2019). Also, PCR techniques are known for detecting Multiple Drug Resistant (MDR) TB, by targeting GC-motive rich PE/PPE gene family which has conserved N-terminal vs. variable C-terminal sequences. Specific primers and probes are being designed to serve this purpose such as Rv0050 and Rv3633 which targets 56 and 44 bp gene fragments (Sinkov, et al., 2020). The present study aims to determine the sensitivity of the Real-Time PCR in comparison to FAST Plaque TB assay for detection of *Mycobacterium tuberculosis* in the pulmonary sample. Overall 90 samples of different age group patients were selected for the present study.

MATERIAL AND METHODS

Sample Collection

90 pulmonary samples were collected from patients of different age groups including both male and female, attending pulmonary of Shri Mahant Indresh Hospital, Dehradun, Uttarakhand and further analysis of research work was done in Central Molecular Research Laboratory Department of Biochemistry, Shri Guru Ram Rai Institute of Medical & Health Sciences, Dehradun, Uttarakhand.

Fast Plaque TB Analysis

FAST Plaque TB is based on the FAST Plaque principle and utilizes Mycobacteriophage to reflect the presence of viable MTB within a sputum specimen (pulmonary sample) and the whole process is carried out by using Fast Plaque TB kit. Firstly, the sputum specimen is decontaminated and concentrated by the addition of FPTB Medium Plus (FPTB Growth Supplement + FPTB Medium) which killed most of the bacteria present in the specimen other than the target mycobacteria. The remaining bacteriophage are protected within viable target mycobacteria which continue to replicate until new progeny phage are released as the cells break open (lyse) and are then amplified by the introduction of Sensor cells followed by the addition of FPTB Medium Plus to neutralize the excess of virucide. Clear areas (plaques) in a lawn of confluent growth of Sensor cells indicate progeny bacteriophage. The number of plaques generated from a given sample is related to the number of viable MTB cells containing mycobacteriophage.

Real-Time PCR Analysis

The sample was concentrated by centrifugation to increase the bacterial load and then DNA is isolated. The primers and probe were designed, based on the sequence homology of the insertion sequence 6110 (IS6110) for all species of the *Mycobacterium tuberculosis* complex (MTBC). The highly conserved region of 123 base pair was chosen as the target region for primer amplification. 20 µl of master mix containing 5 µl of the DNA samples were added in different RT-PCR tubes which were placed in the thermocycler and programmed for MTB. In stage I; the Initialization step consists of heating the reaction to a temperature of 94–96 °C which is held for 1–9 minutes. Heating the reaction to 94–98 °C for 20–30 seconds causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules marks the denaturation

step. The reaction temperature is lowered to 50–65 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. In the Extension/ elongation step, the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by Adding dNTPs that are complementary to the template in 5’ to 3’ direction, condensing the 5’-phosphate group of the dNTPs with the 3’-hydroxyl group at the end of the nascent (extending) DNA strand at temperature 75–80 °C. Final elongation was performed at a temperature of 70–74 °C for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended and a final hold is given at 4–15°C. In stage II; Again Denaturation step is performed at 95°C for 10 seconds followed by Annealing reaction at a temperature 60°C for 1 min. The denaturation reaction temperature is again increased up to 95°C for 15 seconds and Annealing is performed at a temperature of 60°C for 15 seconds. The real-time PCR results are interpreted on the basis of MELT CURVE for detecting mycobacterium.

RESULT AND DISCUSSION

TB samples were collected from those patients who were showing positive symptoms of Tuberculosis and had a positive result on the diagnosis. There were total 90 pulmonary samples and the sample type collected were Bronchial alveolar lavage (BAL), Pleural Fluid (PF), Sputum and Lung Tissue and among the patients, 49 were males and 41 were females as shown in Table 1. There were 40 positive results when diagnosed through Real-Time PCR and 12 positive results when diagnosed through the Fast Plaque Assay Technique.

Percentage positivity of total samples through RT-PCR is 44.4% and of FPA is 13.33%. Percentage positivity of female patients is 14.6% of 6 positive samples out of 41 total female samples (Figure 1), whereas percentage positivity of male patients is 12% of 6 positive samples out of 49 samples in FPA detection. Percentage positivity of female patients is 43.9% of 18 positive samples out of 41 total female samples, whereas the percentage positivity of male patients is 44.8% of 22 positive samples out of 49 samples in RT-PCR detection (Figure 2). The percentage positivity rate of different samples; Lung tissue (85%), sputum (64%), BAL (52.1%), Pleural fluid (40.85%) as per RT-PCR detection and percentage positivity rate of different samples; Lung tissue (21.4%), sputum (17.8%), BAL (8.6%), Pleural fluid (8%) as per FPA (Figure 3 and Figure 4), respectively. Percentage positive rate of patients aged between 41–50 years are more prone to tuberculosis disease as their percentage positivity is 60% as illustrated in Figure 5.

