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Progress in polymerase chain reaction technology and its contribution to the tuberculosis diagnostic ecosystem in India: A mini review

Krishna Himmatbhai Goyani [1, 2] and Pratap Narayan Mukhopadhyaya [1, 2, *]

[1] *Department of Infectious Diseases, TB Research Section, Wobble Base Bioresearch Private limited, Pune, India.*
[2] *Institute of Science, Department of Biosciences (Biotechnology), Shri Jagdishprasad Jhabarmal Tibrewala University, Jhunjhunu, Rajasthan, India.*

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Abstract

The advent of Polymerase Chain Reaction (PCR) has revolutionized the human molecular diagnostic scenario. Its ability to exponentially generate desired stretch of DNA from chromosomal or complimentary DNA has laid the foundation for sensitive and specific detection of a pathogen or an aberrant stretch of DNA. Importantly, PCR technology has helped generate diagnostic immunity from phenotypic variation of pathogenic targets. The technological evolution of conventional end-point PCR to its real time version further added clinically important features such as enhanced sensitivity and quantification of target DNA molecules. Two important chemistries became prominent in the real-time PCR domain. The intercalating dye-based chemistry comes with convenience, economy and feature such as melt curve analysis but also carries the disadvantage of overestimation of signal due to unwanted signal from primer dimers. Dual labelled probe on the other hand provides unprecedented sensitivity and specificity. Isothermal method of genome nucleic acid amplification has also evolved as a parallel and convenient method of amplification-based detection of nucleic acid targets. Tuberculosis diagnostics has immensely benefitted from these technologies. However, unmet need exists in sputum handling methods and in its adaptation for molecular diagnostics in resource limited settings. Significant dependence for diagnostic needs on the western world is a matter of concern in India. The country will benefit from rapid and indigenous research and development of molecular diagnostic solutions by way of formulating kits for rapid extraction of DNA and RNA from various clinical sources and developing detection technologies that can compete international products in its category. A solution to address sputum transportation, storage and DNA extraction that has enhanced advantages compared to current methods will also be helpful in the efforts to make India free from tuberculosis.

Keywords: DNA; Purification; Extraction; Detection; Tuberculosis.

1. Introduction

Kary Mullis discovered the technology of Polymerase Chain Reaction (PCR) in the year 1984. This achievement came as one of the most important and significant revolution in modern biological science. It provided the key to biological photocopying of DNA into millions of identical replicas thus making possible the critical and detailed analysis of minute quantity of DNA with ease and comfort. In the current times, an overwhelming number of research publications are available that mention PCR or its variants in some context or the other [1].

The world has seen a great scientific stride by way of successful completion of the human genome mapping project [2]. The vast array of data that emerged from this program is now used by clinicians as well as biological researchers across the globe to rapidly diagnose diseases, analyze gene sequences and run complicated quantitation studies on human

* Corresponding author: Pratap N. Mukhopadhyaya.

genome to learn more about human health. At the heart of this mammoth progress is the PCR technology. The particular source of DNA that initiates the process of PCR and often called the 'template', can be from a wide range of human system including blood, tissue and other body fluids.

The Polymerase Chain Reaction is primarily an enzyme driven chemical event that occurs in a tube. As a result of this reaction, a specific fragment of DNA gets copied exponentially. The major components of a PCR reaction are the template DNA, the oligonucleotide primers and a polymerase enzyme. This enzyme is one of the key components as it is responsible for linking the individual nucleotides with each other. The oligonucleotide primers impart the much-needed specificity of the reaction and decide the exact region of the template DNA that is to be exponentially amplified.

2. Conventional and Real Time PCR

Although conventional PCR paved the way for widespread use of the technology for DNA amplification, the next most important milestone was ushering of the idea of monitoring the process of DNA amplification in real time by tracing the quantum of a signal that accumulated during the process [3,4]. In one of the most popular formats of real time PCR, the oligonucleotide primers are 'tagged' with fluorophore. With completion of every thermal cycle, accumulation of the products of PCR correspond to parallel accumulation of fluorescence. This is because the dye-tagged oligonucleotide primers are compulsorily an integral part of the products of the amplification process.

