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Perigrinations of Real Time PCR

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Abstract: ***Polymerase chain reaction (PCR) is a diversifying molecular biology technique in nucleic acid analysis. Real time PCR is the peregrinating form of polymerase chain reaction with accurate quantitation of the cells of individual bacterial species in given dental samples providing the bacterial etiology of endodontic and periodontic infections. Real-time PCR offers efficient, sensitive and reliable approach to quantitation. In addition to snap shot of state of art of real time PCR, here we review the principles of real time PCR, methods of quantitative detection and applications of Real-Time PCR in dentistry.***

Keywords: Polymerase chain reaction-PCR, real time PCR, Reverse transcription PCR – RT-PCR, Endodontic infection, bacterial identification, double-stranded DNA (dsDNA), Fluoroprobes, Amplicons, Quencher.

1. Introduction

Since a decade, Real-Time PCR has peregrinated in clinical diagnostics and research because of its sensitivity, specificity and quantitative results. Real time PCR shows both qualitative and quantitative results whereas conventional PCR holds on to qualitative results[3].Minute quantities of specific DNA (or RNA after reverse transcription – RT-PCR) are enzymatically amplified by real time PCR. Clinical samples used for real time PCR analysis include blood, single hairs, sputum, semen and saliva.[1]

In Real Time PCR technique, fluoroprobes bind to specific target regions of amplicons to produce fluorescence. This fluorescence is detected in a PCR cycler with an inbuilt filter flurometer. The Principles of Real -Time PCR are:

- Based on the complete detection and quantification of a fluorescent reporter.
- Correlating CT - threshold cycle (first significant increase in the PCR product) to the initial amount of target template.

Real-Time PCR has a thermocycler with an optical system where fluorescence is captured by software followed by data analysis of the reaction. The emission of fluorescence is directly proportional to the PCR product generated. Fluorescence values are recorded during each cycle which also represents amplified PCR product. The fluorescent probes used are *SYBR Green and* TaqMan etc.[2, 3] Advantages of Real-Time PCR include the ease of quantification, sensitivity, rapid analysis, better control of quality in the process, reproducibility, precision and a lower risk of contamination.[3, 4] Real time PCR is not influenced by non-specific amplication and the amplication is monitored real time. It has ultra-rapid cycling ranging 30 min - 2 hrs at 10 [10] fold amplification. We just need 1000 fold less RNA in real time PCR when compared to conventional PCR. The aim of this review is to outline the principles and applications of Real-Time PCR in dental sciences.

2. Principles of Real-Time quantitative PCR Technology

The fluorescence is proportional to the PCR product generated by the fluorescent dyes, which are specific to ds-DNA or by sequence-specific oligonucleotide probes. The three general methods of the quantitative assays are;

1) **Hydrolysis probes technique:** The hydrolysis probe is conjugated with a quencher fluorochrome, where it absorbs the fluorescence of the reporter fluorochrome. Intact probes do not emit fluorescence because they are quenched. Upon amplification of the target DNA, the hydrolysis probe is displaced and gets hydrolyzed by Taq polymerase which results in separation of the reporter fluorochrome and increased fluorescence. Eg; TaqMan Probes, Molecular beacons, Scorpion primers and Sunrise primers.
   - **TaqMan Probes:** TaqMan probe is a short oligonucleotide that contains a 5′terminal reporter fluorophore and a 3′terminal quencher. Two events must occur to generate a fluorescent signal. At 60ºC Probe first binds to a complementary DNA strand and Taq polymerase cleave the 5′terminal TaqMan probe, separating the fluorescent dye from quencher dye. The quencher is released from the fluorophore, which now fluoresces after excitation.[5]
   - **Molecular beacons:** beacons combine an oligonucleotide which is capable of forming a stem-loop structure with the quencher-reporter pair. Through hybridization with the target DNA, the stem - loop structure opens up resulting in separation of the fluorophore and quencher thereby enhanced fluorescence.[6]
   - **Sunrise primers:** Sunrise Probes function similar to TaqMan probes. Like conventional TaqMan, Sunrsire primers require a new probe for each amplification. In the first phase, the Sunrise primer is extended and this extended product serves as the template for the reverse primer in the second phase.[7]
   - **Scorpion primers:** Scorpion primers similate molecular beacons. They present themselves in two different

formats: 'stem-loop' and 'duplex'. The basic elements of scorpions are: (i) PCR primer (ii) PCR stopper to prevent the cross-reaction (iii) sequence-specific probe and (iv) fluorescence detection system.[8]

