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Review Article

Localization of *Porphyromonas gingivalis* and Tannerella Forsythia *In vivo* and *In vitro*

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Abstract

Porphyromonas gingivalis and *Tannerella forsythia* have been identified as pathogenic bacteria associated with periodontal disease. Also, there are reports describing possible association of periodontal disease with other systemic diseases and some studies have revealed the presence of these bacterial species or their genomic content in sites distal to oral cavity in human body. There have been various studies investigating on possible pathogenic mechanisms that these bacteria device in healthy and disease conditions. Majority of the studies have been focused on possible molecular mechanisms and investigating the end products that are released as a result of bacteria coming into contact with the human tissues/ cells. Up to date only a limited number of studies have visualized direct histo-pathological and cellular events of these bacteria with time lapse information. In this mini review we tried to narrow down our focus on the visualization *P. gingivalis* and *T. forsythia*, starting from the oldest possible publications to current advancements, their contribution on understanding the histo-morpho-pathological changes during bacterial invasion, the pros and cons in these methods and how future research should be shaped to reveal the possible translocation of these bacteria from periodontal tissues to distal sites of the human body.

Keywords: Localization; Periodontal pathogens; *P. gingivalis*; *T. forsythia*

Introduction

Periodontology as a discipline has evolved very rapidly during the last few decades along with the advancements in science & technology, which has led to very rapid improvements in treatment modalities, diagnostic methods and materials used for treatment. With these advancements, pathological understanding of periodontal diseases has improved over the years. According to currently available evidence Periodontitis is a multifactorial inflammatory disease that results in compromising the integrity of tooth supporting tissues, which eventually results in loss of teeth [1,2]. Out of the multiple factors periodontal bacteria plays a vital role that contributes for the inflammatory process. *Porphyromonas Gingivalis* (Pg) and *Tannerella Forsythia* (Tf) along with *Treponema denticola* have been considered as red complex group of periodontal bacteria due to their virulent properties and frequent association with deep periodontal pockets [3]. Out of these bacteria, Pg has been considered as a keystone pathogen in recent publications due its ability to manipulate the host immune response and induce dysbiotic microbial communities [4-6]. In addition, Tf has been considered as a "key-stone like pathogen" due to increased bone loss when it acts in synergism with Pg [6,7].

Several scientific reports have established that periodontitis can affect the general health of an individual. The evidence given by these reports vary from association of periodontitis to conditions such as type 2 diabetes mellitus [8], increased risk for atherosclerotic cardiovascular diseases [6,9], adverse effects on pregnancy [10,11], rheumatoid arthritis [12], respiratory diseases [13] and peripheral arterial diseases [14,15]. Therefore, the types of samples that have been analyzed to detect Pg and Tf are not only limited to intra-oral samples but includes a variety of extra oral samples. The intra-oral samples that have been analyzed include plaque, saliva, buccal swabs, tongue scrapings and tissue specimen such as gingival and subgingival granulation tissues and oral mucosa. The extra-oral specimen varies from vascular tissues (including arteries with atheromas and veins), lymph nodes, blood/ serum samples, cerebral tissues and lung aspirates. These specimens have been analyzed for Pg and Tf using several techniques varying from microbial culture to immunoassays to hybridization techniques to next generation sequencing over the time. The techniques using such molecular analysis methods contributed to identifying the presence of these bacteria along with the density in relevant samples at different levels in disease progression. Also second generation sequencing helped in identifying distinct bacterial profiles in health and disease as well as uncultivable bacterial species present in the oral cavity [16,17]. However, the direct observation of these bacteria under microscopy *in vivo* or *in vitro* has a lot of insightful information that would help in understanding the pathogenic impact of these bacteria relevant to histopathology, immunopathology, disease progression (of periodontitis) and translocation.

In this mini review we narrow down our focus on imaging of Pg and Tf in different types of specimen in novel experimental designs that have focused on imaging these bacteria as part of their data and how they have contributed to the advancement in understanding of the impact of microbial pathology in periodontal disease. These imaging methods include light microscopy, Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM), immuno-fluorescence microscopy, confocal microscopy and Fluorescence *In Situ* Hybridization (FISH) using different antibodies and fluorescing agents in different experimental designs.

