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Review Article

Apical Periodontitis - Virulence Factors of *Enterococcus faecalis* and *Candida albicans*

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Abstract

Introduction: Apical periodontitis is an inflammatory disease of the dental periradicular tissues triggered by bacteria colonizing necrotic root canals. It is known that a few species are more frequently isolated from persistent cases compared with primary cases. These include the *Enterococcus faecalis/faecium* group, enteric Gram-negative facultative rods, i.e., coliforms and *Pseudomonas* species. *Enterococcus faecalis* has prevalence ranging from 22% to 77% of post treatment cases analysed. Incidence of *Candida albicans* is 17% in endodontic infections.

Data Collection: Articles from 1950-2020 were studied and relevant articles were included in this review.

Source: The Pub met data base search revealed that the reference list for *Enterococcus faecalis* and *Candida albicans* featured 16, 270 and 40, 445 articles respectively. A forward search was undertaken on study article, author names and contemporary endodontic microbiology text.

Study Selection: Review articles on microbiology and commonly used medicaments were included. Articles from 1950-2020 were taken into consideration.

Conclusion: To make the dental fraternity aware of two main microorganisms which may lead to endodontic failure, to know about the virulence factors and the recent strategy to combat such a microbial disease.

Keywords: Apical periodontitis; Biofilms; *Candida alcibans*; *Enterococcus faelacis*; Virulence factors

Introduction

Apical periodontitis is an inflammatory disease of the dental periradicular tissues triggered by microorganisms colonizing necrotic root canals. Primary apical periodontitis results from the microbial colonization of necrotic pulp tissues. Secondary apical periodontitis results from a persistent infection of incorrectly treated root canals. Persistent apical periodontitis occurs when root canal treatment of apical periodontitis has not adequately eliminated intraradicular infection. Problems that lead to persistent apical periodontitis include inadequate aseptic control, poor access cavity design, missed canals, inadequate instrumentation, debridement and leaking temporary or permanent restorations. Another classification of Apical Periodontitis is based on duration-Acute Apical Periodontitis and Chronic Suppurative Apical Periodontitis. Acute Apical Periodontitis (AAP) is an acute inflammation of the apical periodontal ligament as a result of irritation *via* the root canal, or from trauma, regardless of whether pulp is vital or non-vital. It is inflammation around the apex of a tooth. The distinctive feature of AAP is microscopic rather than roentgenographic, symptomatic rather than visible [1]. Chronic Supputative Apical Periodontitis is similar to acute alveolar abscess. It also results from pulpal necrosis and is associated with chronic apical periodontitits that has formed an abscess. The abscess has burrowed through bone and soft tissue to form a sinus tract stoma on the oral mucosa.

The various routes by which the microorganisms reach the pulp are [2]: dentinal tubules, open cavity, periodontal membrane, blood stream, faulty restoration. Essentially endodontic infection in dental root canal system is the major etiologic agent of apical periodontitis. During the 1990s, a series of investigations have shown that there are six biological factors that lead to asymptomatic radiolucencies persisting after root canal treatment [3]. These are: (i) intraradicular infection persisting in the complex apical root canal system; (ii) extraradicular infection, generally in the form of periapical actinomycosis; (iii) extruded root canal filling or other exogenous materials that cause a foreign body reaction; (iv) accumulation of endogenous cholesterol crystals that irritate periapical tissues; (v) true cystic lesions and (vi) scar tissue healing of the lesion. Almost 700 bacterial species can be found in the oral cavity, with any particular individual harboring 100-200 of these species [4]. Several virulence factors are responsible for the persistence of these microorganisms in the oral cavity [5]. They are namely lipopolysaccharide and peptidoglycan in the cell wall of gram negative and gram-positive bacteria respectively, capsule, fimbriae, exotoxins, etc. All these organisms are present on the tooth surface in the form of biofilm. Biofilm is an organized aggregate of microorganisms living within an extracellular polymeric matrix that they produce and irreversibly attached to fetish or living surface, which will not remove unless rinse quickly [6]. Formation of Extracellular Polymeric Substances (EPS) occurs in the attachment stage of a biofilm to the surface. Usually thickness of EPS matrix

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is 0.2-1.0 *μm*, however the size of the biofilm does not exceed 10-30 nm. Typically 5-35% of the biofilm volume is constituted by the microorganisms while the remaining volume is extracellular matrix. This extracellular matrix is partially or mostly composed of proteins. Endodontic microbiota is established to be less diverse compared to oral microbiota. Endodontic bacterial biofilms can be categorized as-intracanal biofilms, extraradicular biofilms, periapical biofilms and biomaterial-centered infections.

