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Life Sciences Revolution: PCR and Its Application In Molecular and Microbiology.

REVIEW ARTICLE

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Abstract:

The polymerase chain reaction and its expanding numbers of modifications have become a mainstay in diagnostic and research medicine. The technique allows amplification of nucleic acid sequences both for the purposes of disease and pathogen detection and also for the preparation of hybridization probes and sequencing templates. PCR mimics the in vivo process of DNA replication with a sensitivity which enables detection of a single target sequence in 106 genomes. The principles of standard PCR, together with its more widely-used variations, are reviewed and their main applications outlined.

Keywords:
PCR, Biotechnology, Molecular Biology, DNA, Genetics, Microbiology.

Introduction:

The development of molecular biology was one of the greatest achievements in biological science in the century XX. The discovery of Polymerase Chain Reaction (PCR) brought enormous benefits and scientific developments such as genome sequencing, gene expressions in recombinant systems, the study of molecular genetic analyses, including the rapid determination of both paternity and the diagnosis of infectious disease. PCR enables the in vitro synthesis of nucleic acids through which a DNA segment can be specifically replicated in a semi-conservative way. It generally exhibits excellent detection limits.

Recently, a technological innovation of PCR, known as Real-Time PCR, has become increasingly important in clinical diagnostics and research laboratories due to its capacity for generating quantitative results. This technique allows accompanying the reaction and presentation of results in a faster and more accurate fashion than conventional PCR, which only displays the qualitative results.

PCR Technology Conventional PCR-

PCR was developed in the 1980s by Kary Mullis, who received the Nobel Prize in 1994. Since its description, this technology has caused a veritable revolution in biological research, establishing the agreement of basic biological processes in applied areas involving diagnoses and genetic improvements for plants and animal. PCR enables the synthesis of specific DNA fragments using a DNA-polymerase enzyme, which takes part in the replication of the cellular

genetic material. This enzyme synthesizes a complementary sequence of DNA, as a small fragment (primer) is connected to one of the DNA strands in the specific site chosen to start the synthesis. Primers limit the sequence to be replicated and the result is the amplification of a particular DNA sequence with billions of copies.

The development of tools for amplifying DNA segments has generated enormous benefits in gene analysis as well as the diagnosis of many genetic diseases and the detection of bacterial, viral and fungal pathogens. Another useful PCR application is the cloning of a particular DNA fragment, which allows the study of gene expression and has considerable potential in forensic medicine.

Real-Time PCR-

The possibility of Real-Time PCR monitoring has revolutionized the quantification process of DNA and RNA fragments. Real-Time PCR allows the precise quantification of these nucleic acids with greater reproducibility. This technique provides a sensitive method for the accurate quantification of individual species, which could be very relevant to the diagnosis of pathogens and genetic diseases. Advantages of Real-Time PCR include the ease of quantification, greater sensitivity, reproducibility and precision, rapid analysis, better control of quality in the process and a lower risk of contamination.

Real-Time PCR requires a thermocycler with an optical system to capture fluorescence and a computer with software capable of capturing the data and performing the final analysis of the reaction. The programs available from diverse manufactures exhibit differences regarding sample capacity, method of excitation and total sensitivity. There are also differences between regarding the data processing. The emission of fluorescence generates a signal that increases in direct proportion with the amount of PCR products. Fluorescence values are recorded during each cycle and represent the amount of amplified product. The fluorescent composites used are SYBR® Green and TaqMan® .

Forms of detection-

The fluorescence signals are proportional to the amount of PCR product generated by the fluorescent dyes, which are specific to double-stranded DNA (dsDNA), or by sequence-specific oligonucleotide probes.

SYBER Green- I dye -

SYBR Green I is the most used dsDNA binding-specific dye in real-time PCR. Its fluorescence is undetectable when not bound to dsDNA. Its binding affinity to DNA is 100 times greater than that of ethidium bromide, which is the most often used dsDNA binder in conventional PCR . The disadvantage to SYBR Green I is that it binds to any dsDNA, such as non-specific amplification products and primer dimers. Amplified non-specific products affect the efficiency of the amplification of specific products. Thus, analysis should be

optimized in such a way that non-specific amplification does not occur. Melting curve analysis after the PCR reaction is a good practice for controlling the formation of dimer primers. Fluorescence is measured as a function of temperature, gradually diminishing with the increase in temperature of the amplified product. However, upon reaching the temperature at which the double-stranded DNA separates, the stain detaches and fluorescence drops off abruptly . Once optimized, detection by SYBR Green I is highly sensitive to the identification of a single molecular target in the reaction mixture. The greatest advantage is that it can be used with various pairs of different primers, making it less expensive than a probe.

