ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Recent advances in the identification of the microbiota involved in Endo-Perio Lesions -A literature review

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Manuscript Info

Manuscript History:

Received: 15 July 2015
Final Accepted: 22 August 2015
Published Online: September 2015

Key words: Endo-perio,PCR, Red complex organism

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Abstract

The aim of this paper is to discuss the communications between the pulp and periodontium, microorganisms involved in endo-perio lesions and the variousmolecular biology techniques available for more comprehensive broad-range investigation of the bacterial communities in endo-perio infections.This review also examines the recent approaches of themolecular principles, providing us with further information on the hidden metabolic diversity of endo-periomicrobiota. Various revolutionary techniques such as Polymerase Chain Reaction (PCR),Immunofluorescent assays, hybridization assays such as Checkerboard. Fluorescence in situ hybridization (FISH), DNA probes and metagenomic studies provides a promising strategy to study the full genetic pool and will provide an evidence based research revealing factors that can contribute to efficient treatment of endo-perio lesions.

INTRODUCTION

The relationship between periodontal and pulpal disease was first described by Simring and Goldberg in 1964.[1] Dental pulp and periodontium have embryonic, anatomic and functional inter-relationships .[2]They are ectomesenchymal in origin, the cells which proliferate to form dental papilla and follicle, which are the precursors of the pulp and periodontium, respectively. They are separated by the formation and development of tooth bud from the overlaying ectoderm into enamel and dentine.[3]The embryonic development gives rise to anatomical connections which remain throughout the life of the tooth.

The periodontal-endodontic lesions have been characterized by the association of the pulp and periodontal disease in a same tooth, which makes its diagnosis complex because a single lesion may present signs of endodontic and periodontal involvement. This suggests that one disease may be the result or cause of the other or even originate from two different and independent processes which are associated by their advancement .[4]

Pathways of Communication Between Pulp And Periodontium

There are two forms of possible pathways for bacteria and their products to invade the two tissues. It can be classified as: Anatomical and Non-physiological pathways.[5]

Anatomical pathways

The major connections between periodontal and pulpal tissue is the apical foramina.[5]As the proliferation of the Sheath of Hertswig continues, the apical foramen decreases in size but it remains patent and serves as the communication on which the pulpal tissues rely for nutrition and nervous innervation .[6]The apical foramen also serves as the prime and most precise route for bacteria, bacterial toxins and inflammatory mediators to exit easily causing periapical pathosis and in case of deep periodontal pockets the vice versa may occur .[7]

Other than these main avenues of communication, there are a multitude of branches such as the accessory or lateral canals connecting the pulp and the periodontal ligament.[8] As the root develops, ectomesenchymal channels get

incorporated, either due to dentine formation around existing blood vessels or breaks in the continuity of the Sheath of Hertswig, to become accessory or lateral canals.[7]The majority of accessory canals are found in the apical part of the root and lateral canals in the molar furcation regions.[9]

In addition to the apical foramina and accessory canals, there is a third possible route of communication, the dentinal tubules.[5] As many as 15,000 dentinal tubules per square millimeter are present on the root surface at the cervical area. [10]Tubular communication between the pulp and periodontium may occur when dentinal tubules become exposed to the periodontium by the absence of overlying cementum.[6] The pulp chamber can thus communicate with the external root surface in case of denuded cementum through these dentinal tubules.[11]

Non-physiological pathways

They include iatrogenic lesions such as root perforations, overfilling of root canals, coronal leakage, trauma, chemical induced root resorption, intra-canal medicaments and vertical root fractures. [12]Root perforations are undesirable clinical complications that may lead to periodontal lesions. When root perforation occurs, communications between the root canal system and either peri-radicular tissues or the oral cavity may often reduce the prognosis of treatment. Root perforations may result from extensive carious lesions, resorption, or from operator error occurring during root canal instrumentation or post preparation.[13,14] At the site of perforation, an inflammatory reaction in periodontal ligament produces a degradation of surrounding tissues and formation of a lesion.[15]