It is important to known that a few species of microorganisms are more frequently isolated from persistent cases compared with primary cases. These include the *Enterococcus faecalis/faecium* group, enteric Gram-negative facultative rods, i.e., coliforms, and *Pseudomonas* species. *Enterococcus faecalis* has prevalence ranging from 22% to 77% of cases analysed [7]. In last decade, incidence of *Candida albicans* is 21% in primary infection, 18% in cases of retreatments [8,9]. Enterococcal cells are spherical or ovoid, occurring in pairs or short chains in liquid media. Endospores are not formed and some species can be motile by scanty flagella. They form creamy whitish colonies, are Gram-positive, catalase-negative and able to grow in 6.5% NaCl, at temperatures ranging from 101°C to 451°C and they can survive 30 min at 601°C and a pH over 9.6 [10]. Most enterococci are facultative anaerobes, but some species are strict aerobes. A wide range of carbohydrates are fermented in glucose broth with the production mainly of lactic acid with a final pH of 4.2-4.6, sometimes with lower values. They are among the most frequent causes of nosocomial infections and are recognized as potential human pathogen. Development of multiple resistances to various antibiotics in Enterococci poses serious therapeutic problems.

Enterococcus faecalis accounts for around 85-90% of all infections caused by enterococci [11]. Nosocomial and community-acquired infections caused by the genus Enterococcus include urinary tract infections, bacteremia, intra-abdominal infections and endocarditis [12,13]. *Enterococcus faecalis* which possess a group D carbohydrate cell wall antigen (Lancefield antigen), which is an intracellular glycerol teichoic acid associated with the cytoplasmic membrane. The cell wall contains a large amount of peptidoglycan and teichoic acid. The peptidoglycan (cross-linked peptide sugar), which is found in most of the bacterial cell walls, helps to maintain the microbe's shape and has a polysaccharide backbone of alternating N-(GlcNAc) and N-Acetylmuramic Acids (MurNAc). Because of the location of the peptidoglycan Acetylglucosamine on the outside of the cytoplasmic membrane and its specificity, the transglycosylation step has been indicated as a potential target for antibacterial medicaments. Enterococci possess a number of virulence factors that permit adherence to host cells and extracellular matrix, facilitate tissue invasion, effect immunomodulation and cause toxin-mediated damage. These factors include- 1) Aggregation Substance (AS), 2) enterococcal surface proteins such as esp, 3) gelatinase, 4) a cytolysin toxin, 5) extracellular superoxide production, 6) capsular polysaccharides and 7) antibiotic resistance determinant. *Enterococcus faecalis* is known to be a resistant species. Its persistence in endodontic infection might be aided by an enhanced resistance to sodium hypochlorite [14].

Enterococcus faecalis biofilms consist of exopolysaccharides, proteins, lipids, and Extracellular Deoxyribonucleic Acid (eDNA). The dense and protected environment of a biofilm may facilitate gene transfer and enhance biofilm stability. The eDNA is released *via* autolysis in a fratricidal or suicidal manner and/or active release through membrane vesicles and nanofibers in *Enterococcus faecalis* biofilms. There is a crucial role of eDNA in the formation, mechanical stability and maturation of bacterial biofilms in general and *Enterococcus faecalis* biofilms in particular. The eDNA is released *via* autolysis in a fratricidal or suicidal manner and/or active release through membrane vesicles and nanofibers in *Enterococcus faecalis* biofilms. Enterococci appeared to respond less well to penicillin due to an inherent tolerance to the killing action of these compounds [15]. It was later found that the addition of aminoglycosides to penicillin produced synergistic activity improving the cure rates for enterococcal infective endocarditis from 40 to 88% [16]. Hence, this became the standard of care for deep-seated enterococcal infections and this combination is still used to the present day. Among the most distinct examples of antibiotic resistance is the acquisition of the genes encoding vancomycin resistance. Vancomycin use was associated with the emergence and spread of Methicillin-Resistant Staphylococcus Aureus (MRSA) in the 1960s. Despite the availability of anti-gram-positive agents (e.g., line-zolid, Quinupristin/Dalfopristin [Q/D], Daptomycin [DAP], tige-cycline). This phenomenon makes the treatment of MDR enterococcal infections a daunting clinical challenge. High-level resistance to ampicillin (MIC 128 *μg/ml* or more) was first associated with increased production of the enzyme, requiring a higher concentration of antibiotic to saturate the active site [17].

