

PRINCIPLES AND APPLICATIONS OF POLYMERASE CHAIN REACTION IN MEDICAL DIAGNOSTIC FIELDS: A REVIEW

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ABSTRACT

Recent developments in molecular methods have revolutionized the detection and characterization of microorganisms in a broad range of medical diagnostic fields, including virology, mycology, parasitology, microbiology and dentistry. Among these methods, Polymerase Chain Reaction (PCR) has generated great benefits and allowed scientific advancements. PCR is an excellent technique for the rapid detection of pathogens, including those difficult to culture. Along with conventional PCR techniques, Real-Time PCR has emerged as a technological innovation and is playing an ever-increasing role in clinical diagnostics and research laboratories. Due to its capacity to generate both qualitative and quantitative results, Real-Time PCR is considered a fast and accurate platform. The aim of the present literature review is to explore the clinical usefulness and potential of both conventional PCR and Real-Time PCR assays in diverse medical fields, addressing its main uses and advances.

Keywords: Conventional PCR; Real-Time PCR; Molecular Biology; Methods of molecular detection

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 (73,99). 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 (19,101).

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 (50,62,73).

The aim of the present study is to outline the principles and applications of conventional PCR and Real-Time PCR techniques in some medical sciences. It also seeks to evaluate and discuss the indications, uses and advantages of these techniques, as well as their advances in various medical areas.

PCR TECHNOLOGY CONVENCIONAL PCR

PCR was developed in the 1980s by Kary Mullis, who received the Nobel Prize in 1994 (14). 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 (101). 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

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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 (66,73).

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 (4,72,73,99). 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 (94).

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 (62,73).

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[®]* (50,73).

Real-Time PCR Technology

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 (64,113). 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 (74). However, upon reaching the temperature at which the double-stranded DNA separates, the stain detaches and fluorescence drops off abruptly (84). 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 (51).

Hybridization probes

Oligonucleotide probes marked with fluorophores are used for the detection of specific sequences (16,115). The amount of the fluorescence may be related to the amount of PCR product through the product-dependent reduction of a quencher fluorophore and a reporter or through an increase in the fluorescent resonance energy transfer (FRET) from a donor fluorophore to a receptor. The donor probes are marked in the 3' terminal portion with a reporter fluorophore (often 6-carboxyfluorescein, FAM) and the acceptor probes are marked in the 5' terminal portion with an acceptor fluorophore (cyan dyes Cy3, Cy5; 6-carboxy-4,7,2',7'-tetrachlorofluorescein, TET; 6-carboxy-N, N, N', N''-Tetramethylrhodamine, TAMRA; 6-carboxyrhodamine X, ROX). Only the donor fluorophore is excited in such a way that no fluorescent acceptor is detected in the free-floating probes. During the annealing phase of the primer, the probes hybridize adjacently to the single-stranded DNA (ssDNA) and the excitation energy is transferred from the donor to the acceptor. Four oligonucleotides are used in this format: two primers and two probes. The amount of fluorescence is proportional to the amount of target DNA generated during the PCR process (10).

TaqMan Probes

The first fluorescent probe developed for real-time PCR was **5' nuclease**, which is commonly referred to by the name *TaqMan*.

A TaqMan probe is a short oligonucleotide (DNA) that contains a 5' terminal reporter fluorophore similar to fluorescein and a 3' terminal quencher. Intact probes do not emit fluorescence because they are bound (quenched). Two events must occur to generate a fluorescent signal. First, the probe must bind to a complementary DNA strand at 60°C. Second, at this temperature, *Taq* polymerase, the same enzyme used for the PCR, must cleave the 5' terminal TaqMan probe (5' nuclease activity), separating the fluorescent dye from a quenching dye. The quencher is released from the fluorophore, which now fluoresces after excitation (28,33).

Molecular beacons

Tyagi, Kramer (106) first evaluated molecular beacons 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 fluorophore 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) (107). The transition between the dark and shining state of the molecular beacon allows the differentiation between bound and unbound probes (49,106,108).

Sunrise primers

Sunrise™ Probes (Oncor, Inc.) (70) function in a similar manner to TaqMan probes. They are also doubly marked with a fluorochrome in the 5' terminal portion. The 3' region of the probe is the specific target and the 5' region is the complementary target, such that when not extended (not incorporated in the amplicon), it forms a hairpin structure that holds the quencher and reporter marker together. When the probe is extended and integrated within a dsDNA molecule, the quencher and reporter are kept apart by a recently copied complementary strand. Like conventional TaqMan, Sunrise primers require a new probe for each amplification (109).

