Isolation, transportation and culture method of *Prevotella intermedia*: A Review

Devika Warrier.E[1], Dr. Caroline Jacob[2]

[1]Undergraduate Student, [2]Senior Lecturer
Saveetha Dental College, Saveetha University Chennai, India.

Abstract: *Prevotella intermedia* is a gram negative obligate anaerobic bacteria widely associated in periodontal disease especially. It has also been associated in greater numbers in women with puberty-associated gingivitis and pregnancy-associated gingivitis due to higher levels of the nutrient obtained from progesterone. Bacterial culture has been the primary diagnostic method widely used in analyzing the composition of dental plaque and is still considered the gold standard when determining the utility of a new microbial test in periodontal microbiology. Various compositions and methods have been provided in the literature but there is a lack of consensus on what may be the most ideal techniques to isolate, transport and culture *Prevotella intermedia*. This information may also be useful to find economical substitutes in laboratories of developing nations to keep abreast with the advances seen in plaque biofilm. Therefore, the aim of this review is to discuss the isolation and culture methods used in detecting the obligate anaerobic periodontal pathogen, *Prevotella intermedia*.

Keywords: Culture methods, culture media,, *Prevotella intermedia*, blood agar, obligate anaerobic pathogen.

INTRODUCTION

The microbial populations involved in periodontal diseases are known to be highly complex and variable and have not yet been fully identified although there are many key organisms which are generally recognised to be associated with disease progression.[1] Anaerobic bacteria are important components of the normal microbiota present in the human host. Anaerobes are present on most body surfaces and mucous membranes; ranging in areas from the mouth to the colon. With the exception of the stomach and esophagus; they are found in larger numbers in the females when compared to males. In most areas, a true symbiotic relationship exists: humans supply the environment for the anaerobes to live and multiply in the presence of food, water, and a hospitable atmosphere; the bacteria aid in digestion of food for metabolism, prevent attachment of more virulent microbes by virtue of their presence in very large numbers, and make up a major component of the innate immunity of the host.[2] Anaerobes can also cause infections when they are in greater number in areas where they are part of the normal microbiota, when they are introduced into a new site in the body or when a non-normal microbiota anaerobic bacterium gains entrance into the host via penetrating wounds as a result of trauma, accidents, or surgical procedures.

Prevotella intermedia is a bacteria that previously known by the name *Bacteroides intermedius*. With advancements in DNA sequencing, researchers discovered that many bacteria previously classified as Bacteroides would be more appropriately fit for a new genus, *Prevotella*, due to their bile-sensitive characteristics. The genus Bacteroides is then re-classified which includes bacteria that are bile-resistant gram-negative bacilli.[3]One of only two strains of the entire *Prevotella* genus, among all species, that has had its entire DNA genome sequenced is *P. intermedia.* [4] As with other pathogenic species of Prevotella, *P. intermedia* has been researched for its various pathogenic effects in humans. It is commonly studied for its role in the oral cavity, and more specifically, periodontal disease. *P. intermedia* is seen as the main cause of many periodontal diseases and is often hard to eliminate in infected areas, due to its ability to form biofilms. Its antibiotic-resistant capabilities have serious implications for human health[5] and its pathogenic impacts on human health are not yet fully understood.

Cell structure

Prevotella intermedia is an anaerobic, gram-negative, rod-shaped bacterium.[6] *P. intermedia* is also classified as a black pigmented bacteria because of its formation of shiny and smooth colonies which appear either a grey, light brown, or black colour on blood agar plates.[7] *P. intermedia* has been found to contain exopolysaccharides which are composed of neutral sugars and mannose and are a major component of biofilm formation. These exopolysaccharides provide *P. intermedia* the ability to evade the innate human immune system.[8]