Penicillin-Binding Proteins (PBPs) are the workhorses of the cell wall synthesis machinery and they can be roughly divided into two groups: class A, which are bifunctional enzymes that possess both D, D-transpeptidase and transglycosylase activity and class B, which possess only the transpeptidase domain and rely on the transglycosylase activity of other enzymes. All enterococci produce at least five PBPs, which can be differentiated by migration pattern on gel electrophoresis and were originally named by convention on the order of migration [18]. Subsequent genomic analysis of both *Enterococcus faecalis* and *Enterococcus faecium* revealed six putative PBP genes, three of which are class A (*ponA, pbpF, pbpZ*) and three class B (*pbp5, pbpA, pbpB*) [19]. Intrinsic tolerance to the action of *β*-lactams (like ampicillin and penicillin) is associated with the presence of a species-specific chromosomal gene, *pbp5*, which encodes a class B PBP with low binding affinity for ampicillin and the cephalosporins [20]. Thus, resistance in these strains was attributed to mutations altering the protein sequence, specifically a Met485 → Ala substitution near the active serine residue and the insertion of a serine residue at position 466 in a loop structure predicted to interact with the active site complex. Sequence variation of PBP5 is sufficient to distinguish two groups of *Enterococcus faecium*, one possessing high-level ampicillin resistance (termed *Pbp5*-R) associated with the hospital environment and a community-associated variant (*Pbp5*-S) that results in lower MICs to ampicillin (usually <64 *μg/ml*), but is unable to completely explain the differing susceptibility profiles observed in clinical practice [21]. Both overexpression of the enzyme and mutations in amino acid sequence have been implicated in higher levels of resistance, though neither method produced changes in MIC as dramatic as seen in *Enterococcus faecium*. Two main

mechanisms have been postulated to mediate DAP (Daptomycin) resistance in enterococci. The first is diversion of the antibiotic from the septum by redistribution of cardiolipin microdomains away from the division plane at the septum level. This mechanism, characterized in *Enterococcus faecalis* only, is initially mediated by substitutions in the LiaFSR signaling system that controls cell envelope homeostasis. The second mechanism, seen in *Enterococcus faecium*, is electrostatic repulsion of the positively charged daptomycin/calcium complex from the cell membrane. Several genes may be involved in this mechanism. DAP.

Candida albicans and to a lesser extent other *Candida* species are present in the oral cavity of up to 75% of the population [22]. Although yeasts have occasionally been reported in untreated cases [23], they have been more associated with cases of failed endodontic treatment. During both superficial and systemic infection, *Candida albicans* relies on a battery of virulence factors and fitness attributes. A number of attributes, including the morphological transition between yeast and hyphal forms, the expression of adhesins and invasins on the cell surface, thigmotropism, the formation of biofilms, phenotypic switching and the secretion of hydrolytic enzymes are considered virulence factors. Additionally, fitness attributes include rapid adaptation to fluctuations in environmental pH, metabolic flexibility, powerful nutrient acquisition systems and robust stress response machineries. Yeast cells adhere to host cell surfaces by the expression of adhesins. Contact to host cells triggers the yeast-to-hypha transition and directed growth *via* thigmotropism. The expression of invasins mediates uptake of the fungus by the host cell through induced endocytosis. Adhesion, physical forces and secretion of fungal hydrolases has been proposed to facilitate the second mechanism of invasion, i.e., fungal-driven active penetration into host cells by breaking down barriers. The attachment of yeast cells to abiotic (e.g., catheters) or biotic (host cells) surfaces can give rise to the formation of biofilms with yeast cells in the lower part and hyphal cells in the upper part of the biofilm. Phenotypic plasticity (switching) has been proposed to influence antigenicity and biofilm formation of *Candida albicans*. In addition to these virulence factors, there is a robust stress response mediated by heat shock proteins (Hsps); auto-induction of hyphal formation through uptake of amino acids, excretion of ammonia (NH_3) and concomitant extracellular alkalinisation; metabolic flexibility.

