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Research Article

TO ANALYZE THE TB-PCR POSITIVITY RATE USING REAL-TIME PCR FOR EARLY DETECTION OF TUBERCULOSIS

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ABSTRACT

Objective: Tuberculosis is one of the leading causes of death worldwide. It is present in all the countries and among all age groups and also seen in both the genders. In investigating the early stage, identification, and rapid detection of tuberculosis, the PCR method (polymerase chain reaction) is one of the fast, safest, and reproducible new approaches. It covers all advantages such as the use of closed systems, reduced risk of carryover contamination, improved sensitivity and reproducibility, reduced turnaround time wide dynamic range of target detection, and feasibility for quantitation making it easy and reliable for the early detection of *Mycobacterium tuberculosis.*

Methods: All samples used for the study are confirmed by conventional microscopic observation using acid-fast staining using ZN STAIN. The pulmonary sputum samples are obtained from clinical and radiological evidence of tuberculosis and these samples are selected for DNA extraction.

Discussion: As per the statistical analysis using SPSS 22 version, it is found that the TB positivity rate (is 29.75%). Out of 119 positive patients (Male: 60.5% and Female: 39.5%). The average and standard deviation for CT values are 23.6 and 2.9, respectively. The 95% confidence interval of CT values for specimens is (22.7, 24.5). Average and standard deviation for CT values are equal in male patients and female patients. Age group-wise average and standard deviation for CT values are nearly equal.

Results: The percentage of 3+ AFB positive grades having CT values between 20 and 24 is more than all other combinations of AFB positive grades and CT values. The average and standard deviation for CT values are 23.6 and 2.9, respectively. The 95% confidence interval of CT values for specimens is (22.7, 24.5). The average and standard deviation for CT values are equal in male patients and female patients.

Conclusion: A significant improvement in specificity with high accuracy was achieved by a real-time PCR assay. Real-time PCR tests prove both a high degree of sensitivity in the upper respiratory sputum samples and for the early detection of the TB infection. The MTB real-time PCR test suitably identified the majority of the AFB positive and bacterial culture confirms TB.

Keywords: Mycobacterium tubercle bacilli, Real-time polymer chain reaction, *Deoxyribonucleic acid.*

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INTRODUCTION

The PCR method is a direct method of non-stop detecting MTB in clinical specimens. PCR method is used for quantification of MTB DNA in pulmonary sputum samples even if we have minutes quantities of DNA or RNA containing samples [1]. We can obtain multiple copies of specific DNA fragments using the PCR technique [2]. It is a revolutionary technique in the field of molecular biology. This methodology is based on the incorporation of the fluorescent dye where the increase in fluorescence signal, generated during the PCR, which includes DNA-intercalating dyes such as SYBR Green or sequence-specific oligonucleotide chemistry such as TaqMan probes [3]. The advantages of this PCR method are the use of closed systems, reduced risk of carryover contamination, improved sensitivity and reproducibility reduced turnaround time, the wide dynamic range of target detection, and feasibility for quantitation [4]. It is highly cost-effective throughout the laboratories and can become a convenient option for many other laboratories also as more indigenous and less expensive commercial kits are made available shortly. The real-time PCR permits for fast and extremely specific detection of infectious diseases, as well as those caused by *Mycobacterium tuberculosis* infection [5]. The innovative RT-PCR permits identification of slow-growing *M. tuberculosis* bacilli, anaerobic bacterium, and viruses [6]. The main idea for RT-PCR diagnostic applications in medical science is the detection of infectious agents and also the sensitivity of non-pathogenic from infective strains by specific genes [7]. It also allowed the detection of a low quantity of disease organisms (both live and dead), in clinical samples [8]. This could be achieved using either external standards or internal standards such as various housekeeping genes, since the data is not obtained in terms of absolute copy numbers, it is called relative quantification. Reverse transcriptase PCR: In clinical microbiology, one has to ideal deal with situations where there is a need to look at the expression levels of cellular transcripts such as cytokines or detection of RNA viruses in body fluids [9]. In these experiments, nucleic acid is available in the form of RNA [10]. The RNA will have to be converted into complementary DNA using enzymes derived from retroviruses and the process is called reverse transcription [11]. The cDNA thus obtained will be then subjected to slandered PCR using the regular protocol. This protocol can be adapted to real-time PCR also for quantification of the data [12]. There are several other variations such as inverse PCR, booster PCR, asymmetric PCR, touchdown, and touch-up PCR that have been used for different purposes.

METHODS

Out of 400 Sputum Samples, 119 patients were found to be positive. The samples have been collected from the Dr. D. Y. Patil Medical College Hospital and Research Institute, Kolhapur. All materials were used without further purification primers and oligonucleotides were purchased from Integrated DNA Technology (IDT), NALC (N-Acetyl-L-cysteine) was purchased from Thomas baker. All samples used for the study are confirmed by conventional microscopic observation using acid-fast staining using ZN STAIN. The pulmonary sputum samples are obtained from clinical and radiological evidence of tuberculosis and these samples are selected for DNA extraction. All samples were treated with 0.5 % N-acetyl-L-Cysteine (NALC)/2% NaOH method for digestion and decontaminated and

concentrated by centrifuging at 3000 rpm for 15 min. The supernatant is decanted and transferred to the sediment in 2 ml of phosphate buffer pH 6.8. The sediment is used for ZN staining and DNA extraction [13,14].