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Taxonomic Affiliation

The earliest records of detection of periodontal bacteria was initially based on getting plaque/ saliva samples from patients and observing them under light microscopy. With the advancement of microbiology, culturing and isolating bacteria by colony morphology became the gold standard for the identification of these bacteria. These isolated bacteria were separated and classified according to their pigmentation characteristics and metabolic characteristics. Pg was first identified as *Bacteroides gingivalis* as a separate species among other Black Pigmented *Bacteroides* by Coykendall et al. due to its ability to produce phenyl acetic acid compared with other non-glucose fermenting (asaccharolytic) black pigmented bacteroides [18]. Later on Shah and Collins proposed three *Bacteroides* species including *B. gingivalis* into a new Genus called *Porphyromonas* depending on their major fermentation products, ability to agglutinate erythrocytes, growth enhancing/reducing factors, cell wall contents and DNA base composition [19]. Tf was first isolated from the deep periodontal pockets of humans [20] and was later proposed by Tanner et al. as a novel species under the genus *Becteriodes* and was identified as *Becteriodes forsythus* in various literature for more than a decade [21]. In 2002 Sakamoto et al. proposed reclassifying of *Becteriodes forsythus* into a new genus called *Tannerella*, and identified the organism as *Tannerella forsythensis* according to the biochemical characteristics and 16s rDNA sequence analysis [22]. In 2008 the Judicial Commission of the International Committee on the Systematics of Prokaryotes corrected the specific epithet to its original form *forsythia* [23].

Culture Conditions

Both the bacterial species are cultured under anaerobic conditions at 37°C on tripticase soy agar plates containing defibrinated horse blood. Tf in addition requires N-Acetyl Muramic Acid (NAM) supplementation for the growth. Pg colonies usually appear as smooth, convex, black pigmented, shiny colonies with 1-2 mm diameter and reaches the state of full-blown colonies within 5-7 days. Pg cells are gram negative, obligately anaerobic, non-sporeforming, non-motile rods or coccobacilli bacteria under the light microscope. Tf colonies are pale speckled-pink, circular and slightly convex on tripticase soy blood agar plates supplemented with NAM discs. Tf cells are gram negative, obligately anaerobic, non-motile fusiforms.

Discussion

The bacteria causative of periodontal inflammatory process exists as plaque or as biofilm intact to tooth surface supra or sub-gingivally. Initial studies that described the architecture of biofilms on epoxy resin crowns and extracted teeth, described bacteria as cocci and filaments with structural architecture of plaque consisting of corn cob-like and test-tube brush like structures under light and electron microscopy [24,25].

In a study done by Zijnge et al. using Fluorescent *In Situ* Hybridization (FISH) Pg and Tf are localized in oral biofilms/ plaque on natural teeth under fluorescent microscopy. These bacteria are observed heterogeneously scattered in the second layer of supragingival plaque, which consisted of four layers. In subgingival biofilm Pg is present as micro-colonies within the top layer and fourth layer. Tf along with *Fusobacterium nucleatum* has been found perpendicularly arranged around lactobacilli, forming fine test-tube brush like morphology. Similar morphology of test-tube brush like formations are seen in a mix of species of bacteria, including Tf, *Campylobacter sp., P. micra, Fusobacteria* and *Synergistetes* group A [26]. In this study, Pg and Tf have been localized and identified *in vivo* using FISH, which has helped in understanding their positional information in intact biofilm and may contribute to the understanding of molecular diversity within the biofilm and bacterial function in relation to periodontal disease. On the other hand, even according to authors, each of these observations are a "snap-shot" of plaque architecture at that specific time-point and does not provide time lapse microscopic data and quantitative bacterial cell count for each species.

In majority of publications that were focused on clarifying the host-parasite interactions in periodontitis, cultured human cell lines (especially human gingival epithelial cells or human gingival fibroblasts), are challenged with pathogenic periodontal bacteria. And the cytokine production and cellular events have been studied up to apoptosis in the cultured human cells. Also, some of the studies have visualized periodontal pathogens extra cellularly, inter-cellularly and intracellularly in relation to cellular events.