The transition between yeast and hyphal growth forms is termed dimorphism and it has been proposed that both growth forms are important for pathogenicity [24]. The hyphal form has been shown to be more invasive than the yeast form. On the other hand the smaller yeast form is believed to represent the form primarily involved in dissemination [25]. Hypha-associated proteins include the hyphal wall protein Hwp1, the agglutinin-like sequence protein Also, the secreted aspartic proteases Sap4, Sap5 and Sap6 and the hypha-associated proteins Ece1 and Hyr1. Deletion of *HGC1*, which encodes a hypha-specific G1 cyclin-related protein, results in cells that grow normally in the yeast form but fail to produce hyphae. Nevertheless, the *hgc1Δ/Δ* mutant cells still express at least four hypha-associated genes (*HWP1, ECE1, HYR1* and *ALS3*) [26]. *Candida albicans* adhesins are the Agglutinin-Like Sequence (ALS) proteins, which form a family consisting of eight members (Als1-7 and Als9). The family of Secreted Aspartic Proteases (Saps) comprises ten members, Sap1-10. Sap1-8 are secreted and released to the surrounding medium, whereas Sap9 and Sap10 remain bound to the cell-surface. Sap1-3 have been shown to be required for damage of Reconstituted Human Epithelium (RHE) *in vitro*, and for virulence in a mouse model of systemic infection [27]. However, the relative contribution of Saps to *Candida albicans* pathogenicity is controversial. Recent results indicate that Saps are not required for invasion into RHE and that Sap1-6 are dispensable for virulence in a mouse model of disseminated candidiasis.28 The family of phospholipases consists of four different classes (A, B, C and D) [29]. Only the five members of class B (*PLB1-5*) are extracellular and may contribute to pathogenicity *via* disruption of host membranes. The third family of secreted hydrolases, the lipases, consists of 10 members *(LIP1-10*) [30]. A *lip8Δ/Δ* mutant had reduced virulence in a mouse model of systemic infection, supporting a role for these extracellular hydrolases in *Candida albicans* pathogenicity. *Candida albicans* is a remarkable pathogen as it can utilize two different mechanisms to invade into host cells: induced endocytosis and active penetration [31]. For induced endocytosis, the fungus expresses specialized proteins on the cell surface (invasins) that mediate binding to host ligands (such as E-cadherin on epithelial cells and N-cadherin on endothelial cells), thereby triggering engulfment of the fungal cell into the host cell. Indeed, even killed hyphae are taken up, indicating that induced endocytosis is a passive process that does not require the activities of viable fungal cells. Two invasins have been identified so far, namely Als3 and Ssa1. Als3 and Ssa1 bind to host E-cadherin and likely induce endocytosis by a clathrin-dependent mechanism; however, macropinocytosis has also been implicated in *Candida albicans* induced endocytosis [32]. In contrast, active penetration is a fungal-driven process and requires viable *Candida albicans* hyphae. *Candida albicans* is not only able to sense and adapt to environmental pH, but can also modulate extracellular pH, actively alkalinizing its surrounding environment under nutrient starvation and, thereby, autoinducing hypha formation. Following adhesion to host cell surfaces and hyphal growth, *Candida albicans* hyphae can secrete hydrolases, which have been proposed to facilitate active penetration into these cells [33]. In addition, secreted hydrolases are thought to enhance the efficiency of extracellular nutrient acquisition. Three different classes of secreted hydrolases are expressed by *Candida albicans*: proteases, phospholipases and lipases. The primary goal of endodontic therapy is the reduction or elimination of micro-organisms and their by-products from the root canal system. Proper canal cleaning, shaping and irrigation, significantly reduce and sometimes eliminate bacteria from canals. The main factor which is needed for successful treatment of pulp and periradicular inflammation is complete removal of the source of infection such as microorganisms and their by products. Oxygenating a canal simply by opening it is detrimental to anaerobes. The drug of choice for endodontic infections is the Penicillin VK because of its spectrum of microbial activity against most of the bacteria associated with endodontic infections and also because of its low toxicity. Amoxicillin because of its wider spectrum than penicillin, clarithromycin and metronidazole for their action against most of the anaerobes found in the root canals, can be used as substitutes. If patient is allergic to penicillin, clindamycin is recommended. Intracanal medicaments play an important role in combating the microorganisms. Irrigation is critical to remove microorganisms from root canal systems. NaOCl is the most frequently used material

for endodontic treatment, it is an antiseptic and inexpensive lubricant that has been used at dilutions ranging from 0.5% to 5.25%. NaOCl is a strong oxidizing agent and may cause significant damage when in direct contact with tissue, including rapid hemolysis and ulceration, inhibition of neutrophil migration, and destruction of endothelial and fibroblast cells. The use of antimicrobial medication has been advocated to disinfect the root canal system. Calcium hydroxide plays an important role in endodontics by its ability to induce hard tissue formation and antibacterial property. When it is used along with sodium hypochlorite, it exaggerates the tissue-dissolving ability of sodium hypochlorite. Calcium hydroxide has been advocated as an intracanal medicament due to its bactericidal properties. Its high pH (of about 11-12.5) has a destructive effect on bacterial cell membrane and protein structure. Intracranial use of calcium hydroxide have shown to increase the efficiency of sodium hypochlorite and also the effectiveness of antimicrobial agent. Calcium hydroxide powder is mixed with water or glycerine to form a thick paste, which is placed in pulp chamber with amalgam carrier or a syringe. This paste is covered with a sterile cotton pellet and access is sealed with temporary restoration. Chlorhexidine gluconate has been shown to be an effective antimicrobial endodontic irrigant. Chlorhexidine does not have the ability to dissolve the pulp tissue. The substantivity property of chlorhexidine may prevent the microbial colonization on the dentinal surface. Studies have been conducted which proved that propolis can be used as an effective antifungal agent [34]. Thus, it can be concluded that for a successful endodontic outcome, one must have awareness of the close relationship between endodontic infections and microorganisms.

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