Isolated DNA from clinical sputum samples is processed for amplification by real-time PCR (Rotor Gene 2000/3000/6000-Corbett Research, Australia) for *in vitro* diagnostic use. Total 25 µl reaction volume of master mix contains 12 µl (R1) super mix, magnesium solution, MTB complex 2.5µl (R2), and internal control IC-1 (R3) RG 0.5 µl and 10 µl extracted DNA samples [15]. The PCR grade water was used as a negative control. Cycling conditions were 1 cycle initial activation at 95°C for 10 min, several cycles 45 cycles, denaturation at 95°C for 15 s, annealing at 60°C for 20 s, and an extension step at 72°C for 15 s (Sawant *et al*. 2018). Real-time PCR conditions are shown in Table 1.

Amplification of MTB species genes with IS6110 primers: According to Genome Diagnostics Pvt. Ltd. PCR, primers were used [11,16]. The probes and primers were purchased from Genome Diagnostics Pvt. Ltd. (ISO 13485, 2012&9001:2008), MTBC-specific primers were used to amplify a target IS6110, the Accession number LC005454.1. An internal control (IC) plasmid genomic DNA was used to detect PCR inhibition in the extracted MTB DNA. The amplification of MTB species shown in the process can be summarized in three steps.

Ethical considerations

Ethics approval to conduct this study was granted by the Institutional Ethical Committee of D. Y. Patil Medical College Hospital and Research Institute, Kolhapur, 416006, Maharashtra, India (DYPMCK/344/2021/IEC).

RESULTS

The majority of the infected cases were found in male patients that are 60.5%. The percentage of 3+ AFB positive grades having CT values between 20 and 24 is more than all other combinations of AFB positive grades and CT values. The average and standard deviation for CT values are 23.6 and 2.9, respectively. The 95% confidence interval of CT values for specimens is (22.7, 24.5). The average and standard deviation for CT, values are equal in male patients and female patients. Patients' age, group-wise average, and standard deviation for CT values are nearly equal. The percentage of specimen's AFB positive grade 3+ is more than all other AFB positive Grade.

Out of the given samples, the majority of the infected cases were found in males patients that is (60.5%).

The percentage of 3+ AFB positive grades having CT values between 20 and 24 is more than all other combinations of AFB positive grades and CT values.

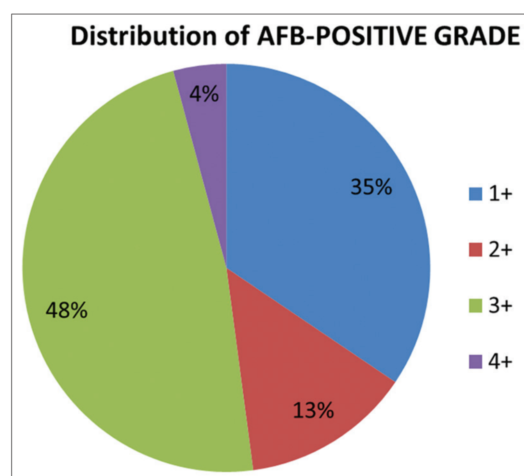

Figure 1 Distribution of AFB positive Grade

Table 1: Real-time PCR conditions

PCR-steps	Cycle	Temperature	Time
Initial activation	1 cycle	95°C	10 minutes
Denaturation	1 cycle	95°C	15 minutes
Annealing	45 cycle	60°C	20 seconds
Extension	45 cycle	72°C	15 seconds

Table 2: Gender wise distribution of TB infection

Gender	Frequency (%)
Male	72 (60.5)
Female	47 (39.5)

The average and standard deviation for CT values is 23.6 and 2.9 respectively. The 95% confidence interval of CT values for specimens is (22.7, 24.5).

From the above table average and standard deviation for CT, values are equal in male patients and female patients.

From the above table age, group-wise average, and standard deviation for CT values are nearly equal.

Percentage of specimens AFB positive grade 3+ is more than all other AFB positive Grade.

DISCUSSION

As per the statistical analysis using SPSS 22 version, it is found that the TB positivity rate (is 29.75%). Tables 1-6 indicates Out of 400 sputum samples 119 positive patients (Male: 60.5% and Female: 39.5%). The average and standard deviation for CT values are 23.6 and 2.9, respectively. The 95% confidence interval of CT values for specimens is (22.7, 24.5). Age group-wise average and standard deviation for CT values are nearly equal. It is present in all the countries and among all age groups and also seen in both the genders (dominant in males). In our zone, we have observed some common patterns among the males, such as smoking, chewing tobacco, alcohol usage causing immunosuppressive effects, various hormonal and physiological factors exposure to polluted areas (specifically we have more handloom mills in our zone) malnutrition, weak immune system, and also the lower socioeconomic group of people these are various factors that contribute to its causes [9,17]. During the study, it was found that male patients are more prone to infection as compared to females due to the exposure to the above-mentioned factors. Over the past decade, new diagnostic tests are continually being developed.