Genomic structure

Prevotella intermedia 17, a strain from the human periodontal pocket, was the first published genome sequence of *Prevotella*. Genes involved in *P. intermedia's* cell envelope structure are highly conserved among multiple strains, including *Prevotella intermedia* 17. AdpB, a binding protein on the cell surface which is thought to be involved in the microbe's adhesion capabilities, is highly conserved and found in all strains of *P. intermedia.* [9] In the process of analyzing different strains of *P. intermedia*, scientists have found that only about 3% of the whole genome typically accounts for alignment between two strains. Despite the small percentage of alignment in nucleotide sequences, scientists found a 49Kb region where gene content is conserved and shared among the different genomes. *Prevotella*, as a genus, is reported to have roughly 165-170 core genes which allow it to thrive in diverse environments.[10]

Metabolic processes

P. intermedia is known as a periodontopathic bacteria whose metabolic activity often results in an accelerated development of oral biofilm-mediated diseases. Its metabolites are known to initiate and promote oral disease, both directly and indirectly.[11] This bacterium is non-acid producing and utilizes amino acids and peptides as metabolic substrates. Fluoride is added into toothpastes in order to inhibit these metabolic substrates that result in disease.[12] P. intermedia is also known to metabolize glucose, allowing it to readily exist in the human oral cavity. Glucose metabolism utilizes the EMP pathway and hexokinase, which tends to increase the anaerobic properties of the organism. *P. intermedia* also favors catabolizing nitrogenous compounds for energy sources. [13] These microbes grow well in media containing trypticase or peptone in addition to yeast extracts. This indicates that they depend primarily on the catabolism of the nitrogenous compounds for their energy production. They degrade these nitrogenous compounds such as proteins by mainly their extracellular proteases and then metabolize the formed peptides and amino acids into short chain fatty acids and ammonia. These metabolic end products are cytotoxic and many nitrogenous compounds are present in their habitat, which includes the periodontal region. Hence the catabolism of nitrogenous compounds are significant for their periodontal pathogenicity.

Prevotella intermedia has serious implications in the pathogenesis of periodontal disease. Periodontal diseases affect over 15% of people in the United States alone.[14] Periodontitis, is characterised by subgingival plaque deposition, loss of attachment and the probing depth beyond the standard ranges [15]. One of the main reasons why P. intermedia has such a virulent impact on the connective tissue of the mouth is because of the biofilm it is capable of creating, resulting in dental plaque.[16] The biofilm that houses P. intermedia can be difficult for innate defense mechanisms to challenge and resists antibiotic penetration.[17] Bactericidal effects with antibiotics such as tetracycline, doxycycline, and ofloxacin are only effective when it is in its planktonic form.[18]
To optimally recover anaerobic bacteria, appropriate collection, transport, and processing procedures must be followed. Adequate transport devices for fluids, aspirates, and tissues should be suggested or provided for sample collection and transportation. The media for optimal anaerobic bacterial recovery should be fresh media and include added vitamin K and hemin, minimally.[19]

Isolation of bacteria

Collection of specimens to avoid contaminating them with normal microbiota and prompt transport to the laboratory for processing are extremely important. Isolating *P. intermedia* from clinical specimens, determining the numbers of anaerobes in the specimen, and establishing the clinical significance all depend on proper collection and transport of the specimen. The laboratory director or supervisor must provide the clinical staff with guidelines for the optimal amount and type of specimen required for the culture and must stress the need to transport the properly collected specimen to the laboratory without delay. Patient care units, clinics, and emergency rooms must be supplied with appropriate collection devices and complete instructions for their use. The clinician, in turn, must provide information regarding specific source, clinical impression, special status of patient, or unusual suspected organisms. Good communication between the clinical microbiology laboratory and the clinical staff will ensure the collection and transport of the best possible specimen for culture [20,21].