Real time PCR is also known as quantitative PCR or qPCR. This nomenclature is often confused with RT-PCR which is intended to convey the term 'Real Time PCR'. This is however, an incorrect nomenclature and is often confused with Reverse Transcription PCR which also abbreviates to RT-PCR.

In real time PCR, as describes above, the oligonucleotide primer-borne fluorescent tag gets incorporated in the PCR amplicon or the product of PCR and with every accumulating amplicon population, there is a rise in fluorescence. However, in practice it is observed that during the initial phases or cycles of PCR, the accumulated fluorescent signals are less and does not equate mathematically and rise as expected with the passing of each thermal cycle. Hence, the fluorescence at this initial stage cannot be distinguished and differentiated from the fluorescence that is present in the back ground. But once the thermal cycles progress beyond a certain number, the accumulated quantity of fluorescent signal increases in a rapid manner with every passing cycle and more importantly, the quantum of signal accumulated becomes directly proportional to the quantity of thermal cycling product or amplicon generated during the process. The point, when this rise of fluorescent signal gains momentum and corroborate with proportional increase in PCR amplicon products, can be mapped with the cycle number at which this happens and is called the cycle threshold or Ct. Once the signal graph touches and then crosses this threshold the quantity of PCR amplicon mathematically match the signal compiled from within the tube [1].

2.1. Advantages of Real Time PCR

One of the major advantages of the use of dye-mediated PCR amplification is the proportionate rise of fluorescent signal with corresponding rise in the quantity of PCR product which has the unique potential to quantify the template. Known quantity of DNA can be added in a fluorescent PCR reaction in decreasing concentration that follow a Log 10 based reduction in concentration and corresponding fluorescent signal can be measured to generate signal quantity data points that can accurately measure the quantity of DNA [5,6,7].

The quantitative PCR technology can also generate semiquantitative results. This can be achieved by using a known reference material into the reaction. A cycle threshold beyond the one obtained from the reference material can provide broad idea of the quantity of the unknown template which would be 'higher or lower than' the reference material. This concept has been extensively used in gene expression studies where quantitative PCR technology is employed [7]. The identification and subsequent measurement of gene arrangement, amplification, deletion and translocations holds a special significance in today's molecular diagnostic sphere [8,9,10].

2.2. Types of Chemistry employed in Real Time PCR

Real Time PCR imparts the unique advantage of monitoring the progress of a reaction as it happens. This is made possible by the use of fluorophores that play crucial role in the process.

The fluorophore molecules can be used in real time PCR by two different strategies.

The first is use of a fluorophore that emit signal when it suffers minor conformational change in its structure. The most popular fluorophore in this category is the Syber Green dye [11]. This dye does not emit any fluorescence when it is

present in its nascent state. However, in presence of double stranded DNA, it integrates within the strands. This results in minor alteration in its structural conformation. This altered conformation results in emission of fluorescence. With every passing thermal cycle there is an exponential increase in the population of double stranded DNA by way of PCR amplicons that are identical replica molecules of the target region that is intended for amplification. These new breeds of linear, synthetic DNA molecules attract the dye molecule that get incorporated within the strands. These trapped dye molecules suffer minor structural conformation. This alteration in the structural conformation results in fluorescence that is captured by the detector of the real time PCR machine. The rise in quantity of the PCR amplicons in the reaction tube then directly corelate with rising fluorescence emanating from the dye molecules trapped within the double stranded PCR amplicons. These dynamics of fluorescence can be monitored in real time on the screen of the real time PCR machine.

One of the major shortfalls of the intercalating dye based real time PCR reaction is its specificity. In PCR reaction one of the byproducts is the formation of primer dimers [12]. These are very short (<100 base pairs) stretch of DNA molecules that are generated by a template independent manner, primarily because of interaction of the forward and the reverse oligonucleotide primers during the process of PCR. These primer dimer products are technically double stranded DNA molecules but they do not represent a contiguous region of the target DNA region. Given the fact that the intercalating dyes does not discriminate between true target and any other, it intercalates within the primer dimers also thereby generating signal. However, these signal does not originate from the double stranded DNA representing the actual target DNA region and hence the signal generated from primer dimers are considered to be false positive signals. They therefore erroneously overestimate the PCR product and are not reliable for any form of quantification studies [6,13]

However, the intercalating dye-based detection chemistry supports a specific method to generate an amplicon footprint that is not possible with any other chemistry. This is made possible by the reversible nature of the dye to fluoresce. The ability of these dye molecules to get trapped within double stranded DNA is a reversible phenomenon meaning that a 'trapped' dye can get released if the double stranded DNA assumes single strand conformation due to heating or any other treatment. More importantly, the ability to fluoresce also is a reversible phenomenon and the dye fluoresce when it is trapped within a double stranded DNA and stops fluorescing when released from this position.