Minor groove DNA binder probes (MGB)-

MGB probes consist of oligonucleotides from 14 to 15 pb in length that carry a fluorescent dye in terminal 5', as well as a non-fluorescent quencher and MBG in terminal 3',, which specifically hybridizes with a target sequence. MGB is released from a probe that binds to the minor groove of the dsDNA (consisting of part of the MGB probe and complementary target sequence by which it is hybridized) related to the nucleotide sequence. The MGB increases binding stability to the amplification probe .

Molecular beacons-

It combine an oligonucleotide capable of forming a stem-loop structure with the quencher-reporter pair. Specifically, an oligonucleotide probe with a binding domain to the antisense target flanked by two short arms of complementary sequences is marked in one terminal with the reporter dye and in the opposite terminal with the quencher dye. In the absence of the target, the short arms anneal to form a hairpin structure (stem-loop), forcing the flourophore toward the quencher. In this conformation, the molecular beacon is 'dark'. Through hybridization with the target sequence, the hairpin structure opens, separating the fluorophore and quencher and resulting in the restoration of fluorescence ('shining' state). The transition between the dark and shining state of the molecular beacon allows the differentiation between bound and unbound probes.

Principles and Applications in Virology-

Recent advances in molecular biology have made possible the detection and characterization of viral nucleic acids. Methods such as PCR enable the amplification of specific regions of interest. Technological improvements in the detection systems of gene sequences provide a complete viral characterization, determining the subtype, genotype, variation, mutation and standards of genotypic resistance of these viruses. The recent development of Real-Time PCR has facilitated the detection and amplification of PCR products. This method is useful in quantifying a larger range of sequences of viral nucleic acids than most quantitative methods. Moreover, the qualitative detection also is possible. Quantification and qualification are carried out automatically.

Examples of the detection and quantification of specific viral regions have been published and this field of study is growing very quickly.

The newer molecular methods are advantageous, mainly in cases for which the viral culture routine is not available. The introduction of molecular biology in clinical diagnosis is important to reducing the use of viral culture techniques. The implementation of automatic extraction and detection, combined with an extensive quality control program, should convince the clinical community that molecular diagnosis is important in clinical virology. The ability to exclude viral infections can help avoid unnecessary therapies, such as powerful antibiotics and antiviral medicines, as well as reduce costs incurred on the part of patients. Thus, these techniques are important to establishing the best therapeutic protocol.

Real-Time PCR is extremely useful in the study of viruses that cause infectious diseases. The majority of published assays show an increase in the frequency of viral detection. This is therefore an attractive technology for many virological fields . It is valued for its quickness in the detection of viral variants and the syndromes caused by these viruses. The method contributes to epidemiological studies due to its capacity to quantify nucleic acids in a single reaction. New chemicals have allowed a better discrimination of multiple viral genotypes within a single reaction and have provided an alternative viral detection method based on morbidity and mortality assays.

For many years, the diagnosis of viral infections has been hampered by the high costs, laboratory time and qualified personnel required in the cell culture process. An additional negative factor is the low sensitivity and slow development of many viruses in artificial mediums. PCR technology facilitates and improves detection, thereby facilitating the diagnosis of a certain number of these viruses.

Mycology and Parasitology-

The ability to accurately identify microorganisms is fundamental to all aspects of fungal epidemiology and diagnosis. In phytopathology, the early identification of disease-causing agents is essential to the recognition of pathogens. In the last ten years, advancements have been made in the molecular diagnosis of fungi through PCR technology. Unlike conventional methods, samples can be tested directly through PCR and isolated without the need for cultures. The technique is fast and highly specific. It can be used to detect trace amounts of fungal DNA from environment samples before symptoms occur. It therefore allows the implementation of early disease control methods. PCR can be performed routinely and does not require specialized skill to interpret the results. The technology can also offer more accurate quantitative data, providing additional information necessary for decision making and the assessment of how effective fungal agents are in

biological control. Since its introduction in the mid 1980s, PCR has become the cornerstone of DNA technology and has cleared the path for the creation of innumerous associated technologies. It is remarkable for its ability to detect amounts of DNA amplified from one or few original sequences. Conventional PCR is not quantitative, but rather qualitative. It has been used to detect, monitor and identify fungi from an entire set of environmental samples and is the core of molecular fungal diagnostics (4). Another example of the use of PCR technology in mycology is in the detection of infection from Aspergillus ssp. in patients with neutropenia. This disease is notoriously difficult to diagnose due to the poor sensitivity of the culture method and the difficulty of finding histopathological specimens in individuals with low platelet counts. Early treatment is essential to achieving the best results. PCR can reduce the time required for the specific diagnosis. Real-Time PCR has been successfully used to quantify the number of pathogens, thereby assisting in decisions regarding how to treat fungal diseases and assess the effects of fungi.