Vertical root fracture is another common artificial pathway between periodontal and pulpal tissues.They are most often caused when a tooth, often weakened due to undermining caries, previous restorative treatment or a non-vital pulp becomes traumatized.[12]In endodontically treated teeth, excessive force used during lateral condensation of gutta-percha can cause vertical root fracture.Leaching of the root canal contents or bacterial contamination of the fracture line may cause an inflammatory lesion in the periodontal tissues.[15]

Microbiota

It is known that in the oral cavity there are more than 600 species of microorganisms, and the anaerobic bacterias have been directly related to both the apical and periodontal lesion. However, the endodontic ones is considered less complex than the periodontal bacteria. [16]In periodontal disease the following bacterial species may be found*: Porphyromonas gingivalis, Tanerella forsythia, Treponema denticola, Aggregatibacter actinomycetemcomitans (Aa) and Prevotellaintermedia*.[4] According to Siqueira Jr. and Lopes,[4] the bacterias that are part of the red complex are of the species *P. gingivalis, Treponema denticola and Tannerellaforshytia,* being related to severe and isolated forms of periodontitis.Most of the endodontic infections are mixed and polymicrobial, with the predominance of strict anerobic microorganisms. Trope et al.[17] found that in the root canal there is the predominance of anaerobic microorganisms, such as some species of *Porphyromonas and Prevotella*.

Kobayashi et al.[18]reported that microorganisms are common to root canals and periodontal pockets. Among them there were: *Eubacteriumand Fusobacterium spp, Porphyromonas gingivalis, Prevotella intermedia, Peptostreptococcus spp, Capnocytophaga spp, Actinomyces spp and Streptococcus spp*. The similarity between the endodontic and periodontal microbiotainclude bacteria such as *Actinobacillu sactinomycetemcomitans, bacteroides forsythus, Ekinella corrodens, Fusobacterium nucleatum, Porphyromonas gingivalis, Prevotella intermedia and Treponema denticola*.[19] This indicates the strong probability of the occurrence of cross infection between the root canal and periodontal pocket.

Various fungal species especially *Candida albicans* are prevalent both in endodontic infections as well as subgingivally.[20] Recent data also suggests that a number of common types of viruses such as *Cytomegalo virus, Epstein-Barr virus, herpes virus* may be involved in pathogenesis of periodontal and endodontic disease ranging from an increase in periodontal pathogens in periodontal pockets to involvement in pulpal and periapical pathologies.[21,22]The high similarity in the organisms present in both root canals and periodontal pockets suggests that the pocket could be a source of root canal infection and vice versa. The above data also demonstrates that endo-periolesions consist of a diverse and complex microbial community.[23]

Rationale For The Use Of Recent Molecular Techniques For Analysis Of Microbiota Involved In Endo-Perio Lesions.

Despite of the vast micobiota involved in endo-perio lesions,a significant proportion of oral bacteria are unable to undergo cultivation by existing techniques. In this regard, for precise identification of bacteria and their phenotypic properties,the use of recent techniques usingmolecular analysis is recommended.Molecular analyses are sufficient to identify organisms that are uncultivable from samples. They allow to us to predict their physiology or pathogenesis by phylogenetic associations.[24]

Molecular studies have included several new species in the list of periodontal pathogens. These studies revealed that 40–60% of the periodontal microbiomeis made up of as-yet-uncultivated species-level phylotypes.[25] Examples of

uncultivated bacteria found in association with periodontal diseases include phylotypes of the genera *Prevotella, Selenomonas, Desulfobulbus,Peptostreptococcus, Treponema, Fusobacterium*, as well as members of the *Lachnospiraceae* family.[25]