Collection methods:

1. From deep pockets – sample sites were isolated using cotton rolls, followed by removal of supra gingival plaque and one sterile paper point was inserted to the depth of each periodontal pocket.[22]
2. Pocket-out collection :- This method is basically a swab technique with a commercially available foam tip with polymer head. The swab was performed by applying a light pressure with the foam tip on the surface of masticatory mucosa between the crest of gingiva and the mucogingival border in the range of two teeth, one of which is the collection tooth for paper point method. The Pocket-out collection method was strictly applied before standard collection method, in order to exclude possible cross-contamination of the supra- gingival mucosa by the paper point with living pathogen cells from the depth of the periodontal pocket. [22]

Specimen transport

1. Transport time depends on the volume and nature of the specimen. Large volumes of purulent material and large pieces of tissue maintain the viability of P inermedia for many hours.[23] Swabs, small volumes of aspirated material, biopsy samples, or curetted samples should be transported in an anaerobic transport device making certain to avoid extremes of heat or cold.[24]If delays are unavoidable, hold the specimen at room temperature until processing. Transport of material for culture in the needle and syringe is not accepted due to risks of needle stick injury as well as the potential for the specimen may to be expelled during transport, creating a threat to personnel and the environment.

Identification of Anaerobic Bacteria - *Prevotella intermedia*

Identification of *P.intermedia* isolates, which can be done presumptively or definitively. It is done to, know which bacterium is causing the infection and can help the physician choose an empiric therapy that is most likely to be effective. The identification of isolate often indicates the likely source of the infective process. It also helps to build a database of information about their role in infections. Presumptive identification of these isolates has become increasingly popular because definitive identification is both costly and time-consuming, and in most cases presumptive identification suffices to help physicians choose appropriate therapy.[25]Presumptive identification is based on colony morphology, Gram stain appearance, and a variety of rapid, inexpensive tests. These mainly include, Fluorescence under long-wave (366 nm) ultraviolet light. The pigmented bacteria, *Prevotella intermedia* appears fluoresce brick-red.[26]

Specimen preparation

1. Centrifuge large volumes of nonpurulent material. Use the sediment to inoculate the media and to prepare the Gram stain
2. Wring out swabs in 0.5 ml of liquid medium (THIO or chopped meat), and then treat them as a liquid specimen. Alternatively, plant swabs directly onto appropriate media, but this option is less desirable because the loss of organisms on each medium will result in a poorer specimen for Gram stain.

Culture media

The choice of media for culturing these bacteria is important for the success of anaerobic bacteriology. The media must contain appropriate nutrients and supplements needed by these microbes. A combination of enriched, non-selective, selective, and differential media should be used for the initial processing, isolation, and presumptive identification of bacteria from clinical specimens. These bacteria have a wide range of nutritional needs; most, however, require hemin and vitamin K. Kanamycin-vancomycin-laked blood agar (KVLB or LKV)is the ideal media used for the culture of *Prevotella intermedia* . Some studies suggest that freshly prepared, properly stored, highly enriched media are essential for recovery of these anaerobes while another study has shown that prereduced anaerobically sterilized (PRAS) media best support the growth of *P.intermedia*.[25] Recent studies have suggested that using media containing oxyrase may be another alternative.[26,27] Media that have been exposed to air contain oxidized products that may delay or inhibit the growth of many anaerobes..

KVLBorLKV contain :

Trypticasepeptone	23g
Glucose	1g
Yeast extract	2g
sodium chloride	5g
Hemin	10ml
vitaminK1	1.0ml
L-cystine	0.5g
Agar	15g
Kanamycin	100mg
Vancomycin	7.5mg
Sheep blood	45.5ml
Distilled water	1000ml

The ideal media used to culture, therefore, are those that have had limited exposure to oxygen.