Table 1: Interpretation for pulmonary samples

Serial no	Age (in years)/ Gender	Sample Type	RT-PCR result	FPA result
1.	25/M	Bal	+ve	-ve
2.	44/M	Pf	-ve	-ve
3.	34/F	Sputum	+ve	+ve
4.	50/M	Pf	+ve	-ve
5.	59/M	Bal	-ve	-ve
6.	35/M	Bal	-ve	-ve
7.	42/F	Lung Tissue	-ve	-ve
8.	41/F	Sputum	+ve	-ve
9.	39/M	Pf	+ve	-ve
10.	33/F	Sputum	-ve	-ve
11.	47/M	Lung Tissue	-ve	-ve
12.	56/M	Pf	+ve	+ve
13.	34/M	Pf	-ve	-ve
14.	30/F	Bal	-ve	-ve
15.	29/F	Sputum	-ve	-ve
16.	30/M	Sputum	-ve	-ve
17.	30/F	Sputum	-ve	-ve
18.	56/M	Bal	+ve	-ve
19.	45/M	Pf	+ve	+ve
20.	44/F	Lung Tissue	+ve	+ve
21.	26/M	Bal	-ve	-ve
22.	60/M	Bal	-ve	-ve
23.	62/M	Bal	-ve	-ve
24.	58/F	Bal	+ve	-ve
25.	49/F	Pf	-ve	-ve
26.	62/M	Bal	+ve	-ve
27.	30/M	Sputum	-ve	-ve
28.	46/M	Lung Tissue	+ve	-ve
29.	38/M	Pf	-ve	-ve
30.	67/F	Sputum	+ve	+ve
31.	69/M	Pf	-ve	-ve
32.	30/M	Sputum	-ve	-ve
33.	58/F	Sputum	-ve	-ve
34.	34/M	Lung Tissue	+ve	+ve
35.	41/F	Pf	-ve	-ve
36.	40/F	Pf	-ve	-ve
37.	49/M	Bal	+ve	-ve
38.	42/M	Bal	+ve	-ve
39.	46/F	Sputum	-ve	-ve
40.	43/F	Bal	+ve	-ve

Serial no	Age (in years)/ Gender	Sample Type	RT-PCR result	FPA result
41.	58/M	Sputum	-ve	-ve
42.	59/F	Pf	+ve	-ve
43.	71/M	Lung Tissue	-ve	-ve
44.	49/M	Lung Tissue	-ve	-ve
45.	58/M	Sputum	+ve	+ve
46.	47/F	Pf	+ve	-ve
47.	67/F	Pf	+ve	-ve
48.	26/F	Pf	-ve	-ve
49.	36/M	Sputum	+ve	-ve
50.	40/M	Lung Tissue	+ve	+ve
51.	40/M	Pf	-ve	-ve
52.	58/F	Sputum	-ve	-ve
53.	48/F	Lung Tissue	-ve	-ve
54.	78/F	Pf	+ve	-ve
55.	55/M	Pf	-ve	-ve
56.	34/F	Bal	-ve	-ve
57.	30/M	Bal	-ve	-ve
58.	66/F	Lung Tissue	-ve	-ve
59.	31/F	Sputum	+ve	+ve
60.	77/M	Pf	-ve	-ve
61.	41/F	Bal	+ve	-ve
62.	30/M	Lung Tissue	-ve	-ve
63.	56/M	Pf	-ve	-ve
64.	62/M	Sputum	+ve	-ve
65.	71/F	Bal	-ve	-ve
66.	67/F	Pf	-ve	-ve
67.	61/M	Bal	+ve	-ve
68.	45/F	Bal	+ve	-ve
69.	33/F	Lung Tissue	-ve	-ve
70.	45/M	Sputum	+ve	-ve
71.	56/F	Pf	-ve	-ve
72.	79/M	Lung Tissue	+ve	+ve
73.	49/F	Pf	-ve	-ve
74.	44/M	Sputum	-ve	-ve
75.	80/F	Sputum	+ve	-ve
76.	55/M	Sputum	-ve	-ve
77.	47/F	Pf	-ve	-ve
78.	44/M	Bal	+ve	-ve
79.	56/F	Sputum	-ve	-ve
80.	32/F	Sputum	+ve	-ve
81.	68/M	Lung Tissue	-ve	-ve
82.	30/F	Sputum	-ve	-ve
83.	22/M	Bal	+ve	+ve
84.	66/F	Bal	+ve	-ve
85.	28/M	Sputum	-ve	-ve
86.	67/F	Bal	-ve	-ve
87.	55/M	Pf	+ve	-ve
88.	46/M	Sputum	-ve	-ve
89.	76/F	Bal	+ve	-ve
90.	34/M	Sputum	+ve	+ve