Yilmaz et al. detected Pg in primary epithelial cells in an *in-vitro* study conducted to assess ATP-dependent activation of inflammasome in Primary Gingival Epithelial Cells (PGEC) infected by Pg. Here the immuno-fluorescence microscopy has been used to localize pro-IL-1β/ IL-1β, Pg and nucei of PGEC using antibodies against them. The confocal microscopic observations along with the analysis of fluorescence intensity using software has helped in clarifying the necessity of extracellular Adenosine Triphosphate (ATP) for the release of pro-IL-1β from cells infected with Pg [27].

In some of these cell invasion assays multiple bacterial species in combination have been used and in some, different strains within one species has been used. Initial studies such as by Lamont et al. using transmission electron microscopy describes invasion of primary gingival epithelial cells by Pg and observed the apparent contact between microfilamentous cellular contents and surface adherence of bacteria and division of bacteria within the epithelial cells. It has been shown that the invasive capacity of wild type Pg (clinical isolates) is higher than the type strain and also that Pg has invasive capacity similar to other invasive gastro intestinal tract pathogens such as *Salmonella, Shigella*, and *Listeria spp*. Authors in this publication have suggested that invading Pg may contribute to the pathology of periodontal diseases [28].

Co-culture of Pg W83 strain and *Filifactor alocis* for the invasion of gingival epithelial cells by Pg revealed that receptor mediated internalization of HeLa cells was found only during the co-culture of bacteria under the transmission electron microscopy. When using a monoculture of bacteria to challenge HeLa cells, Pg W83 invasion occurred without an external envelope or covering and variation of cytoskeleton could be observed through formation of microvilli, filamentous projections of the epithelial cells facilitating invasion by the bacteria [29]. Deshpande et al. have demonstrated that according to the Pg strain, invasion capacity varies. In this study Fetal Bovine Heart Endothelial Cells (FBHEC), Bovine Aortic Endothelial Cells (BAEC) and Human Umbilical Vein Endothelial Cells (HUVEC)

have been challenged with Pg A7436, Pg 381 and Pg fim A mutant DPG3 strains and observed under Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM). Results show that both these wild type strains had similar invasion efficiencies with BAEC and in contrast Pg fim A mutant DPG3 adherence was not observed. Imaging under TEM showed that Pg being adhered to these epithelial cells and presence of microvilli protruding from the epithelial cells surrounding the attached cells and the authors suggest that this process may represent the formation of a cytoplasmic vacuole. Under SEM, changes in the normal surface architecture has been observed with long microvilli surrounding large bacterial lumps. These observations were not seen in epithelial cells in the presence of Pg fim A mutant DPG3 [30].

Major drawback in such experimental design is that bacteria present in the culture media are in a planktonic state and bacteria may not represent the pathogenic characteristics, functions and behavior that is present in the intact natural subgingival biofilm in oral cavity.

In a study by Guggenheim et al. an attempt to replicate the natural biofilm *in vitro* is reported. An artificial production of subgingival oral biofilm model comprising of nine periodontal pathogens developing on a hydroxy apatite disc has been produced and used in co-culture with primary Human Gingival Epithelial Cells (HGEC). In this study, bacterial biofilm structure has been visualized using Confocal Laser Scanning Microscopy (CLSM), Transmission Electron Microscopy and multiplex FISH. In multiplex FISH, Locked Nucleic Acid (LNA) incorporated DNA probes have been used to increase FISH fluorescence intensity compared to the conventional DNA probes with similar DNA sequence and four bacterial species have been observed at a time. The same biofilms have been used for CLSM which interestingly has produced coronal and sagittal sections of the artificial biofilm in addition to the transverse sections and in all three dimensions Pg and Tf are localized within the thickness of the biofilm. Even though the biofilm-HGEC co-culture system has been used in this study, the focus has been to observe and analyze the effects of the *in vitro* biofilm on HGEC in inducing inflammatory response, triggering apoptosis and biofilm mediated cytokine degradation [31]. Bacteria-HGEC interactions have not been visualized in terms of extracellular, intercellular and intra-cellular pathology.

An ultrastructural study using electron microscopy has revealed a direct interaction between human platelets and Pg along with bacterium-induced platelet aggregation. In this study, the authors have elaborated that the addition of Pg to platelet rich plasma induces platelet aggregation, and the bacteria are internalized in the resulting aggregates. Also, it has been revealed that Pg was localized not only on the surface between adherent platelets, but also in the engulfment vacuoles of aggregated platelets. Furthermore, the authors have concluded that human platelets engulf Pg in the channels of the open canalicular system of the platelets, and then internalize it into the cytoplasm by phagocytosis during the bacteria induced platelet activation [32].