Similar to periodontal diseases, the breadth of bacterial diversity in endodontic infections has been substantially expanded by culture-independent molecular methods. Clone library analyses of different types of endodontic infections reveal that a significant proportion of the microbiome consists of not-yet-cultivated bacteria. Sakamoto et al. reported that uncultivated phylotypes accounted for approximately 55% of the taxa found in root canals of teeth with apical periodontitis. Uncultivated phylotypes from several genera have been identified, *including Dialister, Treponema, Prevotella, Solobacterium, Olsenella, Fusobacterium, Eubacterium,Megasphaera, Veillonella, and Selenomonas* as well as phylotypes related to the family *Lachnospiraceae*. One of the most prevalent as-yet-uncultivated phylotypes found in endodontic infections is *Bacteroidaceae* spp. [25]

Various reports have shown that molecular methods, in addition to being a rapid method, is much more sensitive than traditional culture methods for detection of putative microorganisms which could justify their utilization over traditional culture methods .[26]

Strategies to Cultivate As-Yet-Uncultivated Bacteriain Endo - Perio Lesions

It must be assumed that no single method or culture medium is suitable for isolating the vast diversity of bacteria involved in endo - perio lesions. There has been a growing trend to develop specific approaches and culture media that allow cultivation of previously uncultivated bacteria.[26]Some of the recent techniques includepolymerase chain reaction (PCR), immunofluorescent assays, enzyme linked immunosorbent assays,hybridization assays such as checkerboard and microarrays,fluorescence in situ hybridization (FISH), DNA probes and metagenomic studies.[26]

One of the most revolutionary of these techniques regarding nucleic acid analysis is the polymerase chain reaction (PCR). PCR is a highly sensitive and specific technique by which minute quantities of specific DNA (or RNA after reverse transcription – RT-PCR) can be enzymatically amplified.These techniques can be used to detect very small amounts of bacterial, fungal, or viral nucleic acid in clinicalspecimens.[26]This process uses multiple cycles of template denaturation, primer annealing, and primer elongation to amplify DNA sequences. It is an exponential process since the amplified products from each previous cycle serve as templates for the next cycle of amplification, thus making it a highly sensitive technique for the detection of specific nucleic acids sequences. Typically, enough amplified product is generated after 20 to 40 cycles of PCR, so that it can be visualized on an ethidium bromide-stained gel. The reaction includes several components: template, primer, reverse primer, reaction buffer, magnesium, dNTP mix, and thermostable DNA polymerase. The template can include purified genomic or plasmid DNA; RNA converted by reverse transcriptase to complementary DNA (cDNA); or unpurified, crude biological samples such as bacterial colonies or phage plaques. The forward and reverse primers determine the sequence and the length of the amplified product. The most frequently used thermo stable polymerase is Taq DNA polymerase.[26]

PCR amplification of the bacterial 16S or 23S rRNA gene (rDNA) or other rDNAs is more sensitive and more efficient than culturing and biochemical identification of endodontic flora. In the root canal microbial environment, PCR was shown to be more accurate than sodium dodecyl sulfate-polyacrylamide gel electrophoresis in differentiating and identifying the two important endodontic pathogens, *Prevotellaintermedia and Prevotellanigrescens*, which could not be differentiated by culturing .[27]Okada, Hayashi, Nagasaka in 2001, assessed the presence of *Prevotellaintermedia, Prevotellanigrescens, Bacteroidesforsythus, Treponemadenticola and Campylobacter rectus* using PCR. The study indicated that *P. intermedia and T. denticola*were more associated with periodontal disease.[28]

Takeuchi, et al. in 2001 usedPCR to identify microorganisms and to clarify the relationship between their presence and the severity of clinical periodontitis. He concluded that *T. socranskii, T. denticola e P. gingivalis*was associated with the severity of periodontal tissue destruction.[29] Kumar, et al. in 2003, evaluated the association of newly identified bacterial species or phylotypes with periodontitis. Targets for investigation included both uncultivated phylotypes and characterized species that were not previously thought to be associated with periodontitis. Species-specific ribosomal 16s primers for PCR amplification were developed for detection of new species. Named species commonly found in subjects with chronic periodontitis included *T. denticola, EubacteriumsaphenumPorphyromonasendodontalis, P. gingivalis, T. forsythensis, Filifactoralocis, Prevotelladenticola, Cryptobacteriumcurtum, Treponema medium, T. socranskii, and Actinomycesnaeslundii*.[30]