Total no. of samples= 90; No. of positive samples through RT-PCR=40; No. of positive samples through FPA=12

Fast and accurate diagnosis is very important when it comes to tuberculosis diagnosis. A better technique for diagnosis can be rapid, accurate and highly sensitive. RT-PCR has been proven to be the more useful molecular technique to diagnose pulmonary and extra-pulmonary TB due to the efficient processing of fluid and tissue samples of patients (Mangayarkarasi, et al., 2019). The Positivity rate in pulmonary samples is much higher in the case of Real-Time PCR Technique which is 44.44% when compared to Fast Plaque Assay which is 13.33% which tells that the Real-Time PCR Technique is a much better technique than Fast Plaque Assay.

The Positivity rate in pulmonary samples like Bronchoalveolar Lavage, Sputum, Pleural Fluid, Lung Tissue, etc. is much higher in the case of Real-Time PCR rather than Fast Plaque Assay. The positivity rate in the Real-Time PCR technique is 44.8 in males and 12 in females whereas the positivity rate in Fast Plaque Assay is 43.9 in males and 14.6 in females which complies that both male and female are nearly equal prone to get TB.

The Lung Tissue sample showed the highest positive result with a positivity rate of 85% from RT-PCR and 17.8% from FPA Technique whereas Pleural Fluid was the least positive sample with a positivity rate of 40% when diagnosed through RT-PCR and 8% when diagnosed through FPA (Table 2).

The positive rate of different samples for TB diagnosis through RT-PCR (Figure 3) clearly indicates that Lung Tissue shows the highest positivity of suffering from TB i.e. samples of Lung Tissue give more positive results rather than other samples like BAL, Pleural Fluid and Sputum. Likewise, Pleural Fluid shows the least positive result for TB disease diagnosis than other samples.

Table 2: Positivity rate on the basis of sample type for FPA and RT-PCR

Serial no	Sample type	Total no. of samples	RT-PCR positive	FPA positive	Positivity rate of RT-PCR (%)	Positivity rate of FPA (%)
1.	Bal	23	12	2	52.1	8.6
2.	Pf	25	10	2	40	8
3.	Lung Tissue	14	12	3	85	21.4
4.	Sputum	28	18	5	64	17.8

Positive rate of different samples for TB diagnosis through FPA (Figure 4) clearly shows that Lung Tissue provides the highest positivity of suffering from TB similarly as was seen in RT-PCR TB diagnosis of different samples (Figure 3) and Pleural Fluid shows the least positive result for TB disease diagnosis than other samples which was a similar case in RT-PCR.

Middle-aged patients (41-50 years) are highly prone to get TB with a positivity rate of 60% when diagnosed through RT-PCR and old patients (71-80 years) are least likely to suffer from TB with a positivity rate of 2% when diagnosed through FPA.

Table 3: Age-wise distribution of positive samples

Serial no	Age (yrs)	Total no. of samples	RT-PCR positive	FPA positive	Positivity rate of RT-PCR (%)	Positivity rate of FPA (%)
1.	21-30	6	3	1	50	16
2.	31-40	21	9	5	42	23
3.	41-50	25	15	2	60	8
4.	51-60	14	6	2	42	14
5.	61-70	19	5	1	26	5
6.	71-80	5	2	1	40	2

RT-PCR provides high positivity rate than FP. Moreover, pulmonary samples of patients aged between 41-50 years are more prone to Tuberculosis as their positivity rate is highest which is 60% when diagnosed by RT-PCR (Figure 5).

18 samples out of 90 samples were such which gave negative results when diagnosed through Fast Plaque Assay but showed positive results when diagnosed through Real-Time PCR Technique (Table 4). FPA results are available in 48 hours whereas RT PCR results are available within 24 hours so we can conclude that RT PCR can render results much earlier than FPA. FPA detects only live bacilli whereas RT-PCR can detect both live as well as dead bacilli. FPA can detect tuberculosis only in samples having bacterial copy number >100/ml while RT-PCR can detect even 10 bacilli /ml of the sample. RT-PCR technique is a more sensitive method for the detection of tuberculosis.