2) Hybridization probe technique: In this technique, one probe is labelled with donor fluorochrome and adjacent probe with an acceptor fluorochrome. When the two fluorochromes are 1-5 nucleotides apart, fluorescence is emitted as donor fluorochrome excites the FRET (fluorescent resonance energy transfer).[9] E.g. light cycler**.**

3) DNA- binding agent:

- **SYBER Green I dye:** It is a ds-DNA binding-specific dye. In real-time PCR, its fluorescence is undetectable when it is unbounded with ds-DNA. The disadvantage of SYBR Green I is that it binds to any ds-DNA, such as non-specific amplification products and primer dimers. Fluorescence is measured as a function of temperature of the amplified product. However, upon reaching the temperature at which the ds DNA separates, the stain detaches and fluorescence drops off abruptly.[10]
- **Minor groove DNA binder probes (MGB):** MGB probes consist of oligonucleotides which carry fluorescent dye in 5á terminal and non-fluorescent quencher in terminal 3′, which hybridizes with a target DNA. MGB is released from a probe that binds to the minor groove of the dsDNA related to the nucleotide sequence. The MGB increases the binding stability to the amplification probe.[11]

3. Applications in Dentistry

Periodontal disease

Diverse methods, such as immune enzymatic assays, protein electrophoresis and DNA-DNA hybridization have been reported earlier for detection of periodontal pathogens. However, these methods exhibit different limitations which lead to false positive results as well as cross-reactions[12].

Real time PCR is adiversified technique in identifying periodontal pathogens in subgingival samples. [10]. With real time PCR investigation, there was prevalence of Human Cytomegalovirus, Epstein-Barr virus Type I and II, Herpes Simples Virus, Human Papillomavirus and Human Immunodeficiency Virus in the crevicular fluid [12]. Using real time PCR, Sakamoto et al. identified Treponema socranskii both in children and adults who were associated with periodontal disease. This was more frequently found in subgingival plaque than in saliva samples. Actinobacillus actinomycetemcomitans, B. forsythus, P. gingivalis, T. denticola were detected using real time PCR.[16] Rreal time PCR has revolutionized knowledge regarding periodontal pathogens. Apart from the diagnosis of known pathogens, they identify new periodontal pathogens.[13]

Dental Caries

Real time PCR method gives specific quantitative determination of Streptococcus cricettus, S. ratti, S. mutans, S. downei , S. sobrinus, S. ferus and S. macacae in dental caries and saliva. Real time PCR allows quick deter mination of unknown bacterium and provides an efficient means for evaluating the risk of caries as well as efficiency of preventative and therapeutic measures[12,17].

Endodontic Infections

Real time PCR identified microbial species in infected root canals that are currently considered important endodontic pathogens which include Dialister pneumosintes, Tannerella forsythia, Treponema socranskii, Treponema denticola and Prevotella tannerae[12.] Using real time PCR the prevalence of P. endodontalis, P. gingivalis, P. intermédia and P. nigrescens, Enterococcus. faecalis, staphylococcus aureus, fungi - Candida albicans were seen[13,17].

Oral Cancer

Diagnoses, prognoses and treatment can be improved by means of immunohistochemistry, PCR and other molecular biology procedures[1,12]. Squamous cell carcinoma of the oral cavity is generally accompanied by aerodigestive tract carcinomas (oropharyngeal and esophageal carcinoma). S. anginosus is detected in squamous cell carcinomas of the oral cavity. Real-time PCR minimizes the risk of contamination, when compared to other molecular methods, thereby becoming the ideal assay for HPV DNA detection.[17]The quantification of the number of genetic copies through real-time PCR has been reported for human tumors, including breast cancer, stomach cancer, prostate cancer, follicular lymphoma and Ewing's sarcoma. In carcinoma of the head and neck, real time PCR technology has been mainly applied for the detection of Epstein-Barr virus in nasopharynx cancer and squamous cell carcinoma in lymph nodes[14].

Limitations of Molecular Methods: Real time PCR is not ideal for multiplexing and there is a chance of inter and intra assay variation. Real time PCR may have DNA contamination in mRNA analysis. Its set up requires high technical skill, support and cost[16].

4. Conclusion

Although qualitative results do not lack significance, the use of benchmark technologies like real time PCR (quantitative molecular assays), can allow inference of given species in the infectious process with enhanced sensitivity and specificity in detecting fastidious or uncultivable microbial species in endodontic and periodontic infections.

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