Siqueira Junior, et al. in 2001 used PCR method to assess the occurence of four black-pigmented anaerobic rods, *T. denticola and A.actinomycetemcomitans*in acute periradicular diseases. The high prevalence of *P. endodontalis, T. denticola, and P. gingivalis* suggested that they could play an important role in the etiology of acute periradicular disease.[31]Using PCR technique, Bogen,et al. in 1999, aimed to determine the prevalence of *Porphyromonasendodontalis, P. gingivalis, P. intermedia and P. nigrescens* in periapical lesions associated with

non-healing endodontically treated teeth.[32]Machado de Oliveira, et al. in 2000, evaluated the occurrence of P. endodontalis in both symptomatic and asymptomatic endodontic infections using 16S rRNA genedirected PCR. The results indicated that, although *P. endodontalis* was commonly detected in symptomatic cases, it could also be present in asymptomatic root canal infections.[33]

In order to assess the expression of mRNA in the development of endo-periolesions RT-PCR [Reverse transcription-polymerase chain Reaction] is used.[26]The procedure combines cDNA synthesis from RNA templates with PCR to provide a rapid, sensitive method for analyzing gene expression. RT-PCR is used to detect or quantify the expression of mRNA, often from a small concentration of target RNA.[34,35,36] The template for RT-PCR can be total RNA or poly (A)+ selected RNA. RT reactions can be primed with random primers, oligo(dT), or a gene-specific primer (GSP) using a reverse transcriptase. RT-PCR can be carried out either in two-step or one-step formats. In two-step RT-PCR, each step is performed under optimal conditions. cDNA synthesis is performed first in RT buffer and one tenth of the reaction is removed for PCR.[35,36] In one-step RT-PCR, reverse transcription and PCR take place sequentially in a single tube under conditions optimized for both RT and PCR.[26]

RT-PCR has also been used in dental research in order to assess the expression of mRNA for proteins involved in the development of periodontal disease.[26]In order to study the role of chemokines in periodontal diseases, Garlet, et al. in 2003 used reverse transcriptionpolymerase chain reaction (RT-PCR) techniques toexamine the expression of chemokines macrophage inflammatory protein-1 alpha and interferon-gamma inducible protein. They concluded that CCR5 and CXCR3 were more prevalent and higher in aggressive periodontitis.[37] Shelburne, et al. in 2002, evaluated the expression of *Porphyromonas gingivalis* virulence factors in periodontitis subjects. They found quantitative (real time)reverse transcription PCR (QRT-PCR) well suited to examine gene expression of *P. gingivalis* in periodontal disease.[38]Periodontal viruses may be identified successfully by using diagnostic DNA microarrays that are able to detect simultaneously Human Herpesviruses (HHV), Human Cytomegalovirus (HCMV), Epstein-Barr Virus (EBV). Multiplex real-time PCR techniques have been used to quantify simultaneously the number of genome-copies. HHV, HCMV and EBV have been isolated from periodontal disease sites and were confirmed through molecular assays. The presence of HHVare also confirmed through DNA probes, Flow cytometryand immunofluorescence assays.[39]

Based on the studies above, molecular methods can be used to characterize the microflora associated with endo-perioinfections without the inherent biases of culture techniques. PCR technique is much more sensitive and fast, allowing for detection of as little as 10 to 10^2 bacteria per human cell.Thus, PCR is considered suitable for detection of pathogens involved in both periodontal and endodontic infections, which could represent a benefit for their treatment.[26]