In the first phase, the Sunrise primer is extended with the forward primer. This extended product serves as the template for the reverse primer in the second phase. In the end, polymerase opens the hairpin structure and a double-stranded PCR product is formed, in which the reporter and quencher are separated (70).

Scorpion primers

Scorpion primers are structurally and functionally related to molecular beacons, but serve as primers in PCR. Two different

formats are possible: 'stem-loop' and 'duplex'. In both cases, the marking mechanism is intra-molecular. The basic elements of scorpions are: (i) a PCR primer; (ii) a PCR stopper to prevent the cross-reaction of a probe; (iii) a sequence-specific probe; and (iv) a fluorescence detection system containing a minimum of one fluorochrome and one quencher. After PCR amplification of the scorpion primer, the resulting amplicon contains a sequence that is complementary to the probe, which is restituted a single strand during the denaturation stage of each PCR cycle. With cooling, the probe is free to bind to this complementary sequence, producing increased fluorescence. Thus, the quencher is not increased in the proximity of the fluorophore (110).

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 (56,71). 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 (39,72).

The implementation of molecular methods has resulted in progress regarding the diagnosis of many viruses and the monitoring of antiviral therapy, especially HIV-1 (Human Immunodeficiency Virus type 1) (92), HBV (Hepatitis B Virus) (79) and HCV (Human Cytomegalovirus) (23). It has also led to the development of amplification assays on nearly all human viruses, including those that are more easily be cultivated, such as HSV-1 and HSV-2 (Herpes Simple Virus type 1 and 2) (25).

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 (46,120). 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 (56,72).

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 (56). It is valued for its quickness in the detection of viral variants and the syndromes caused by these viruses (27). The method contributes to epidemiological studies due to its capacity to quantify nucleic acids in a single reaction (46,120). New chemicals have allowed a better discrimination of multiple viral genotypes within a single reaction (44) 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 (100).

PRINCIPLES AND APPLICATIONS IN 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 (60). 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 innumerable 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).

Fluorescent PCR *in situ* utilizes fluorescently marked primers or probes to detect and locate fungi in fixed environmental samples following semi-permeabilization (102). The fluorescence of the primers or probes is detected using a confocal microscope. This technique allows the direct detection of the organism in

the sample. It also shows the spatial distribution, interactions with the host and other organisms. Bago, Piche, Simon (5) used *in situ* PCR to detect and locate infections caused by Arbuscular Mycorrhiza fungi. The scope of PCR is infinite (5). It can be used to investigate either a single species or entire communities (22,35,80).

Pneumocystis jiroveci (a fungus previously denominated *Pneumocystis carinii*) can cause severe pneumonia in patients infected with HIV or otherwise immunosuppressed, but its detection is restricted to the microscopy of specimens in the respiratory tract. Microscopy for the detection of *P. jiroveci* generally involves the use of stains. Immunofluorescence is more sensitive than these stains, but is more expensive and requires specialized facilities. PCR is more sensitive, especially in patients not infected with HIV, and can therefore be of considerable usefulness (36). PCR specificity is limited, but as this microorganism is an omnipresent commensal, it can be detected through PCR in the absence of the disease (37).

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 (111). Real-Time PCR has been successfully used to quantify the number of pathogens (7,13,21, 112), thereby assisting in decisions regarding how to treat fungal diseases and assess the effects of fungi (57).

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 (99)

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 (114). 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 (9) and Variola Major (26), 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 (18).

Bacteriology

Anaerobic bacteria are involved in a broad range of infections that are commonly associated to considerable morbidity and mortality rates (98,116). 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 (2). This occurs mainly because conventional identification is complicated, expensive and time-consuming. It is also not reliable, as it is based on antiquated taxonomy (97).

Molecular detection methods are a powerful means of identifying these pathogens in the study of the parasite-host relationship, clarifying the taxonomic positions of known pathogens. Thus, there is a growing trust in genotyping for microbial characterization. Genotypes are more specific and more easily quantified and standardized between different organisms than traditional phenotype markers. In the last ten years, a number of advances have been made in molecular bacterial diagnostics, including the greatest discovery: PCR technology (97,116). Real-time PCR has recently emerged and has been used in the detection and quantification of anaerobic bacteria. This provides users with the ability to amplify DNA as well as detect and confirm the specific sequences of microorganisms. Each cycle in real time also provides greater sensitivity.

PCR for the detection of *Lactobacillus*, *Gardnerella vaginalis* and *Mycoplasmas hominis*, genital tract bacteria was used (119). In another study, Aliyu *et al.* (1) reported the importance of *Fusobacterium necrophorum* as a cause of acute pharyngitis. There are a considerable number of studies using real-time PCR in the characterization of bacterial communities in the human intestine (6,40,58).