The melting temperature of every stretch of linear double stranded DNA is a function of its GC content and is directly proportional to each other. Since the GC content of any two double stranded linear DNA is unlikely to be identical, the specific temperature at which it 'melts' or becomes single stranded from its double stranded conformation is also specific and unique signature of a particular linear double stranded DNA such as a synthetically amplified, double stranded PCR amplicon.

Real Time PCR platforms are equipped with software that command incremental heating of a PCR product and meticulously trace the fluorescence of the intercalating dye present during the reaction. This fluorescence exhibits a sudden fall when the double stranded DNA 'melts' which is represented by an inverted bell-shaped curve where the X axis shows the precise temperature at which the DNA 'melted' and the Y axis shows the quantum of fluorescence dropped due to the process. The X axis value, which is the temperature at which the fluorescence falls, is a thermal fingerprint of the PCR amplicon analyzed and has diagnostic value. It mostly assists in tracing presence of nonspecific amplification if any and is occasionally used for diagnostic purpose by monitoring formation of the desired PCR amplicon by way of the melt temperature profile [47].

The other popular technology platform for real time PCR-based detection of targets is the 5' nuclease assay. Both the intercalating dye and the 5' nuclease assay-based technologies were developed parallelly [3,4]. In the later method, the enzyme called *Taq* Polymerase "chews up" a dual labelled probe that anneal midway between the forward and the reverse primers. This dual labelled probe is a short oligonucleotide that is tagged with a fluorescent dye at one end and a quencher dye at the other. The natural fluorescence of fluorescent probe is quenched by the Quencher dye due to the phenomenon of fluorescence resonance energy transfer or FRET [14]. During polymerization event executed by the enzyme *Taq* polymerase the enzyme proceeds by synthesizing a new strand of DNA using a bottom single strand DNA as template until it encounters the annealed dual labelled probe. By virtue of its nuclease activity, it breaks apart the nucleotide bases that comprise the dual labelled oligonucleotide probe. As a result of this, the extreme 3' base harboring the fluorescent dye gets detached from the main oligonucleotide structure. This destroys the precise physical distance between the fluorescent dye-labelled base and the one that is tagged with the quencher dye. Due to increase in this distance, the energy of the fluorescent dye is no longer quenched by the quencher dye thereby resulting in fluorescence. This phenomenon occurs with every amplifiable template and with every thermal cycle. As a result of this, there is gradual accumulation of fluorescence that can be traced in real time.

The dual labelled probe based real time PCR has several advantages. It is immune to formation of primer dimers. This is because the primer dimers do not facilitate annealing of the dual labelled probe between them and therefore do not contribute for any signals. Further, the target is interrogated by three different oligonucleotide primers thereby raising the specificity. These are the forward and the reverse primers and the dual labelled probe which plays the role of the third annealing primer.

There are several other versions of real time PCR chemistry that uses the FRET phenomenon but not the nuclease feature of the *Taq* DNA polymerase. One such technology, called the contact quenching [15] uses a common fluorophore-tagged oligonucleotide primer and another that has the complimentary sequence to the fluorophore-tagged primer but is tagged with a quencher. Under non amplifying conditions, they anneal to each other thereby executing FRET phenomenon and emitting no fluorescence. However, when the process of amplification occurs, a primer-specific tag assist in annealing of the dye-tagged probe with the amplification primer during thermal cycling process. This dissociates it from the quencher probe thereby increasing the distance between the quencher and the fluorophore resulting in fluorescence.