Other methods like fluorescence in situ hybridization (FISH) approach and its derivations permit that uncultivated bacteria be directly visualized in clinical specimens. By using oligonucleotide probes designed to target specific phylotypes, one can have information about the morphology of the cells, their spatial location in the tissues, as well as their physical relationship to the host tissues and other bacteria in a multispecies community. [25]However, the use of specific DNA probes limits the boundaries of the detection technique, as it assumes that these probes target the species of importanceand do not account for any uncultivated bacteria or uncultivable biotypes of known species.[40]

Furthermore the use of Checkerboard DNA-DNA hybridization is a high-through-put method to analyze large numbers of DNA samples by use of a wide range of DNA probes on a single nylon membrane. The quantity of bacteria in the samples is an important factor in the checkerboard DNA-DNA hybridization technique. The level of detection is about 10^4 bacterial cells of a given species. Since samples from endodontic pathologies often contain very few bacterial cells it may be below the level of detection of the checkerboard method. Another method is the use of multiple-displacement amplification (MDA), the process allows uniform amplification of the whole genome of DNA targets. MDA also provides enough amplified DNA to perform multiple analyses of the same sample by use of different DNA probe sets.[41]

The aforementioned molecular techniques have theirlimitations. These approaches detect species only if they have been previously characterized. Perhaps more importantly, quantitative information is incomplete with these methods since the total number of bacteria is not easily determined with these approaches. Thus, it is possible that pathogens remain undiscovered with such approaches. To advance our understanding of oral biofilm communities and disease processes, it is necessary identify the microbiota involved in endo-perio lesions more comprehensively .[42]Several types of further analysis such as: construction of cDNA libraries, analysis of gene transcription profiles, cloning of new genes, sequence completion of partially sequenced genes, restriction enzyme mapping, allele-specific oligonucleotide hybridization and template generation for DNA sequencing are under research study.[26]

Conclusion

The development of efficient treatment strategies for endo-perio lesions depends on accurate characterization of the microbial communities.The use of only cultivation-based techniques causes a significant proportionof the microorganisms to go undetected. In this context, use of advanced molecular techniques will provide a trustworthy tool for determining bacterial diversity.Howevercomprehensive clinical studies are required for better understanding of the pathogenesis and to design targeted therapies for endo-perio lesions.