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 gonorrhoeae* 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. gonorrhoeae* (116).

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. gonorrhoeae*, etc. (100).

PRINCIPLES AND APPLICATIONS IN DENTISTRY

The recognition of the universality of the genetic code in living organisms has been essential to the development and application of genetic technologies. A number of methods have been employed in dentistry to better understand and diagnose infectious agents that lead to maxillofacial infections, thereby obtaining an evaluation of risk patients might have to caries, periodontal disease and other oral conditions (47). These methods include cultures, microscopy, immunofluorescence assays and DNA probes. More recently, polymerase chain reaction has been introduced. Many different types of clinical samples have been used for PCR analyses, including blood, sweat, semen, strands of hair and saliva. Saliva is a potential source of genetic material for diagnostic tests in oral and systemic diseases. With the recent interest in associations between systemic disease and the mouth as well as the successful use of saliva in molecular diagnostics, saliva sampling may soon become part of the routine dental exam. Furthermore, as a medium where biologically active proteins and exogenous substances are found, saliva is also a source of patient DNA (82,88,103) as well as cariogenic and periodontopathogenic microorganisms (32,42,48,76).

The publication of the PCR technique was a revolutionary watershed for medicine and science. It has recently become a standard diagnostic and research tool in dentistry. The literature reports the uses of PCR for the study of factors involved in periodontal disease, dental caries, endodontic infections and oral cancer (90).

Periodontal disease

Diverse methods have been reported for the rapid detection of periodontal pathogens, such as immunological and immunoenzymatic assays, protein electrophoresis and DNA-DNA hybridization. However, these methods exhibit different limitations that can lead to false positive results as well as cross-reactions (3,17).

PCR is an excellent tool for directly identifying periodontal pathogens in subgingival samples. Due to its sensitivity and

specificity, it is also a rapid, efficient method for detecting, identifying and differentiating organisms, but appropriate standardization is necessary (24). Diverse molecular means are often used to identify periodontal pathogens, but PCR is considered the easiest and fastest method in clinical samples (83). PCR may soon become the ideal detection method for periodontal pathogens due to its greater ease of use in comparison to cultures associated with biochemical identification tests. It also demonstrates excellent detection limits with few cross-reactions under ideal conditions (19).

In researching the possible involvement of human viruses in periodontal disease, Parra, Slots (77) determined the prevalence of Human Cytomegalovirus (HCMV), Epstein-Barr Virus Type I and II (EBV 1 and 2), Herpes Simplex Virus (HSV), Human Papillomavirus (HPV) and Human Immunodeficiency Virus (HIV) in the crevicular fluid of individuals with various forms of periodontal disease. Viral identification was carried out using the PCR technique. The study provided evidence of human viruses in the crevicular fluid of the most advanced adult periodontal lesions. Ssygun *et al.* (91) confirmed the frequent presence of HCMV in crevicular samples of chronic periodontal lesions and suggested a strong relation between HCMV and EBV-1 in subgingival areas with deep furrows accompanied by a loss of insertion.

In studying the expression of virulent factors of *Porphyromonas gingivalis* in periodontitis, Shelburne *et al.* (93) modified methods of quantitative measurements and gene activation, and discovered (real-time) quantitative reverse transcription of PCR (QRT-PCR) to examine gene expression *in vivo* in the oral cavity of the anaerobe *P. gingivalis*. The authors described these initial results with QRT-PCR using a selection of virulent factors. The effectiveness of the PCR method in the detection of *P. gingivalis* from saliva samples was compared to bacterial cultures performed by Matto *et al.* (59), demonstrating that PCR detected the bacteria in saliva three time more frequently than the culture method.

The PCR was also used by Sakamoto *et al.* (89) for the detection and identification of *Treponema socranskii* associated to periodontal disease. This pathogen is related to an inflammation in the gingiva in both children and adults. *T. socranskii* was detected more frequently in subgingival plaque samples than in saliva samples.

Studies have detected *Actinobacillus actinomycetem-comitans* using PCR in the most distinct patterns in a population with (70%) and without (19%) periodontal disease. These studies have also shown the sensitivity and specificity of PCR in comparison to traditional culture methods (65,104). Okada *et al.* (75) used PCR to detect the presence of *A. actinomycetem-comitans* and *P. gingivalis* in dental plaque samples from children using their toothbrushes. The research indicated that such bacteria are rarely present in the oral cavity of healthy children. In 2001, the researchers also detected the presence of *P.*

Intermedia, nigrescens, B. forsythus, T.denticola and *C. rectus* in periodontal disease.