Table 4: Comparison of FPTB results with RT-PCR

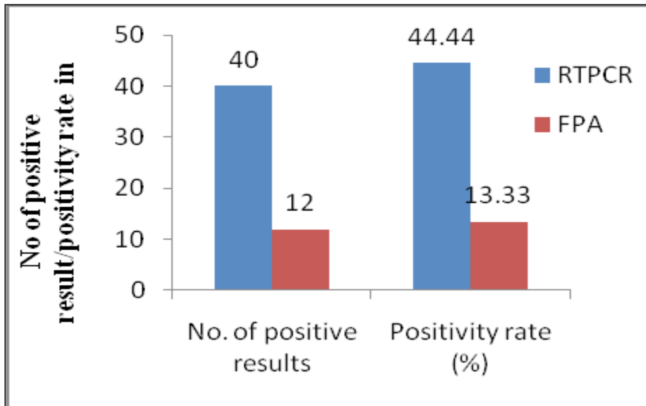
	RT-PCR +ve	RT-PCR -ve	Total
FPTB +ve	12	0	12
FPTB -ve	18	50	68
Total	40	50	90

Real-time PCR is the more reliable, sensitive, fast and accurate technique when compared to Fast Plaque Assay, as FPA is conducted manually so there is a possibility of not getting the accurate results due to contamination and mishandling of the sample, whereas in RT-PCR no such possibilities are there because the sample is processed by the instrument itself.

Recently developed and used RT-PCR is much more efficient as it has the ability to apply smear microscopy, culture, and conventional PCR tests with the help of the single sample aliquot and it is efficient and sturdy to provide the scarce amounts of tissue and the proteinaceous nature of pleural fluid (Mangayarkarasi, et al., 2019). Early detection of TB might not be specific but recently developed genetic markers which are based on IS6110 sequence is very specific for *M. tuberculosis*, although there have been some conflicts that there are some strains of *M. tuberculosis* which lack such sequence (Rahman, et al., 2020).

TB detection need a fast and accurate approach in order to treat patient efficiently and quickly but this becomes a major problem if TB symptoms are not diagnosed properly or undiagnosed or even more hazardous, diagnosed lately. Such conditions have been proved to be fatal in the case of MDR TB, in which timely diagnosis has become the major concern for doctors and health workers. Many other fine and accurate technique has come into play naming MTBDRplus, loop-mediated isothermal amplification (LAMP), line probe assay (LPA), GeneXpert, whole-genome sequencing (WGS) and next-generation sequencing (NGS) (Acharya, et al., 2020). Besides all this, RT-PCR is still used for the diagnosis MDR TB but it is also known as a resource-limited setting with some technical difficulties and other logistics which are needed to be improved for its better functioning (Babafemi, et al., 2019). PCR which might has developed some drawbacks during this period of urgency but most of the diagnosis is

based on RT-PCR (Dheda, et al., 2020). In India, more than 21 domestic manufacturers are involved in the production of RT-PCR reagent and standard RT-PCR kits to meet its increasing demand with every passing day (Singh, 2020).



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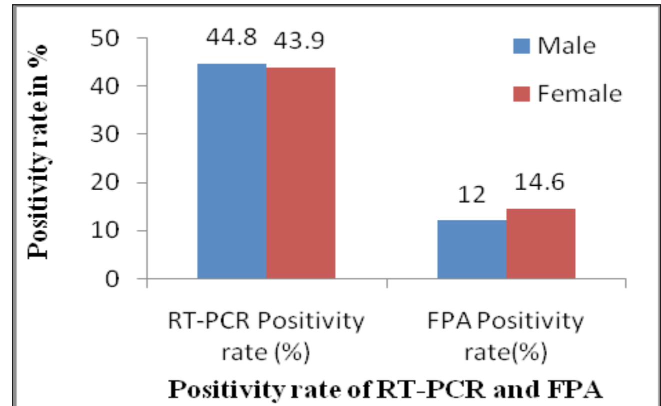

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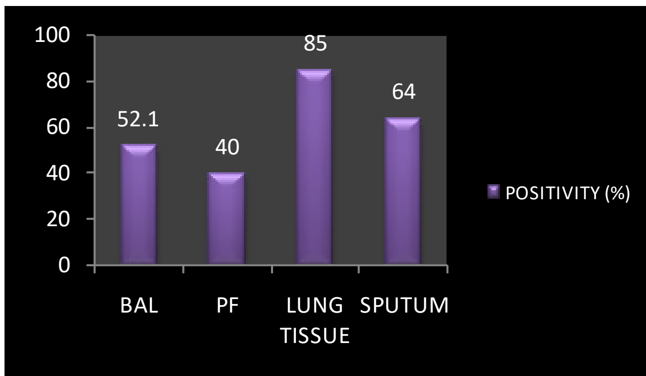