References

1.Simring M., Goldberg M. (1964): The pulpal pocket approach: retrograde periodontitis, J Periodontol 35:22.
2.Mandel E, Machtou P, Torabinejad M. Clinical diagnosis and treatment of endodontic and periodontal lesions. Quintessence Int 1993; 24(2): 135-9.
3. Ten Cate A R. Oral histology, development, structure and function. 4th ed. Philadelphia: Mosby; 1994.
4. Carmen Mueller Storrer, Giuliana Martina Bordin, Tarcísio Tavares Pereira. How to diagnose and treat periodontal-endodontic lesions?.RSBO. 2012 Oct-Dec;9(4):427-33.
5. Zehnder M, Gold SI, Hasselgren G. Pathologic interactions in pulpal and periodontal tissues. J ClinPeriodontol 2002;29:663-71.
6. Solomon C, Chalfin H, Kellert M, Weseley P. The endodontic-periodontal lesion: a rational approach to treatment. J Am Dent Assoc 1995; 126(4): 473-9.
7. Rotstein I, Simon JHS. (2004): Diagnosis, Prognosis and decision-making in the treatment of combined Periodontal-endodontic lesions. Periodontology 2000, 34: 165-203.
8.Shenoy N, Shenoy A. Endo-perio lesions: Diagnosis and clinical considerations. Indian J Dent Res 2010;21:579-85.
9.Whyman RA. Endodontic-periodontic lesions. Part I: Prevalence, aetiology, and diagnosis. N Z Dent J 1988; 84(377): 74-7.
10. Harrington GW, Steiner DR. (2002): Periodontal-endodontic considerations. In:Walton RE, TorabinejadM,editors, Principles and practice of endodontics, 3rd edn. Philadelphia:W.B.Saunders Co., 466-484.
11. Rotstein. I, James H.S. (2006). The endo-perio lesion: a critical appraisal of the disease condition. Endodontic Topics, 13: 34-56.
12. Jenkins WM, Allan CJ. Guide to Periodontics. 3rded. California: Wright Publishing Company; 1994.p. 146-52.
13.Torabinejad M, Lemon RL. Procedural accidents. In:Walton RE, Torabinejad M, eds. Principles and Practice of Endodontics, 2nd ed. Philadelphia: W.B. Saunders; 1996. p. 306-23.
14.Kvinnsland I, Oswald RJ, Halse A, GronningsaeterAG. A clinical and roentgenological study of 55 cases of root perforation.IntEndod J 1989; 22(2): 75-84.
15. Lindhe J. Clinical periodontology and implant dentistry.3rd ed. Denmark: Munksgaard Intl Pub; 1997. p.296-328.
16. Mastromihalis N, Goldstein S, Greenberg M, Friedman S. Applications for guided bone regeneration in endodontic surgery. N Y State Dent J. 1999;65:30-2.
17.Trope M, Rosenberg E, Tronstad L. Dark field microscopic spirochete count in the differentiation of endodontic and periodontal abscesses. J Endod. 1992;18(2):82-6.
18. Kobayashi T, Hayashi A, Yoshikawa R, Okuda K, Hara K. The microbial from root canals and periodontal pockets of non-vital teeth associated with advanced periodontitis. IntEndod J. 1990;23:100-6.
19. Rupf S, Kannengiesser S, Merte K, Pfister W, Sigusch B, Eschrich K. (2000): Comparison of profiles of key periodontal pathogens in the periodontium and endodontium. Endo Dent Traumatol. 16:269-275.
20. Slots J, Rams TE, Listgarten MA. (1988): Yeasts, enteric rods and pseudomonas in the subgingival flora of severe adult periodontitis. Oral MicrobiolImmunol. 3:47-52.
21. Sabeti M, Simon JH, Nowzari H, Slots J. (2003): Cytomegalo virus and Epstein-Barr virus active infection in periapical lesions of teeth with intact crowns. J. Endod. 29:321-323.
22. Contreras A, Nowzari H, Slots J. (2000): Herpes viruses in periodontal pocket and gingival tissue specimens. Oral MicrobiolImmunol. 15:15-18.
23.Hong Li, Rui Guan, Jinghua Sun, and BenxiangHou.Bacteria Community Study of Combined Periodontal-EndodonticLesions Using Denaturing Gradient Gel Electrophoresis and Sequencing Analysis.Journal of Periodontology October 2014, Vol. 85, No. 10, Pages 1442-1449.
24. Ashraf F. Fouad, Jody Barry, Melissa Caimano, Michael Clawson, Qiang Zhu, Rachaele Carver, KarstenHazlett and Justin D. Radolf.PCR-Based Identification of Bacteria Associated with Endodontic Infections.J. Clin. Microbiol 2002;40: 3223–3231.
25. José F. Siqueira, Jr* and Isabela N. Rôças.As-yet-uncultivated oral bacteria: breadth and association with oral and extra-oral diseases. J Oral Microbiol.2013; 5: 10.

