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Competitiveness of Polymerase Chain Reaction to Alternate Amplification Methods

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ABSTRACT

PCR is the first method developed for amplification of DNA/nucleic acids *in vitro*. It was the method of choice since it's invent. PCR has many limitations which gave birth to alternative methods such as Loop Mediated Isothermal Amplification (LAMP), Nucleic Acid Sequence Based Amplification (NASBA), Self Sustained Sequence Replication (3SR), Rolling Circle Amplification (RCA) etc. A comprehensive review of the literature on the principles, limitations, challenges, improvements and alternatives of PCR was performed. Though PCR encountered some limitation afterwards and a no of technically improved alternatives has been devised worldwide, it still holds its standings as a gold standard for nucleic acid amplification preventing it from being obsolete. PCR has gone through some phenomenal modifications making continuous improvement of this method. Although, latter techniques offer better sensitivity and advantages where PCR has limitations, it is still commonly applied in the field of molecular biology for its extensive information all over the world, availability of reagents and methods, widespread validation and very good technical understanding among researchers. PCR is not obsolete despite of threats faced from other methods; rather it is continuously being modified to overcome the limitations. It will remain one of the ultimate methods of choice especially in developing countries for long.

Key words: Loop mediated isothermal amplification, rolling circle amplification, self sustained sequence replication, amplification of DNA

INTRODUCTION

Polymerase chain reaction (PCR) is the method which revolutionized molecular biology through its capability to amplify (creating multiple copies of) DNA independent of a living organism. PCR is routinely used in medical diagnostic and biological research laboratories around the world. PCR is used in a variety of areas, such as detection of inherited diseases, identification of genetic fingerprints, diagnosis of infectious diseases, diagnosis of viral diseases, molecular epidemiology, cloning of genes, sequencing, probe generation, microbial taxonomy, forensic investigations and paternity testing (Alshamary and Abuljadayel, 2009). PCR derives its name from one of its key components, DNA polymerase which is an enzyme catalyzing the DNA replication (Brown, 2001). PCR technique, invented in 1983 by Mullis (1990), allowed scientists and researchers to make millions of copies of a scarce amount of DNA. The approach has revolutionized many aspects of current research, including the diagnosis of genetic defects and the detection of

HIV in human cells (Alshamary and Abuljadayel, 2009). This study has provided a comprehensive review of the literature on the principles, limitations, challenges, improvements and alternatives of PCR.

POLYMERASE CHAIN REACTION (PCR)

Discovery of PCR: In 1971, a new method that uses enzymatic assay and primers to replicate a short DNA template was described first by Kleppe *et al.* (1971). Early manifestation of PCR did not attracted much attention. The invention of the polymerase chain reaction in 1983 is eventually credited to Kary Mullis (Saiki *et al.*, 1985). Kary Mullis developed PCR in 1983 while working as a chemist at the Cetus Corporation, a biotechnology firm in Emeryville, California, USA (Mullis, 1990). When performed manually, Mullis's PCR method was slow and labour-intensive. Therefore, Cetus scientists began looking for ways by which the process could be automated (Rabinow, 1996). The DNA polymerase originally used for the PCR was extracted from the bacterium *E. coli*. However, after each cycle of DNA synthesis, the reaction must be heated at a relatively higher temperature (94-95°C) in order to denature the double stranded DNA product. Unfortunately, heating also irreversibly inactivated this polymerase, urging prompt addition of new enzyme at the start of each cycle (Scharf *et al.*, 1986). The discovery in 1976 of *Taq* polymerase (a DNA polymerase purified from the thermophilic bacterium, *Thermus aquaticus,* which naturally occurs in hot environments (50 to 80°C) paved the way for dramatic improvements of the PCR assay (Chien *et al.*, 1976). The purification of the *Taq* polymerase resulted in the need for a machine to cycle more rapidly among different temperatures. The first thermocycling machine, Mr. Cycle was developed by Cetus engineers to address that need to add fresh enzyme to each reaction tube after the heating and cooling process (Bartlett and Stirling, 2003). In 1985, Cetus formed a joint venture with the Perkin-Elmer Corporation in Norwalk, Connecticut and had introduced the DNA Thermal Cycler by 1988. In 1991, Cetus sold the PCR patent to Hoffman-La Roche for a price of $300 million (Bartlett and Stirling, 2003). Finally in 1993, Kary Mullis was awarded the Nobel Prize in chemistry for his ground-breaking discovery of PCR (Lauerman, 1998).