In all the studies described as examples up to this point had one major drawback, that is the inability to differentiate between live metabolically active and dead bacterial cells in infection assays. This drawback has been overcome in a study by Choi et al. who have genetically constructed Pg with oxygen-independent Flavin Mononucleotide (FMN)-Based Fluorescent Proteins (FbFPs) that could be visualized under fluorescence microscopy without staining since PgFbFP transformants produced bright green fluorescence. The constructed PgFbFP has been used to study and quantify the co-localization of Pg within the Endothelial Reticulum (ER) of the Gingival Epithelial Cells (GECs) to clarify the actual existence of live Pg surviving intracellularly within ER compared to the Pg cells that underwent lysosomal degradation [33].

In a study by Rudney et al. rRNA Fluorescent *in Situ* Hybridization and Confocal Laser Scanning Microscope (CLSM) was used to localize Pg and *Aggegatibacter actinomycetemcomitance* in buccal mucosal (epithelial) cells to determine the presence of periodontal bacteria at sites remote from the gingival crevice. Three-dimensional reconstruction of buccal epithelial cells from the serial microphotographs have helped in clarifying the intra/inter-cellular presence of bacteria [34].

Most of the human tissue and cell samples obtained from patients to analyze for these bacteria have been analyzed using molecular analysis methods to clarify the presence and density of each species. Only a few studies have chosen to visualize Pg and Tf in human samples along with the histology of tissues. Observing these bacteria in human samples, especially in formalin fixed and paraffin embedded samples that are used for routine histopathologic examination could be a difficult task due to unavailability of species-specific monoclonal antibodies. In a publication with our collaborative research group, we have reported successful production of species-specific novel monoclonal antibodies against Pg and Tf and histologic localization of these bacteria for the first time. Using these novel monoclonal antibodies on formalin fixed and paraffin embedded gingival and subgingival granulation tissues we have located both Pg and Tf extracellularly on the epithelial surface as aggregates or within bacterial plaques, and intracellularly in the stromal inflammatory cells, squamous epithelium, and capillary endothelium of the granulation tissues. Furthermore, immunohistochemistry revealed that extracellular Pg existed only when Tf was present, whereas Tf was detected even in the absence of Pg. Also, the density of Tf in tissues when detected by immunohistochemistry, was remarkably higher in samples with extracellular Pg compared to those without extracellular Pg. In many samples with extracellular co-localization of Pg and Tf, intracellular Pg was only found in the presence of intracellular Tf. However, intracellular Tf was found even in the absence of intracellular Pg which is suggestive of the cell-invasiveness or tissue-invasiveness of Pg which may also be supported by the cell-invasiveness or tissue-invasiveness of Tf. The disruption in the epithelial cell barrier caused by intracellular Tf infection supported by intercellular co-localization with Pg and other unknown factors, permits extracellular Tf and Pg invasion into tissue stromal space. This results in tissue inflammation accompanied by many stromal inflammatory cells that have ingested Tf and Pg. Since only one section for each sample was used for IHC detection analysis, the results may be biased especially for intracellular positivity. Nevertheless, samples with any combination of intracellular bacteria (in stromal inflammatory cells, squamous epithelium, or capillary endothelium of the granulation tissue) were always accompanied by extracellular detection of that bacterium. Interestingly, intracellular detection of bacteria in the capillary endothelium of granulation tissues was always accompanied by

intracellular detection of the same species in stromal inflammatory cells. This is suggestive of the fact that the tissue-invading bacteria, many of which are phagocytosed by stromal inflammatory cells, can invade into capillary vessels. Immunohistochemistry based detection of intracellular bacteria in the capillary endothelium is suggestive of bacterial translocation *via* the bloodstream [35]. Further studies including the immunohistochemical analysis of extraoral tissues elaborating the histopatholgical events in relation to Pg and Tf are necessary to confirm this fact.

Conclusion

The number of studies that have visualized Pg in experiments are higher compared to Tf. This could be due to fastidious growth requirements, slow growth rate and absence of any other cultivable species in genus *Tannerella*. Studies combining histopathological visualizing methods and second-generation sequencing may support in revealing the role played by this bacterium in periodontal disease and translocation.

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