26. Santos C F, Sakai V T, Machado M A A M, Schippers D N, Greene A S.Reverse transcription and polymerase chain reaction: principles and applications in dentistry. J Appl Oral Sci 2004; 12(1): 1-11.
27. Baumgartner, J. C., K. S. Bae, T. Xia, J. Whitt, and L. L. David. 1999. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and polymerase chain reaction for differentiation of Prevotellaintermedia and Prevotellanigrescens. J. Endod. 25:324–328.
28. Okada M, Hayashi F, Nagasaka N. PCR detection of 5 putative periodontal pathogens in dental plaque samples from children 2 to 12 years of age. J ClinPeriodontol 2001 Jun; 28(6): 576-82.
29. Takeuchi Y, Makoto U, Sakamoto M, Benno Y, Huang Y, Ishikawa I Treponemasocranskii, Treponemadenticola, and Porphyromonasgingivalisare associated with severity of periodontal tissue destruction. J Periodontol 2001 Oct; 72(10):1354-63.
30. Kimura S, Ooshima T, Takiguchi M, Sasaki Y, Amano A, Morisaki I, et al. Periodontopathic bacterial infection in childhood.J Periodontol 2002 Jan; 73(1): 20-6.
31. Siqueira Junior JF, Rocas IN, Oliveira JC, Santos KR. Detection of putative oral pathogens in acuteperiradicular abscesses by 16S rDNA-directed polymerase chain reaction. J Endod 2001a Mar; 27(3): 164-7.
32. Bogen G, Slots J. Black-pigmented anaerobic rods in closed periapical lesions. IntEndod J 1999 May; 32(3): 204-10.
33.Machado de Oliveira JC, Siqueira Junior JF, Alves GB, Hirata R Jr, Andrade AF. Detection of Porphyromonasendodontalis in infected root canals by 16S rRNA gene-directed polymerase chain reaction. J Endod 2000 Dec; 26(12): 729-32.
34. Linderman JR, Greene AS. Distribution of agiotensin II receptor expression in the microcirculation of striated muscle. Microcirculation 2001 Aug; 8(4): 275-81.
35. Santos CF, Oliveira EB, Salgado MCO, Greene AS. Molecular cloning and sequencing of the c DNA for rat mesenteric arterial bed elastas-2, an angiotensin II-forming enzyme. J CardiovascPharmacol 2002 May; 39(5): 628-35.
36. Santos CF, Caprio MAV, Oliveira EB, Salgado MCO, Schippers DN, Munzenmaier DH, Greene AS. Functional role, cellular source and tissue distribution of rat elastase-2, an angiotensin II-forming enzyme. Am J Physiol (Heart CircPhysiol) 2003 Aug; 285(2): H775-83.
37. Garlet GP, Martins Junior W, Ferreira BR, Milanezi CM, Silva JS.Patterns of chemokines and chemokine receptors expression in different forms of human periodontal disease. J Periodont Res 2003; 38: 210-7.
38. Shelburne CE, Gleason RM, Germaine GR, Wolff LF, MullallyBH, Coulter WA, et al. Quantitative reverse transcription polymerase chain reaction analysis of Porphyromonasgingivalisgene expression in vivo. J Microbiol Meth 2002 Apr; 49(2): 147- 56.
39. Parashar A, Sanikop S, Zingade A, Parashar S, Gupta S (2015) Virus Associated Periodontal Diseases: Futuristic Implications.J Dent Oral DisordTher 3(1): 1-5.
40. H. J. Rolph, A. Lennon, M. P. Riggio, W. P. Saunders, D. MacKenzie, L. Coldero and J. Bagg.Molecular Identification of Microorganisms from Endodontic Infections.J. Clin. Microbiol. September 2001 vol. 39 no. 9 3282-3289.
41. L. C. N. Brito, F. R. Teles, R. P. Teles, E. C. Franc¸A. P. Ribeiro-Sobrinho, A. D. Haffajee, and S. S. Socransky.Use of Multiple-Displacement Amplification and Checkerboard DNA-DNA Hybridization to Examine the Microbiota of Endodontic Infections.J. Clin. Microbiol.Sept. 2007, Vol. 45, No. 9,p. 3039–3049.
42. Purnima S. Kumar, Ann L. Griffen, Melvin L. Moeschberger and Eugene J. Leys.Identification of Candidate Periodontal Pathogens and Beneficial Species by Quantitative 16S Clonal Analysis J. Clin.Microbiol. August 2005vol. 43 no. 8 3944-3955.