Real-Time PCR offers a sensitive, efficient, faithful approximation for quantification. Using the TaqMan® systems, Lyons *et al.* (54) were able to determine both the amount of *P. gingivalis* and total number of bacteria present in bacterial plaque without the use of cultures. Furthermore, this allowed the authors to determine the percentage of *P. gingivalis* in a complex sample.

Sakamoto *et al.* (88) compared conventional PCR, real-time and the culture method in the detection and quantification of periodontopathogenic bacteria, including *A. actinomycetem-comitans, B. forsythus, P. gingivalis, T. denticola* and *T. socranskii* in both saliva and subgingival plaque. There was satisfactory agreement between the results from conventional and real-time PCR for all the saliva samples. The use of real-time PCR optimally simplified the process and was able to determine the amount of periodontopathogenic bacteria within one hour. These bacteria were more frequently detected in the saliva than the subgingival plaque. The study suggests the saliva is as good, or better, than subgingival plaque for the detection and quantification of periodontal bacteria in the oral cavity.

Therefore, PCR has revolutionized understanding regarding periodontal pathogens. These studies not only permit the diagnosis of known pathogens, but also contribute toward the identification of new pathogens involved in periodontal disease (47). PCR is expected to be successful in the microbiological diagnoses and periodontal disease (89,90).

Dental Caries

The group *mutans*, which encompasses cariogenic bacteria, has seven species: *Streptococcus cricettus, S. ratti, S. mutans, S. sobrinus, S. downei, S. ferus* and *S. macacae* (69,108), of which *S.mutans* and *S. sobrinus* are more frequently isolated from the human oral cavity (38,108,117). For epidemiological studies on caries, a rapid, sensitive, simple method is needed for the identification and differentiation of these microorganisms. A number of methods have been used to identify these isolated species in the oral cavity, such as biochemical, immunological and genetic methods (41). Different techniques are used in conjunction in order to achieve reliability in species identification. This requires time and considerable ability, and the results are often unsatisfactory. Species characterization based on DNA testing is currently widely accepted, as phenotyping is a reflection of gene expression (105). As mentioned above, PCR was recently introduced to solve these problems. Tests related to genotyping are currently being used due to the possibility of its being more sensitive and specific (118).

PCR appears to be convenient for studying the epidemiology of disease in isolated individuals. It may prove useful in the identification of species associated with dental caries and their

location in the ecological niches, thereby helping to clarify the progression of the carious process (82). PCR has the potential to replace conventional identification methods, such as biochemical and immunological tests (43). The discriminative power of PCR in the differentiation of *S. mutans* and *S. sobrinus* serotypes and lineages was investigated by Saarela *et al.* (87), who found that PCR exhibited good results in differentiating *S. mutans* lineages and the technique is appropriate for epidemiological studies on this bacterium.

Rupf *et al.* (86) presented a competitive PCR method for the specific quantitative determination of *S. mutans*. This method allowed a quick, exact determination of unknown quantities of this bacterium and provided an efficient means for evaluating the risk of caries in patients as well as monitoring the efficiency of preventative and therapeutic measures.

Endodontic Infections

With the development of molecular methods based on the detection of specific genomic regions, it became possible to identify microbial species in infected root canals that had never been seen by means of the conventional culture procedure. Examples of bacterial species that were only detected in canals through molecular methods and that are currently considered important endodontic pathogens include *Treponema denticola*, *Dialister pneumosintes*, *Filifactor alocis*, *Tannerella forsythia*, *Treponema malthophilum*, *Treponema socranskii* and *Prevotella tanneriae* (96). Based on current studies, molecular methods can be used to characterize the micro-flora associated to endodontic infections (47). Using PCR, Bogen, Slots (12) sought to determine the prevalence of *P. endodontalis*, *P. gingivalis*, *P. intermedia* and *P. nigrescens* in 20 with refractory periapical lesions. The authors concluded that bacteria that produce black pigment do not appear to constitute the majority of microorganisms in these cases. The relatively lower occurrence of these bacteria may help explain the relative stability and chronic nature of this condition.

Molecular methods such as PCR are more sensitive and specific than the conventional culture method, which is based on identification through the phenotypic characteristics of each microbial species (97). Molecular methods, however, are based on the detection of specific genomic sequences for each microbial species and can even discriminate clones within a single species (85). Although studies have determined that bacteria are the greatest etiologic agent in pulp and periradicular disease, fungi have also been associated to root canal infection. Baumgartner *et al.* (8) used PCR to assess the content of infected root canals as well as cellulite aspirations and abscesses of endodontic origin for the presence of *Candida albicans*. The results indicate that PCR is an extremely sensitive molecular method and can be used to identify *C. albicans* directly in samples of endodontic infection.