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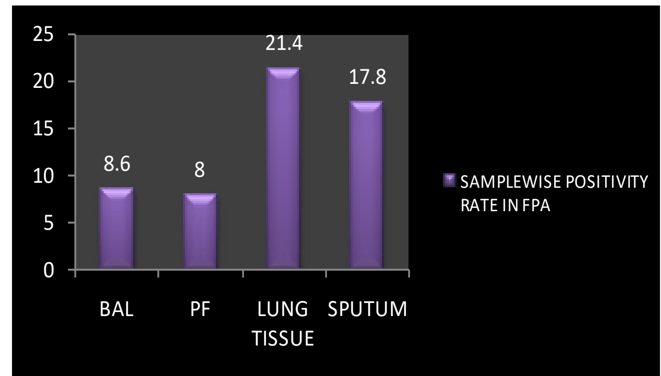

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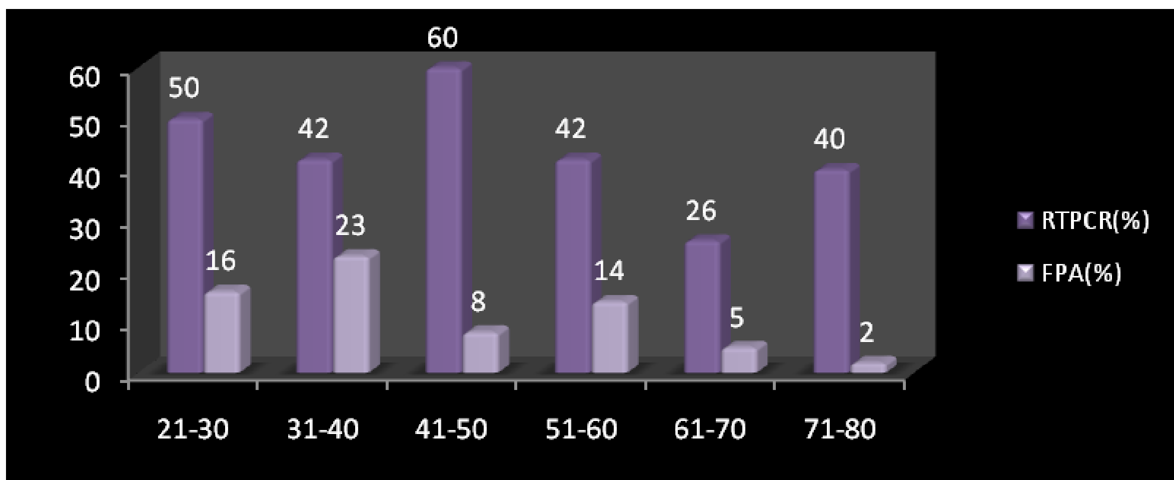

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A. Figure 1: Analysis of positivity rate in pulmonary samples

B. Figure 2: Analysis of positivity rate of TB in males and females

C. Figure 3: Chart analysis of positivity rate of TB through RT PCR

D. Figure 4: Chart analysis of positivity rate of TB through FPA

E. Figure 5: Age-wise positivity rate for FPA and RT-PCR

Although, there has been some conflict about the association of COVID-19 infection and tuberculosis diagnosed disease to be comprehended or not (Walaza, et al., 2020). All these studies are only statistical-based and hence not much can be outdrawn from them.

CONCLUSION

Tuberculosis posed a major disease burden among the Indian population which demands quick diagnosis to commence treatment as soon as possible to decrease the probable morbidity and mortality rate. For this, a definitive and appropriate method for diagnosis with feasible cost and energy is needed to be procuring in the health system. The method should be reliable, cost-effective, precise, rapid and easy to use to decline the level of difficulty to become an obstacle while diagnosis. The method should conquer such an approach so that minimal error could occur. RT-PCR has proven to be such a method when compared with the conventional method of FPA. FPA fails to diagnose the low viral load due to low sensitivity; RT-PCR with high sensitivity has provided positive results which were found negative when tested by FPA. As RT-PCR is a molecular technique, it uses biological markers, designed primers and probes which are very specific for *M. tuberculosis*, making it a more reliable technique than FPA. Due to the very specific nature of biological markers, diagnosis can be confirmed without any fault by the doctors and health workers. Although, this method has some short comes while diagnosing MDR-TB but still, it is the most popular and widely used method of diagnosis amongst molecular diagnostic laboratories. Especially in the time of pandemic due to coronavirus, when TB has been liked with COVID-19 as co-infection, RT-PCR has been used as the most consistent method for immediate diagnosis.

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