Inoculation of media

Media for P. intermedia culture includes

Brucella agar
Kanamycin-vancomycin-laked blood agar
Phenylethyl alcohol
Chopped meat broth
PRAS

a. Brucella agar with 5% sheep blood supplemented with vitamin K and hemin.[28]
b.Kanamycin-vancomycin-laked blood agar for the selection of pigmented *Prevotella*.[29]
c. Phenylethyl alcohol (PEA)-sheep blood agar for the inhibition of overgrowth of bacteria..
PEA also reduces the spreading or swarming characteristic of these anaerobes.[30]
d. Chopped meat broth or THIO (supplemented with vitamin K and hemin).
f. Freshly prepared or prereduced anaerobically sterilized (PRAS) media are preferred. PRAS media have a prolonged shelf life and are superior to commercial media that have been reduced 24 h before use. [30]

Incubation

When inoculated media cannot be placed immediately into an anaerobic atmosphere, it would be best to batch process the specimens so that multiple specimens will be inoculated and placed into the anaerobic environment at once. Holding the clinical specimen in an appropriate transport device and batch processing the inoculation to media is preferred to processing specimens one at a time and leaving them in holding jars until time permits to place them at once in an anaerobic environment(either bags, jars, or an

anaerobic chamber). The viability of anaerobes can be maintained for hours in a good anaerobic specimen collection device. Anaerobic systems.

(i) Coy anaerobic chamber. The Coy anaerobic chamber consists of a flexible glove box filled with 85% N2±10% H2±5% CO2 and heated palladium catalyst pellets. Anaerobiosis of the chamber was monitored by using a BBL disposable anaerobic indicator strip (Becton Dickinson).

(ii) BBL GasPak system. The GasPak system includes a 2.5-liter jar with palladium catalyst pellets and a GasPak anaerobic envelope. Pellets were heated in a 125°C oven for 2 h before each use. Prior to the incubation of the blood agar plates, the GasPak anaerobic envelope was activated by adding 10 ml of water to the envelope. The final CO2 concentration was 4 to 10% [31]. The anaerobic conditions were monitored, after 60 min of incubation, by using the BBL disposable anaerobic indicator strip.

(iii) AnaeroPack system. The AnaeroPack system includes a rectangular container (9.5 by 6.75 by 3.25 in.; 2.5 liters) and one AnaeroPack sachet. The sachet was opened and placed into the container along with inoculated blood agar plates and a BBL disposable anaerobic indicator strip. After 60 min of incubation, the oxygen concentration was less than 1% and the CO2 concentration was approximately 18% To ensure quality, lids for the jars and containers of the BBL GasPak and the AnaeroPack systems were inspected and sealed as described in the manufacturer's instructions. Also, catalyst pellets for the Coy anaerobic chamber and the GasPak system were reactivated before each use.. It is not uncommon for a test species not to grow in one or more of the anaerobic culturing systems. This led to a distribution that could not be analyzed by standard parametric methods.

The Coy anaerobic chamber can process high volumes of bacterial plates and exhibits good recovery for most sub gingival anaerobic organisms but can be expensive to purchase and maintain.

The BBL GasPak system is limited to processing a few bacterial plates at a time and it s small enough to fit a medium size incubator whereas the Coy anaerobic chamber requires a relatively large space.

A direct smear can be gently heat fixed or fixed in absolute methanol for 1 min and then stained by standard Gram stain procedure and reagents. Alternatively, use basic fuchsin in place of safranin to enhance the staining of these gram negative anaerobes. Gram stain reveals the types and relative numbers of microorganisms and host cells present and serves as a measure for the adequacy of these techniques. Correlation of specimen type with bacterial morphology on the Gram stain can provide the clinician with rapid presumptive information about the identity of the bacteria present. The pigmenting *Prevotella intermedia* shows Gram negative coccobacillary forms on Gram staining.

DNA-extraction

In this method first the nucleic acids are extracted within 24 to 48 hours from the collected specimen. The samples are vortex-mixed and centrifuged to collect the cells. The pellet is then suspended in 300 µl of lyses buffer (50 mM Tris, 10 mM EDTA and 10% SDS) plus lysozyme (5 mg/ml) and incubated at 37°C for 1 h. Then, 125 µg of proteinase-K is added and after 1 hour incubation at 65°C, the DNA is extracted with phenol and chloroform-isoamyl alcohol treatment. Nucleic acids are precipitated in alcohol, washed with 70% (vol/vol) alcohol and suspended once more in sterile water. The DNA extracted from each sample is assayed by multiplex PCR for the detection of *P. intermedia*.[31]