PCR has been widely used to identify microbial species that

are difficult or impossible to cultivate, as well as colonies within a species that exhibit a different phenotypic behavior and are therefore difficult to identify in culture procedures. PCR has a greater detection pattern than traditional microbiological identification methods and exhibits greater specificity under optimized conditions. Thus, the use of identification methods based on the knowledge of molecular biology has revolutionized medical microbiology and is broadening the horizons with regard to the actual profile of endodontic infection (85,95).

Oral Cancer

One of the uses of PCR in dentistry is the detection of markers in the diagnosis and prognosis of some types of oral cancer (45,52,81). For this purpose, PCR is a fast, easy method with a relatively low cost (45). Diagnoses, prognoses and treatment can be improved through the study and use of genetic markers identified by means of immunohistochemistry, PCR and other molecular biology procedures (67).

Squamous cell carcinoma of the oral cavity is generally accompanied by other types of aerodigestive tract carcinomas, such as oropharyngeal and esophageal carcinoma. *Streptococcus anginosus* is a bacterium that may be isolated in different parts of the body and has been isolated in squamous cell carcinoma of the head and neck. Through real-time PCR, *S. anginosus* can be detected with greater sensitivity and specific approximation in squamous cell carcinomas of the oral cavity (63).

Pre-malignant lesions of the head and neck have been studied extensively through genetic alterations. A genetic progression model has been established based on histological alterations that occur in the interior of the pre-malignant epithelium (15). HPV (Human Papillomavirus) has been employed in tumor progression in humans based on data from patients with cancer (29). Determining the moment of viral infections in these pre-malignant lesions could clarify the role of HPV in carcinogenesis and help guide future strategies for the prevention and early detection of squamous cell carcinoma of the head and neck. For such, a large number of studies have been conducted to detect the presence of HPV in head and neck epithelia through the use of a variety of laboratorial methods. Different techniques have been employed, including PCR, *in situ* hybridization, etc. Real-time PCR minimizes the risk of contamination, thereby becoming the ideal assay for HPV DNA detection (31).

The quantification of the number of genetic copies through real-time PCR using either DNA or RNA in studies on gene expression has been reported in the literature for human tumors, including breast cancer (11,53,61), follicular lymphoma (30), stomach cancer (68), prostate cancer (34) and Ewing's sarcoma (78). In carcinoma of the head and neck, this new technology has been mainly applied for the detection of Epstein-Barr virus in nasopharynx cancer (55) and squamous cell carcinoma in lymph nodes (20).

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 a 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, favoring the early recognition and optimized treatment;
- In dentistry, molecular methods enhance knowledge regarding the diagnosis of infectious agents that lead to maxillofacial infections, thereby favoring the assessment of patients at risk for conditions such as caries, periodontal disease, endodontic infections and oral cancer. The publication of the PCR technique was a revolutionary watershed for medicine and science. It has become a standard diagnostic and research tool in dentistry, permitting the early diagnosis of the above-mentioned diseases.

RESUMO

Princípios e aplicações da reação em cadeia de Polimerase em áreas médicas diagnósticas: uma revisão

O advento dos métodos moleculares tem, nos últimos anos, revolucionado a detecção e caracterização dos microorganismos em diversas áreas médicas diagnósticas, tais como virologia, micologia, parasitologia, microbiologia e odontologia. Dentre as técnicas baseadas em biologia molecular, a PCR (Polymerase

Chain Reaction) trouxe enormes benefícios e desenvolvimentos científicos, se mostrando como um excelente caminho para a rápida detecção de patógenos, até mesmo aqueles de difícil cultivo. Derivada da PCR convencional, a PCR em Tempo Real se mostra como uma inovação tecnológica e vem conquistando espaço nos diagnósticos clínicos e nos laboratórios de pesquisa por apresentar a capacidade de gerar, além de resultados qualitativos, resultados quantitativos, se mostrando de forma mais rápida e precisa. Este trabalho de revisão tem por objetivo explorar a utilidade clínica da técnica de PCR convencional e em Tempo real nas diversas áreas médicas supracitadas, abrangendo seus principais usos e avanços, direcionando para o cotidiano profissional.

Palavras-chave: PCR convencional; PCR em Tempo Real; Biologia molecular; métodos de detecção molecular.

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