3. Nucleic acid extraction, PCR and human molecular diagnostics

Successful extraction of nucleic acids paved way for development of a wide range of molecular diagnostic solutions related to human healthcare. Interestingly, most of these diagnostic protocols rely on PCR as the backbone technology for amplification of the target genes which is followed by different detection methods. There has been a dominance of commercial nucleic acid extraction kits for extraction of nucleic acid from human cells as well as a wide range of pathogens. Major international companies in this domain include Qiagen (Tegelen, Netherlands), ThermoFisher Scientific (Waltham, Massachusetts) and Roche (Penzberg, Germany). These kits are known for their consistency and efficacy and are widely adopted for DNA and RNA extraction in the field of human molecular diagnostics.

It is the need of the hour for indigenous research work to develop similar kits in the country through innovation such that critical stages of extraction such as purification and precipitation of the analyte are specifically focused upon and developed in a way that they are not only efficient but also economical and indigenous in nature. This will reduce the dependency of such materials on foreign supplies and lead to significant savings of foreign currency in the country.

Molecular diagnostics has spread across all domains of human healthcare like never before. Since the days of conventional PCR based detection of *Mycobacterium tuberculosis* and Human Immunodeficiency virus 1 (HIV-1) pathogens [16,17] molecular diagnostics has evolved to real time PCR and several chemistry working on this platform and contributing to sensitive qualitative and quantitative detection of a wide range of pathogens.

The dual labelled probe chemistry, popularly known as the Taqman chemistry has played a special role in healthcare diagnostics. The popularity of this platform is mainly for few important reasons. These are its ability to ignore signals generated from primer dimers which are formed during PCR, sensitivity, ability to quantify a template when standards are available, hassle-free post PCR processing, improved analysis facility of data and potential to run semi quantitative assays typically required for gene expression analysis. This is the reason for its massive popularity in molecular diagnostics of human infectious and non-infectious diseases. These features have led to a silent revolution in clinical practice in the area of infectious diseases. Real time PCR fits well in settings of acute care where timely report is essential for satisfactory patient care and favourable treatment outcome.

Use of DNA probe is integral to advanced nucleic acid-based assays [18,19,20]. They target the genome or its transcribed products directly by the phenomenon of nucleic acid hybridization and are essentially immune to phenotypic aspects of the pathogen. It was in the year 1980 when the pathogenic enterotoxigenic *Escherichia coli* was first detected using DNA probes thereby raising the hope that the technology will likely be a viable alternative to traditional methods of detecting pathogens [21].

On the contrary, traditional, culture-based methods of identification of pathogen rely on phenotypic characters for detection of the pathogen and often lacks discriminatory power. Further, these methods are culture-based and hence take significant time to generate results. Also, they are not useful when pathogens are refractive to culture techniques or cannot be cultured at all.

A large number of reagent sets, often dubbed as “Kits” are available for detection of clinically important pathogens as well as human genetic aberrations that are associated with health disorders. Qiagen, a multinational company with its headquarters at Tegelen, Hulsterweg 82, Netherlands is a prominent kit manufacturing company. It provides high-end assay-technologies related to molecular diagnostics as well as applied research (www. Qiagen.com). Another

multinational company with similar product profile is the Thermo Fisher Scientific. Head quartered at Waltham, Massachusetts, United States, this company offers optimized dual labelled probes for detection of various clinically important infectious diseases. These assays require a real time PCR platform for its operation. One such product, called the Applied Biosystems TrueMark Infectious Disease Research Panel, is a classical two plex Taqman assay available as reagent pre-spotted plates. These panels assist in rapid categorization of infectious agents into respiratory, urinary, vaginal, wound-derived, sexually transmitted or gastrointestinal origins.

However, most of these kits are imported in India. While the advantage of this is access to high quality reagents for molecular diagnostics, the disadvantages are reliability on multinational companies and erosion of foreign exchange from the country.