PCR-detection

Multiplex PCR is performed using specific primers for the 16S rRNA gene of this bacterium.PCR amplification reactions are carried out in a reaction mixture in a final volume of 100 µl consisting of 10 µl of DNA sample, and 90 µl of reaction mixture containing 30 pmol of each primer, 200 µM of a mixture of deoxynucleosidetriphosphates, 1.5 mM MgCl2, 1 x PCR buffer. The PCR protocol was as follows: 98°C for 15 min followed by 40 cycles of 95°C for 30 s, 60°C for 1 min, 72°C for 1 min, and a final step of 72°C for 10 min. PCR amplification is performed in an iCycler System (BioRad Laboratories Srl, Segrate, Milan, Italy). Amplicons are detected by electrophoresis of 20 µl of samples from each PCR tube in a 2% agarose gel in TAE (Tris-Acetate-EDTA buffer) for 2 h at 80 V. The amplification products are visualized and photographed under a UV light trans illuminator (Gel Doc 2000, BioRad) after 30 min of ethidium bromide (1 µg/ml) staining. A positive or negative identification is based on the presence of clear bands of the expected molecular size using a commercial DNA molecular weight marker.. Each assay is performed once, and in case of disagreeing results, the assay is then repeated once more.[32]

At the beginning, as a point of comparison for multiplex PCR, the culture examination was chosen because it has long been considered the gold standard. The new technique shows a low sensitivity with higher values of specificity, with scarce possibilities therefore of false positives in the search for *P. intermedia*[33].

In a study conducted by Riggio et al in 1996 calculated the diagnostic validity of culture using PCR as the standard reference. The culture method shows a high precision in the possibility of identifying false positives (high specificity), superior to that shown in the contrary hypothesis, in the determination of *P. intermedia*. In the absence of a standard of reference, each technique has the same probability of demonstrating the truth. To determine the degree of accuracy between the two methods, they analyzed according to the perspective that both methods are procedures of reference and test procedures of the examined microorganisms. This can be considered a good strategy, which however observes the problem of evaluation of the characteristics of a diagnostic test in the absence of a gold standard (a very common situation, considering that the presence of a standard is an exception)[34].

Culture methods have the advantage of being able to detect a wide variety of species; the characterization of all isolates may allow the identification of unexpected or new species. This technique enables us to search for all the microorganisms present in a non-specific way and it remains the most objective technique (gold standard). However, taxa present in low proportions might be missed unless selective culture techniques are used, but such techniques are often too suppressive.

PCR can by-pass many of the restrictions of anaerobic cultures and have a lower detection limit than non-selective cultures, but they are only applicable to pre-selected target species for which antibodies of known specificity must be available[35]. One significant advantage of this multiplex PCR is the multi target analysis, i.e. it can detect more than one bacterial species at a time.

The choice could depend on the demand in time for the analysis. PCR can provide results in 2h, whereas anaerobic cultures require 7-8 days to confirm the presence of these pathogens. Although interest in the culture technique remains relative, because of the presence of biofilm and the complexity of the oral flora, we still have no other tested scientific method[36].

CONCLUSION

For oral microbiology laboratories that process a limited number of anaerobic samples, the GasPak anaerobic culture system seems to offer a convenient and effective method for recovering periodontal pathogens. The ideal technique for accurate detection of *P.intermedia* in sub gingival plaque samples has yet to be developed. The high sensitivity and specificity of multiplex PCR justifies its use in detection of these pathogens from periodontal diseases. Both these techniques can detect this particular bacterial species coincidentally, but the bacterial cultures allow the determination of antibiotic resistance and is more effective.