Principle of PCR: PCR has three basic steps as follows: (1) Template denaturation: DNA fragments are heated at a high temperature, which forms single strands of the nucleic acids by breaking hydrogen bonds of the double-helical structure. These strands become accessible to added primers. (2) Primer annealing: The reaction mixture is cooled down to a temperature close to that of the melting temperature (Tm) of the oligonucleotides. Primers anneal to the complementary regions in the DNA template and double strands are formed again between primers and complementary sequences of DNA fragments. (3) Primer extension: The DNA polymerase synthesizes a complementary strand at a higher temperature (72°C). The enzyme reads the opposing strand sequence and extends the primers by adding nucleotides in the order in which they can pair. The whole process is repeated over and over for a maximum number of cycles as long as the enzyme is active for the reaction and the amplification reaches a desired limit (Saiki *et al.*, 1985; Sherfi *et al.*, 2006).

Limitations of PCR: PCR is the most widely used nucleic acid amplification method. Nevertheless, it has several limitations such as: 1. The usefulness of PCR methods can be limited by the presence of PCR inhibitors in the analysis of real biological samples (including food stuff). The wide range of inhibitors (including organic and inorganic substances such as detergents, antibiotics, phenolic

compounds, enzymes, polysaccharides, fats, proteins and salts) has been found to reduce or inhibit the amplification efficiency (Al-Soud and Radstrom, 1998) 2. PCR is labour-intensive and often requires extensive sample preparation to eliminate amplification inhibitors. 3. PCR methods are also sensitive to direct or carry-over contamination (Corless *et al.*, 2000) 4. High-cost, sophisticated instruments for amplification and detection of amplified products (amplicons) are required 5. Minimal 1 h is needed for the procedure (Yeh *et al.*, 2005) 6. Another disadvantage of PCR is the length of the region that can be amplified. PCR works well over short stretches of DNA up to about 2 kb. 7. One of the most significant limitations of PCR is that one need to have some DNA sequence information before one begins to attempt the assay. To overcome these limitations, development of a new technique has become urgent (Fakruddin, 2011; Fakruddin *et al.*, 2012a).

ALTERNATIVES OF PCR

Several alternative amplification methods have been developed already. These are Loop Mediated Isothermal Amplification (LAMP) (Notomi *et al.*, 2000), Self-sustained Sequence Replication (3SR) (Guatelli *et al.*, 1990), Nucleic Acid Sequence Based Amplification (NASBA) (Compton, 1991), Strand Displacement Amplification (SDA) (Walker *et al.*, 1992; Chamani-Tabriz *et al.*, 2007) and Rolling Circle Amplification (RCA) (Lizardi *et al.*, 1998), Ligase Chain Reaction (LCR) (Wiedmann *et al.*, 1994) etc.

Comparison of PCR with its alternatives: Each of the nucleic acid amplification methods has its own prospects and constraints. Most of the alternative approaches are still not available and used as a replacement of PCR. A comprehensive summary of the comparison of PCR with other alternative methods with regards to their various important features is depicted in Table 1 and 2.

PROMISING ADVANCEMENTS IN PCR

Through extensive research, the basic PCR method has been modified in many ways so as to overcome its limitations and to make it more competent to the demands of modern day research. Many advanced types of PCR have been designed and in use. Some of the advancements of PCR are described as follows.