Consequently, there are often several tests available for the diagnosis of a condition. RT-PCR assay is commonly used to determine whether DNA or a sequence of the MTB is present in a sample and detects amplified DNA as the reaction progresses in real-time [18,19]. This value is called cycle threshold (Ct).the PCR-based technique has revolutionized the field of molecular biology and is widely used in microbiology, biotechnology, genetics molecular diagnostics, and clinical forensic environmental laboratories among other several applications [20]. Real-time PCR is a recent modification of the standard PCR technique which combines the objectivity of fluorescence detection with the simplicity of basic PCR assay [21]. There are variants of PCR used today. Hot start PCR, Universal PCR, Multiplex PCR, Nested PCR, Quantitative PCR, and Reverse transcriptase PCR are a few of them [22]. Real-time PCR is accepted as the gold standard for diagnosis of several pathogens and quantification of viral load in clinical samples and indicator of active infection, disease progression, and therapeutic response to antiviral drugs. According to Table 1, out of the given samples, majority of the infected cases were found in male patients that are (60.5%) and 39.5% in females which are relative to the lower side. According to the pie chart (Fig. 1), percentage of specimens with AFB positive grade 3+ is more than all other AFB positive Grade. Average and standard deviation for CT values are equal

Table 3: AFB positive grade wise distribution OF Ct values

AFB-positive grade	CT values, frequency (%)		
	20–24	25–29	30–35
1+	23 (19.3)	12 (10.1)	6 (5.0)
2+	10 (8.4)	4 (3.4)	2 (1.7)
3+	35 (29.4)	12 (10.1)	10 (8.4)
4+	5 (4.2)	0	0

CT: Computed tomography, AFB: Acid-fast bacilli

Table 4: Mean and standard deviation for CT values

CT VALUES	Mean	23.6
	Standard Deviation	4.9

Table 5: Mean and standard deviation for CT values

Gender	Mean	SD
Male	24.5	3.7
Female	24.5	3.7

SD: Standard deviation

Table 6: Gender wise Mean and standard deviation for CT values

Age group	CT values		
	Frequency	Mean	SD
14–30	47.0	24.6	3.7
31–59	47.0	24.5	3.7
60+	25.0	24.4	3.7

SD: Standard deviation, CT: Computed tomography

in male patients and female patients. The real-time PCR permits for fast and extremely specific detection of infectious diseases, as well as those caused by MTB infection. The main idea for RTPCR diagnostic applications in medical science is to detect infectious agents and also the sensitivity of non-pathogenic from infective strains by specific genes [7]. The molecular TB diagnosis with real-time PCR test has great potential to develop the physicians for the early-stage diagnosis of TB. This will confirm the first stage of therapeutic TB management and also prevention of further transmission of infection. The speed and ease of use, sensitivity, specificity, and robustness of PCR have revolutionized molecular biology, the most widely used and powerful technique with a great spectrum of research and diagnostic applications. These improvements have led to more rapid implementation of the best therapy for given TB patients for diagnosis and the early identification of drug-resistant TB. It allowed for the rapid and highly sensitive and specific detection of MTB and it also identify mycobacterium species with liquid cultures without the post-PCR process, which included only 1 h of sample preparation and 1.5 h for target DNA amplification because thermal cycling is much faster and amplicons detection is performed in real-time. However, further future work is needed for improving more specificity and sensitivity of molecular tests and to create it low-cost and user-friendly device.

CONCLUSION

A significant improvement in specificity with high accuracy was achieved by a real-time PCR assay. Real-time PCR tests prove both a high degree of sensitivity in upper respiratory sputum samples and for the early detection of the TB infection. The MTB real-time PCR test suitably identified the majority of the AFB positive and bacterial culture confirms TB. The test allows for a finding of TB in around 2 h. The efficacy of this assay for MTB diagnosis was equal to bacterial culture. Thus, it can be implemented in tertiary care settings. More sophisticated DNA extraction procedures are required for MTB cell lysis. As per the statistical analysis using SPSS 22 version, it is found that the TB positivity rate (is 29.75%). Out of 119 positive patients (Male: 60.5% and Female: 39.5%). The average and standard deviation for CT values are 23.6 and 2.9, respectively. The 95% confidence interval of CT values for specimens is (22.7, 24.5). Average and standard deviation for CT values are equal in male patients and female patients. Age group-wise average and standard deviation for CT values are nearly equal.

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AUTHORS CONTRIBUTIONS

All authors certify that they have participated satisfactorily in the work to make public responsibility for the content, including involvement in the concept, design, analysis, writing, or revision of the manuscript.

CONFLICTS OF INTERESTS

The authors have declared that they have no conflicts of interest.

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