REFERENCES:

[1] Slots, J., and M. A. Listgarten. 1988. Bacteroides gingivalis, Bacteroides intermedius, and Actinobacillus actinomycetemcomitans in human periodontal diseases. J. Clin. Periodontol. 15:85-93.
[2] Kamma J.J., Nakou M., 2004. Microbiological profile of early-onset aggressive periodontitis patients. OMIJ 19, 314-321.
[3] Somer, H. J., and Summanen, P. (2002). Recent Taxonomic Changes and Terminology Update of Clinically Significant Anaerobic Gram-Negative Bacteria (Excluding Spirochetes). JCID , 35(1): 17–21.
[4] Sakamoto, M., and Ohkuma, M. (2012). Taxonomy of the Genus Prevotella. Names for Life. IJSR- 10.160180424.
[5] Moon, J.-H., Kim, M., and Lee, J.-H. (2016). Genome sequence of Prevotella intermedia SUNY aB G8-9K-3, a biofilm forming strain with drug-resistance. BJOM 48(1): 5–6.
[6] Roux, V., Robert, C., and Raoult, D. (2014). Non-contiguous finished genome sequence of Prevotellatimonensis type strain 4401737T. SIGS, 9(3): 1346–1353.
[7] Shah, H.N., and Collins, D.M. (1990). *Prevotella*, a new genus to include bacteroidesmelaninogenicus and related species formerly classified in the genus bacteroides. IJSEM, 40(2): 205-208.
[8] Yamanaka, T., Furukawa, T., Matsumoto-Mashimo, C., Yamane, K., Sugimori, C., Nambu, T., Mori, N., Nishikawa, H., Walker, C.B., Leung, K.P., and others. (2009). Gene expression profile and pathogenicity of biofilm-forming *Prevotella intermedia* strain 17. BMC Microbiology, 9(11).
[9] Ruan, Y., Shen, L., Zou, Y., Qi, Z., Yin, J., Jiang, J., Guo, L., He, L., Chen, Z., Tang, Z., and others. (2015). Comparative genome analysis of *Prevotella intermedia* strain isolated from infected root canal reveals features related to pathogenicity and adaptation. BMC Genomics. 16(122): 1-21.
[10] Hahnke, et al. (2016). Taxonomy of the Genus *Prevotella intermedia*. Names for Life. IJSR :10.1601-8042
[11] Ishiguro, K., Washio, J., Sasaki, K., and Takahashi, N. (2015). Real-time monitoring of the metabolic activity of periodontopathic bacteria, JOMM, 115(C): 22–26.
[12] Takahashi, N., and Yamada, T. (2000). Glucose metabolism by *Prevotella intermedia* and Prevotellanigrescens-JMOM. 15(3):188-195.
[13] Slots, J. 1986. Rapid identification of important periodontal microorganisms by cultivation. JOMI, 1:48-55.
[14] Dorn, B. R., Leung, K.-P., and Progulske-Fox, A. (1998). Invasion of Human Oral Epithelial Cells by *Prevotella intermedia*. Infection and Immunity, JSRR66(12): 6054–6057
[15] Arjunkumar R, Sudhakar U, Jayakumar P, Arunachalam L, Suresh S, Virupapuram P. Comparative analysis of gingival crevicular uid neopterin levels in health and periodontal disease: A biochemical study. Indian J Dent Res 2013;24:582-6.
[16] Eke, P.I., Dye, A.B, Wei, L., Thorton-Evans, G.O., Genoco, R.J, Beck, J., Douglass, G., and Page, R. (2012). Prevalence of Periodontitis in Adults in the United States: 2009 and 2010. JDR, 91(10): 914–920.
[17] Chandki, R., Banthia, P., and Bathia, R. (2011). Biofilms: A microbial home. JISP, 15(2): 111–114.
[18] Wu, M., Chen, S., and Jian, S. (2015). Relationship between gingival inflammation and pregnancy. Mediators in Inflammation.JSRR (1): 1-11.
[19] Takahashi, N., Ishihara, K., Kimizuka, R., Okuda, K., and Kato, T. (2006). The effects of tetracycline, minocycline, doxycycline and ofloxacin on Prevotella intermedia biofilm. JOMI, 21(6): 366–371.
[20] How, K. Y., Song, K. P., and Chan, K. G. (2016). Porphyromonasgingivalis: An Overview of Periodontopathic Pathogen below the Gum Line. Frontiers in Microbiology, JISP7(53): 1-14.
[21] Meredith, F. T., H. K. Phillips, and L. B. Reller. 1997. Clinical utility of broth cultures of cerebrospinal fluid from patients at risk for shunt infections. J. Clin. Microbiol. 35:3109-3111.
[22] Morris, A. J., S. J. Wilson, C. E. Marx, M. L. Wilson, S. Mirrett, and L. B. Reller. 1995. Clinical impact of bacteria and fungi recovered only from broth cultures. J. Clin. Microbiol. 33:161–165.
[23] Okada, M., Hayashi, F. and Nagasaka, N. (2001) PCR detection of 5 putative periodontal pathogens in dental plaque samples from children 2 to 12 years of age. Journal of Clinical Periodontology 28, 576–582.
[24] Al-Soud, W.A. and Radstrom, P. (2001) Purification and characterization of PCR-inhibitory components in blood cells. Journal of Clinical Microbiology 39, 485–493.
[25] Parthsarathy H, Gad MR and Jacob CA: Identification of Aerotolerence Gene Bat A and Bat B in Subgingival Microflora: Aggregatibacter Actinomycetemcomitans, Tannerella Forsythia and Prevotella Intermedia. Int J Pharm Sci Res 2016; 7(2): 689-96.doi: 10.13040/IJPSR.0975-8232.7(2).689-96.
[26] Beem JE., Besmit W E ,Identification of hemolytic activity in Prevotella intermedia. Oral microbiol immunol 1998 Apr;13(2):97-105.