4. Dependence of the country on medical device imports

As per the legal firm 'Legalease Ltd' (188 Fleet Street, London, EC4A 2AG), with regard to medical devices, India is heavily dependent on imports. With an increasing population load in the country coupled with spiraling lifestyle diseases, the need and demand for affordable healthcare was never felt as acutely as now. Currently, almost 80% of the medical devices in India comes from abroad suppliers. The dependency is maximum on five nations, namely, USA, Netherland, Singapore, China and Germany. In the period between financial year 2021-2022 and 2020-2021, the import of medical devices in India demonstrated a robust growth of almost 42% with a rise from ₹44,708 crore to a high of ₹63,200 crore rupees (1crore = 10 millions). Despite all efforts by the Indian government, China continues to remain one of the biggest exporters of medical devices in India. This was particularly prominent during the COVID-19 pandemic when certain specific medical devices were at huge demand in the country. With emergence of large hospitals such as the Fortis, Max, Apollo and those established by the Hinduja group, the import of these foreign medical devices is expected to further rise.

This calls for a specific focus on indigenous development and optimization of medical devices in the molecular diagnostic domain in the country that can significantly reduce the dependence of imports from other countries. Products so developed should match the efficacy of the imported substitutes. The development of such products should not be random but match the specific need of the healthcare industry of the country.

5. Tuberculosis in India

The incidence of tuberculosis is still seen worldwide and the disease continues to be a prominent reason for mortality and morbidity across the globe. Large number of new cases emerge every day and a major part of this is recorded in India. Reviews show that around 0.48 million people embraced death due to tuberculosis in the year 2015 [22]. More importantly, over a million number of tuberculosis patients are known to be missing and the government has nil record regarding them [23].

In the country the prevalence and incidence of multi drug resistant tuberculosis is alarming. These cases were seen in around 2.5% of the new and 16% of the earlier-treated patients in the country. As per one estimate, around 130,000 fresh cases of multi drug resistant (MDR) tuberculosis emerge in India every year. But out of this large number only around 79,000 are notified among which 36% are properly diagnosed and 36% are subjected to initial treatment where the success rate of the treatment is only about 46% [22].

Yet another major concern is the alarming rise of cases of extensively drug resistant or XDR tuberculosis in the country. XDR tuberculosis is a major concern across the world and specially for countries and areas within a country, where the incidence rate of Human Immunodeficiency Virus -1 (HIV-1) is high. Almost 9.5% of all MDR tuberculosis cases are found to be also of XDR tuberculosis variety. As on this date as many as 117 countries across the world has reported cases of XDR tuberculosis in their territory [22]. In our country, around 1.5 to 11% of MDR cases were also found to be XDR tuberculosis variants [24,25,26,27]. A major worry for MDR and XDR tuberculosis cases is the fact that there are very restricted options for treatment. Adding to this is the high cost of treatment that puts additional burden on the country's healthcare related funds. If these cases are not addressed and handled properly, the progress achieved to make India TB-free will suffer major setback.

5.1. Tuberculosis in rural India

In the light of these serious constraints, accurate diagnostics for tuberculosis play a crucial role. In India a large section of the population resides in village settings where resources are limited. As per Globaladata.com website, India recorded

a high of 867 million people staying in rural settings of the country as on 2021. Between the year 2018 and 20211, there is an average growth of 0.9% in this population.

Very few studies exist on incidence rate of tuberculosis in rural parts of India. In one such report [28] that reviewed rural parts of Southern India published an annual rate of infection of tuberculosis of 1% in children who were infected for the first time. Overall, a high of 30% of all infected cases were recorded in patients who were not previously infected by the disease. Further, the prevalence of the disease was around 4 times higher compared to incidence. Around 50% of the disease population died within a span of 5 years while those who survived continued to release the pathogen in the air even after a period or 5 years thereby infecting newer population. In view of such studies, from tuberculosis diagnostics point of view a few important observations can be made. There should be a robust method of tuberculosis diagnostics that can be successfully adapted specifically to rural settings of India.

6. Sputum handling for Tb testing

The traditional method of collection of sputum needs to be relooked. Sputum is traditionally collected in screw cap or similar containers and transported from remote locations of the country to central testing facilities. Despite the fact that this method of transportation of sputum for detecting pulmonary tuberculosis has been practiced for ages, there are a few distinct disadvantages.

One of the major disadvantages is the possibility of breakage or leakage of the infected sputum container that can cause unintended spread of the disease within the handler community of the samples. The clinical sample transportation network in India is broadly unorganized. This is the reason why samples collected from rural parts of India sometimes take significant time in reaching centralized facilities for tuberculosis testing.