Table 1: Comparison of PCR with alternate amplification techniques

Parameter	PCR	LAMP	3SR	SDA	LCR	NASBA	RCA
High sensitivity	<10	<10	10	10	<10	10	<10
High specificity	+	+	+	+	+	+	+
Allow quantification	+	+	+/-	+/-	-	+/-	-
No. contamination risk	+/-	+/-	+/-	+/-	-	+/-	+/-
Live vs. dead microorganisms	+	+	+	-	-	+/-	-
Easy to perform	+	+	+	+	+	+/-	-
Commercial availability	+	-	+/-	+/-	-	-	-
Linear dynamic range	7	6	7	ND	ND	7	ND
Multiplexity	+	-	+	-	+	+	+
Amplification on microarrays	-	-	-	-	-	-	+
Isothermal condition	-	+	+	+	+	+	+
DNA amplification	+	+	+	+	+	+	+
RNA amplification	+	+	+	+	+	+	+
No of enzymes	1	1	3	2	2	3-Feb	1
Primer design	simple	complex	simple	complex	simple	simple	complex

+: Application is demonstrated, -: Application is not possible, +/-: Limited application, ND: No data

Table 2: Comparison of PCR with alternate amplification techniques

Parameter	PCR	LAMP	3SR	SDA	LCR	NASBA	RCA
Tolerance to biological compounds	-	+	-	-	-	-	-
Need to template denaturation	+	-	+	+	+	+	-
Denaturing agents	Heat	Betaine	RNase H	Restriction enzymes; Bumper primers	Helicase	RNase H	Strand displacement property of Ø29 DNA polymerase
Product detection method	Gel electrophoresis, Real time, ELISA	Gel electrophoresis, Turbidity, Real time	Gel electrophoresis, Real time, ECL, ELISA	Gel electrophoresis, Real time	Gel electrophoresis, Real time	Gel electrophoresis, ELISA, Real time, ECL	Gel electrophoresis, Real time

+: Application is demonstrated, -: Application is not possible, ND: No data, Dynamic linear range: Range of target concentrations over which the method performs in a linear manner (quantitative) with an acceptable level of accuracy and precision, Expressed in orders of magnitude, ELISA: Enzyme linked immunosorbent assay, ECL: Electrochemiluminiescence

Allele-specific polymerase chain reaction: Allele-specific PCR is based on single base differences in DNA sequences i.e., single nucleotide polymorphisms (SNPs). It is used as a diagnostic or cloning tool. Prior knowledge of a DNA sequence such as differences between alleles is required. It uses primers whose 3' ends encompass the SNP. PCR amplification under stringent conditions is much less efficient in the presence of a mismatch between template and primer. Successful amplification with an SNP-specific primer signals presence of the specific SNP in a sequence (Singh *et al.*, 2004; Yin *et al.*, 2011).

Hot start PCR: Hot start PCR reduces non-specific amplification during the initial set up stages of the PCR. It is performed manually by heating the reaction components to the melting temperature (e.g., 95°C) before adding the polymerase. Specialized enzyme systems have been developed that inhibit the polymerase's activity at ambient temperature, either by the binding of an antibody or by the presence of covalently bound inhibitors that only dissociate after a high-temperature activation step. Hot-start/cold-finish PCR is achieved with new hybrid polymerases that are inactive at ambient temperature and are instantly activated at elongation temperature (Pirmez *et al.*, 1999).

Methylation-specific polymerase chain reaction: Methylation specific PCR (MSP) is used to identify patterns of DNA methylation at cytosine-guanine (CpG) islands in genomic DNA. Target DNA is first treated with sodium bisulfite, which converts unmethylated cytosine bases to uracil, which is complementary to adenosine in PCR primers. Two amplifications are then carried out on the bisulfite-treated DNA: One primer set anneals to DNA with cytosines (corresponding to methylated cytosine) and the other set anneals to DNA with uracil (corresponding to unmethylated cytosine). MSP used in Q-PCR provides quantitative information about the methylation state of a given CpG island. Methylation is determined by the ability of the specific primer to achieve amplification. The assay has successfully detected hypermethylation of the GSTP1 promoter, not only in tissue samples from patients with prostate cancer, but also in a high proportion of serum and plasma samples, which contain much lower amounts of DNA (Athale *et al.*, 2001).

Reverse transcription polymerase chain reaction: This is a sensitive method for the detection of mRNA expression levels. RNA reverse transcribes into complementary DNA (cDNA) by Reverse

transcriptase, which is then amplified by PCR. RT-PCR is widely used in expression profiling, to determine the expression of a gene or to identify the sequence of an RNA transcript, including transcription start and termination sites. If the genomic DNA sequence of a gene is known, RT-PCR can be used to map the location of exons and introns in the gene. The 5' end of a gene (corresponding to the transcription start site) is typically identified by RACE-PCR (Rapid Amplification of cDNA End-PCRs) (Siddique *et al*., 2008; Feligini *et al*., 2007).