[27] Citron, D. M., andP.R.Murray.1991.General processing of specimens for anaerobic bacteria, p. 488–494. In A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), Manual of Clinical Microbiology, 5th ed. American Society for Microbiology, Washington, D.C
[28]. Citron, D. M., Y. A. Warren, M. K. Hudspeth, and E. J. C. Goldstein. 2000. Survival of aerobic and anaerobic bacteria in purulent clinical specimens maintained in the CopanVenturi Transystem and Becton Dickinson Port-A-Cul transport systems. J. Clin. Microbiol. 38:892–894.
[29]Mangels, J. I., M. E. Cox, and L. H. Lindberg. 1984. Methanol fixation—an alternative to heat f ixation of smears before staining. Diagn. Microbiol. Infect. Dis. 2:129–137.
[30]Mangels, J. I. 1994. Anaerobic transport systems: are they necessary. Clin. Microbiol. Newsl. 16:101–104.
[31]Collee,J.G.,B.Watt,E.B.Fowler,andR.Brown.1972.An evaluation of the GasPak System in the culturing of anaerobic bacteria.J.Appl.Bacteriol.35:71-82.
[32]Fukushima H, Moroi H, Inoue J, Onoe T, Ezaki T, Yabuuchi E, Leung KP, Walker CB, Clark WB, Sagawa H. Phenotypic characteristics and DNA relatedness in Prevotella intermedia and similar organisms.Oral Microbiol Immunol. 1992 Feb;7(1):60–64
[33]Ledder.R., Gilbert P., Husw S.A (2006) Molecular analysis of the sub gingival microbiota in health and disease. Applied and Environmental Microbiology. 73, 516-23.
[34]Riggo M.P., Lennon A., Smith A.J., Kinane E D. (1996). Comparison of polymerase chain reaction and culture methods for detection of Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis in sub gingival plaque samples. JOPR 31, 496-501.
[35] Teomani., Ozcan G. (2007) Comparison of different methods to detect Helicobacter pylori in the dental plaque of dyspeptic patients. Clinical Oral Investigations. 11, 201-205.
[36]Socransky, S. S., A. D. Haffajee, G. L. F. Smith, and J. L. Dzink. 1987. Diffculties encountered in the search for the etiologic agents of destructive periodontal diseases. J. Clin. Periodontol. 14:588-593.