Warm and humid weather dominates a major part of the Indian sub-continent. Therefore, the sputum samples are prone to contamination with other microbes by the time they reach the testing laboratory. For Nucleic Acid based testing (NAAT) contaminating microbes can contribute to significant quantity of background DNA thereby negatively impacting sensitivity and specificity of an assay. This is particularly important because in human sputum samples, the epithelial and other human cells present in the sputum by default already contribute significant background DNA apart from nucleic acid from other microbes that might be present in the sputum bio flora.

Once sputum samples reach a testing laboratory, they are required to be stored under refrigerated condition until processed. This calls for a cold chain, not only during transportation but also upon arrival at the laboratory unless they are immediately processed which is not always the case.

Novel methods are now reported that support transportation of sputum at room temperature by maintaining cent percent biosafety aspect of the process [48]. It also allows archival of the nucleic acid contents of the sputum for an extended period of time at room temperature (> 6 years) and provide rapid and economical methods of DNA-release for cartridge based nucleic acid amplification tests (CB-NAATs) or its variants. The sensitivity of this technology when used in conjunction with CB NAAT platforms is also acceptable [49, 50].

7. NAAT and MTB diagnostics

NAAT is a superior method for diagnostic testing of *Mycobacterium tuberculosis*. As discussed earlier, the traditional method of bacteriological diagnosis of tuberculosis disease has a range of limitations which is one of the primary reasons for the rapid surge in use of molecular diagnostics for detection of *M. tuberculosis* [29]. The NAAT are rapid with very low turnaround time thereby reducing the loss to follow up cases in a very significant manner [30]. For a major part of the NAAT, the target is the insertion element IS6110 of the *M. tuberculosis* genome. This is because of its multi copy presence apart from selective presence in genomes of only in *M. tuberculosis* complex members. This provides the much-needed specificity to the tests [32]. The other popular NAAT targets for detection of *M tuberculosis* are the MTB ribosomal DNA or RNA [33]. NAAT can be done most often from samples without the need for specific culture barring a few cases such as drug resistant testing of *M. tuberculosis* by next generation sequencing platform [31]. NAAT has very high sensitivity even in smear negative sputum [30]. At present NAAT is recommended by the World Health Organization by way of Xpert/RIF MTB assay [34]. The other NAATs that are also recommend by WHO are the Amplified *Mycobacterium Tuberculosis* Direct (MTD) Test (Gen-Probe, Inc) [35,36,37] and the Amplicor *Mycobacterium tuberculosis* Test (Roche Molecular Systems, Inc) [38,39,40].

While all the NAAT described above function on the polymerase chain reaction principle that require real time PCR environment, yet another category of NAAT is the isothermal assay. These tests are commercially available and are known to impart high degree of adaptability to rural India because of the lack of requirement of expensive thermal cyclers to run the assay protocol [41,42,43]. Other non-PCR MTB testing platforms include the cross-priming amplification-based assay for TB detection [44,45] and the Genedrive® *Mycobacterium tuberculosis* iD® assay [46].

One of the advantages of the non-PCR based NAAT is their potential to get adapted to resource limited settings. This is because the test requires a single temperature incubation (isothermal) and further, the amplification products can be detected by a wide range of methods including color-based visual detection using strip based lateral flow devices. The tests do not require highly trained manpower and can be operated with near nil infrastructural requirements including power sources. Being a NAAT test, the sensitivity of isothermal MTB assays is acceptable and often discussed as a potential replacement for microscopic method of detection of *M. tuberculosis*.

8. Conclusion

There is a need to critically analyze the shortfalls and disadvantages that are bottlenecks of current molecular diagnostic scenario in the country with special reference to *M. tuberculosis* diagnostic ecosystem. A concerted effort needs to be adapted to develop a range of reagents and lab wares that can be used for rapid extraction of high-quality nucleic acid from a wide range of human biological sources with clinical significance. Such developments will prove to be useful for molecular diagnosis of prominent human pathogens as well as genome mutations using indigenous technologies and formulations.

Molecular diagnostics of *M. tuberculosis* require a relook specially in the area of sputum transportation, storage and DNA release methods that are safe and economical and align well with detection methods such that they can seamlessly adapt to resource limited settings in the country.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

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