Quantitative polymerase chain reaction: This approach is implemented to measure the quantity of a PCR product (commonly in real-time). It quantitatively measures starting amounts of DNA, cDNA or RNA. Q-PCR is commonly used to determine whether a DNA sequence is present in a sample and the number of its copies in the sample. Quantitative real-time PCR has a very high degree of precision. QRT-PCR methods use fluorescent dyes, such as Sybr Green, EvaGreen or fluorophore-containing DNA probes, such as TaqMan, to measure the amount of amplified product in real time. It is also sometimes abbreviated to RT-PCR (Real Time PCR) or RQ-PCR (Saaid *et al*., 2009; Huang *et al*., 2011).

Multiplex-polymerase chain reaction: Multiplex PCR allows the simultaneous amplification of more than one target sequence in a single PCR reaction (Fakruddin *et al*., 2012b). The reaction consists of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences. By targeting multiple genes at once, additional information may be gained from a single test run that otherwise would require several times the reagents and more time to perform. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction and amplicon sizes, i.e., their base pair length, should be different enough to form distinct bands when visualized by gel electrophoresis (Wong *et al*., 2012; Fakruddin *et al*., 2012b).

Assembly polymerase chain reaction or polymerase cycling assembly: PCA artificial synthesis of long DNA sequences by performing PCR on a pool of long oligonucleotides with short overlapping segments. The oligonucleotides alternate between sense and antisense directions and the overlapping segments determine the order of the PCR fragments, thereby selectively producing the final long DNA product (Suppian *et al*., 2006).

Helicase-dependent amplification polymerase chain reaction: It share many characteristics with traditional PCR but employs a constant temperature rather than cycling through denaturation and annealing/extension cycles. DNA helicase is used to unwind DNA. *Clostridium difficile* tcdA toxin gene has been amplified using helicase-dependent amplification (Chow *et al*., 2008).

Inverse polymerase chain reaction: Inverse PCR is commonly used to identify the flanking sequences around genomic inserts. It involves a series of DNA digestions and self ligation, resulting in known sequences at either end of the unknown sequence. Inverse PCR has numerous applications in molecular biology including the amplification and identification of sequences flanking transposable elements and the identification of genomic inserts (Engelstad *et al*., 2011).

Intersequence-specific polymerase chain reaction: It amplifies the region between simple sequence repeats to produce a unique fingerprint of amplified fragment lengths. It is an efficient tool in phylogenetic classification of prokaryotic genomes in general and diagnostic genotyping of microbial pathogens in particular. *Vibrio cholerae* pathogenic and nonpathogenic can be identified by using Intersequence-specific PCR (ISSR). *Vibrio cholerae* strains including 15 O1 El Tor, nine O139 and 21 non-O1/non-O139 strains were analyzed using eight ISSR primers. Only two sero groups O1 and O139 were identified to cause the disease (El-Assal and Gaber, 2012; Sane *et al.*, 2012; Rahman, 2007).

Ligation-mediated polymerase chain reaction: This PCR uses small DNA oligonucleotide 'linkers' (or adaptors) that are first ligated to fragments of the target DNA. PCR primers that anneal to the linker sequences are then used to amplify the target fragments. This method is deployed for DNA sequencing, genome walking and DNA footprinting A related technique is Amplified Fragment Length Polymorphism (AFLP), which generates diagnostic fragments of a genome. Ligation-mediated PCR was use to report a new case of Chronic Myeloid Leukemia (CML) with an e19a2 transcript, the fusion at the DNA level between BCR and ABL gene (Boeckx *et al.*, 2005).

Miniprimer polymerase chain reaction: This reaction uses a thermostable polymerase (S-Tbr) that can extend from short primers (smalligos) as short as 9 or 10 nucleotides. This method permits PCR targeting to smaller primer binding regions and is used to amplify conserved DNA sequences, such as the 16S (or eukaryotic 18S) rRNA gene (Isenbarger *et al.*, 2008).