Parasitological diagnostics can be assisted by molecular methods. Many parasites are not cultivable in laboratory and diagnosis principally relies on serology and relatively less sensitive microscopy. Microscopy remains a support to the diagnosis of malaria, but due to its greater sensitivity, PCR can diagnose this illness in even in difficult situations. Plasmodium species can also be detected in different infections, which can hinder microscopic discernment.

Principles and Applications in Microbiology-

Conventional PCR has been used for over a decade in clinical microbiology laboratory research for the identification of microbial pathogens . However, for a number of reasons, this technique has been restricted to the detection of microorganisms that either have slow growth or cannot be cultivated. Most tests based on conventional PCR involve multiple steps and, therefore, require careful expertise. These assays often require both time and culture-based methods, thereby increasing the costs. Conventional PCR also involves an open-reaction system, which is more susceptible to contamination from foreign amplified DNA. Conventional PCR assays have been developed for Bacillus anthracis, the Anthrax agent and Variola Major, but clinical validation of these assays is limited due to the unavailability of the human specimen. Real-time PCR has important, immediate implications to diagnostic tests in the clinical microbiology laboratory. The enhanced sensitivity, ease of use and quickness of this technology make it an attractive alternative for detecting microorganisms in humans.

Bacteriology-

Anaerobic bacteria are involved in a broad range of infections that are commonly associated to considerable morbidity and mortality rates .Although different types of these bacteria are frequently found in diverse infections,

evidence suggests that a number of these clinically important pathogens are as yet poorly characterized due to the inadequacy of conventional anaerobic bacteriological methods and phenotype tests. According to recent studies, 50 to 75 percent of anaerobic bacteria are satisfactorily characterized and 27 percent of laboratories indicated that they have never identified such bacteria . This occurs mainly because conventional identification is complicated, expensive and time-consuming. It is also not reliable, as it is based on antiquated taxonomy.

The diagnosis of infections due to specific bacteria has greatly benefited from molecular detection. Many of these bacteria are public health concerns, such as Micobacterium tuberculosis, Chlamydia Trachomatis, Neisseria gonorrheae and Bordetella pertussis. Tests based on molecular methods have the advantage of avoiding days or weeks of delay and allow early recognition and treatment. Commercial assays are available for M. tuberculosis, Micobacterium avium complex, C. trachomatis and N. gonorrheae.

Due to the increased sensitivity, the use of molecular detection methods for sexually transmitted bacteria has led to an increase in the proportion of cases confirmed in laboratory of the diseases such bacteria cause. Traditional sexual health exams require the use of a speculum in women and a urethral swab in men. These exams require special equipment and can cause both embarrassment and discomfort. Molecular detection is a non-invasive method, which increases trust and reduces discomfort. Molecular testing also encompasses a range of genital pathogens, such as C.Trachomatis, N. gonorrheae, etc.

Conclusions:

Based on the items research and described above, the following may be concluded:

Methods associated to molecular biology have made excellent progress, with clear usefulness in diverse fields of medical science. The discovery of Polymerase Chain Reaction (PCR) introduced an technological advancement that is relevant for the detection of microorganisms, increasing the sensitivity, precision and accuracy of the diagnosis,

In virology, the molecular detection and characterization of viral nucleic acids makes a complete viral characterization possible, thereby providing greater knowledge regarding the behavior of the virus and selected infectious processes. In the diagnosis of viruses, this enhances therapeutic treatment as well as clinical and epidemiological virus studies, thereby avoiding unnecessary treatments and reducing overall costs for patients,

In mycology and parasitology, PCR technology favors the early identification of microorganisms, thereby enhancing epidemiological studies as

well as the diagnosis of fungi and parasites, which is essential to the recognition of pathogens. Molecular tests assist in the decision-making process regarding fungal and parasitological diseases by assessing the effects of these microorganisms,

In microbiology, PCR permits important, immediate observations for diagnostic tests in the detection of microorganisms. Genotyping allows the study of bacteria such as Mycobacterium tuberculosis. This is of tremendous worth for public health, favouring the early recognition and optimized treatment.

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