Solid phase polymerase chain reaction (SP-PCR): It encompasses multiple meanings, including colony amplification (where PCR colonies are derived in a gel matrix, for example), Bridge PCR (where primers are covalently linked to a solid-support surface), conventional SP-PCR (where asymmetric PCR is applied in the presence of solid support bearing primer with sequence matching one of the aqueous primers) and Enhanced SP-PCR (where conventional SP-PCR can be improved by employing high Tm and nested solid support primer with optional application of a thermal step to favour solid support priming). During the solid-phase PCR (SP-PCR), DNA oligonucleotides complementary to a soluble template are immobilized on a surface are extended in situ. Although primarily used for pathogen detection, SP-PCR has the potential for much broader application, including disease diagnostics, genotyping and expression studies (Calvo *et al.*, 1998).

Touchdown polymerase chain reaction: Step-down is a variant of PCR that aims to reduce nonspecific background by gradually lowering the annealing temperature as PCR cycling progresses. The annealing temperature at the initial cycles is usually a few degrees (3-5°C) above the T_m of the primers used, while at the later cycles, it is a few degrees (3-5°C) below the primer T_m. The annealing temperature is decreased by one degree centigrade every cycle or every second cycle until a specified or touchdown annealing temperature is reached. The touchdown temperature is then used for the remaining number of cycles. The higher temperatures give greater specificity for primer binding and the lower temperatures permit more efficient amplification from the specific products formed during the initial cycles (Schiavoni *et al.*, 2010).

Asymmetric PCR: It preferentially amplifies one DNA strand in a double-stranded DNA template. It is used in sequencing and hybridization probing where amplification of only one of the two complementary strands is required. PCR is carried out as usual, but with a great excess of the primer for the strand targeted for amplification. Because of the slow (arithmetic) amplification later in the reaction after the limiting primer has been used up, extra cycles of PCR are required. A recent modification on this process, known as Linear-After-The-Exponential-PCR (LATE-PCR), uses a limiting primer with a higher melting temperature (Tm) than the excess primer to maintain reaction efficiency as the limiting primer concentration decreases mid-reaction (Szilvasi *et al*., 2005).

Digital PCR: This PCR simultaneously amplifies thousands of samples, each in a separate droplet within an emulsion (Vogelstein and Kinzler, 1999).

Nested PCR: Nested PCR increases the specificity of DNA amplification, by reducing background due to non-specific amplification of DNA. Two sets (instead of one pair) of primers are used in two successive PCRs. In the first reaction, one pair of primers is used to generate DNA products, which besides the intended target, may still consist of non-specifically amplified DNA fragments. The product (s) are then used in a second PCR with a set of primers whose binding sites are completely or partially different from and located 3' of each of the primers used in the first reaction. Nested PCR is often more successful in specifically amplifying long DNA fragments than conventional PCR, but it requires more detailed knowledge of the target sequences (Rahimi and Doosti, 2012; Obeidat *et al*., 2012).

Universal fast walking: Universal Fast Walking (UFW) is used for genome walking and genetic fingerprinting using a more specific 'two-sided' PCR than conventional one-sided approaches (using only one gene-specific primer and one general primer - which can lead to artefactual noise) by virtue of a mechanism involving lariat structure formation. Streamlined derivatives of UFW are LaNe RAGE (lariat-dependent nested PCR for rapid amplification of genomic DNA ends), 5'RACE LaNe and 3'RACE LaNe (Myrick and Gelbart, 2002).

Why PCR still competitive?: From above discussions, it is clear that PCR is still as competitive as other nucleic acid amplification methods. The reasons of its competitiveness are-1. Availability of reagents and equipments. 2. Availability of enough literatures and methods to consult. 3. Availability of extensively validated methods. 4. Easy to understand principles. 5. Convenient detection method of amplicons. 6. Suitability to be used in nucleic acid sequencing, even with the newer sequencing methods such as pyrosequencing (Fakruddin and Chowdhury, 2012; Fakruddin *et al*., 2012c).

CONCLUSION

Though it is evident that PCR will be replaced by any of the future methods, the impact it has created on molecular biology in a short time span will be credited forever. Future automation and incorporation of new computer algorithms into different polymerase chain reaction will help in deciphering the entire genome of various organisms and generate more information on the evolutionary relation between organisms.

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