Viral-Bacterial Interactions in the Pathogenesis of Human Endodontic Disease

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ABSTRACT

In this study, we hypothesized that herpes viruses, EBV and/or HCMV contribute to endodontic disease via herpes virus–bacterium–host response interactions. In our *in vitro* study, we investigated potential pathogenic interactions between Epstein-Barr virus (EBV) and the Gram-positive bacterium, *Enterococcus faecalis*. In our *in vivo* study, we hypothesized that individuals with symptomatic endodontic disease would have elevated bacterial/viral replication and inflammation compared to that of asymptomatic individuals.

In our *in vitro* study, EBV reactivation within latently infected cells was assessed following exposure to *E. faecalis* metabolic end products and cell wall by quantitative PCR of media for EBV virions and of cellular mRNA for viral gene expression. To determine potential mechanisms of EBV reactivation, the effect of *E. faecalis* mediated activation of latent EBV was evaluated when the cells were simultaneously exposed to bacterial products and specific pharmacologic inhibitors of signal transduction pathways. The growth and virulent gene expression of *E. faecalis* when exposed to lymphoid cells with and without latent EBV infection were evaluated using real-time PCR. To assess the role of viral and bacterial interaction on the host response, transcription of cellular inflammatory genes was determined using real-time RT-PCR.

In our *in vivo* study, twenty pulp tissue samples were collected from patients diagnosed with irreversible pulpits, 10 with symptoms of self-reported severe pain (>7 on 10-point pain scale, Symptomatic) and 10 without self-reported pain (Asymptomatic), respectively. As controls, ten pulp tissue samples were collected from extracted healthy 3rd molars, which had no fracture, caries, or periodontal disease. Additionally, two

periapical tissue samples were collected during apicoectomy from patients with persistent periapical infection after root canal treatment. Total DNA and RNA were extracted from pulp tissue using DNeasy and RNeasy kit (Qiagen), respectively. Quantification of total bacteria, *Streptococcus sp.*, *Lactobacillus sp.*, *Fusobacterium sp.*, *Actinomyces sp.*, *E. faecalis*, EBV and Human Cytomegalovirus (HCMV) were assessed using quantitative PCR (qPCR) for consensus or organism specific DNA. Total bacterial RNA was used to indicate transcriptional activity and was evaluated using quantitative reverse-transcriptase PCR (RT-PCR). The expression of inflammatory genes including IL-6, IL-10, TNF-α, TNF-β and IFN-β was evaluated using quantitative reverse-transcriptase PCR.

The result from our *in vitro* study showed that lipoteichoic acid (LTA), a cell wall component of *E. faecalis*, can re-activate latent EBV. *E. faecalis* LTA -mediated induction of lytic EBV infection was significantly reduced by both a TLR2 antagonist and an inhibitor of the NF-kB/Ikb pathway. Interestingly, the growth of *E. faecalis* increased 5-fold with presence of EBV. The expression of *E. faecalis* virulence genes were significantly increased in presence of EBV. Proinflammatory cytokine expression was also significantly higher in the presence of EBV.

The result from our *in vivo* study showed that a greater than 5-fold increase was detected in bacterial DNA levels from pulp tissue of symptomatic patients compared to controls (P=0.004). These differences were not detected between asymptomatic patients and the control (P= 0.275). Likewise, total bacterial activity (RNA) was 5-fold higher in symptomatic patients than the asymptomatic (P=0.027). *Lactobacillus sp.*, *Fusobacterium sp.* and *Actinomyces sp.* were present in inflamed pulp tissue, but not in healthy pulp tissue. Among the samples with *Streptococcus sp.,* asymptomatic pulp

tissues had 30 fold higher levels, and symptomatic pulp tissues had 5 folds higher levels detected compared to controls (P=0.001, P=0.003 respectively). Interestingly, while EBV was widely detected, CMV was detected only in periapical tissues. Greater than a 30-fold increase in EBV DNA was detected in pulp tissue from symptomatic individuals compared to controls (P<0.001). Further, a 30-fold difference was shown in EBV detection in pulp tissue from symptomatic compared to asymptomatic (P=0.028) patients. The quantity of EBV in the pulp tissue from asymptomatic patients was comparable to the control (P=0.403). Real-time RT-PCR analysis of inflammatory gene expression in pulp tissues detected expression in disease but not within healthy controls. The IL-6 gene was expressed at levels 11-fold higher in symptomatic pulp than in asymptomatic pulp (P=0.026). Regression analysis indicated that inflammatory gene expression levels were associated with bacterial transcription and with EBV viral load.

Collectively, our *in vitro* results show that *E. faecalis* and EBV may have synergistic effect during inflammatory process. Our *in vivo* results suggest a significant association between bacteria, EBV and inflammation in symptomatic disease within the pulp tissue. CMV is more tightly associated with chronic infection than acute infection. These results support a paradigm shift in which elevated bacterial/viral replication and inflammation are associated with endodontic infection.

To my family

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1.1 Endodontic disease is prevalent and one of the major causes for dental pain.

Endodontic disease denotes inflammation within pulp tissue and around periapical tissue. Endodontic disease is prevalent. Endodontic therapy was required by 13.4% of the Air Force recruits in England in order to maintain the new recruits dentally fit for their first 5 years in the service (1). The needs for endodontic therapy was as high as 31% in Moscow student in a recent epidemiologic report (2). Periapical disease is prevalent and was evident in 4.1% of all teeth in a US population (3). Furthermore, endodontic disease is considered a major cause for dental pain (4). Dental pain is highly prevalent among the general population. Its reported prevalence in community dwelling adults ranges from 12 to 40% (5). According to the Executive Summary of the Surgeon General's report published in 2000 (6), 22% of adults reported some form of orofacial pain in the past 6 months. Every year, about 15 million working days are lost because of dental pain in the United States (6). In addition to direct costs for absenteeism, the overall burden must be considerably higher because of pain affecting function as well as quality of life. The majority of dental pain is attributable to endodontic diseases (7-9). In approximately 90% of dental emergencies with pain as a symptom, the pain is pulpal or periapical (10).

1.2 The diagnosis and classification of endodontic diseases

The classification and diagnosis of endodontic diseases is as follows according to the American Board of Endodontics' Pulpal and Periapical Definitions (http://www.aae.org/certboard/currentnews/).

Pulpal

Normal pulp. A clinical diagnostic category in which the pulp is symptom free and normally responsive to vitality testing.

Reversible pulpitis. A clinical diagnosis based on subjective and objective findings indicating that the inflammation should resolve and the pulp return to normal.

Irreversible pulpitis. A clinical diagnosis based on subjective and objective findings indicating that the vital inflamed pulp is incapable of healing.

Additional Descriptions

Symptomatic. Lingering thermal pain, spontaneous pain, referred pain.

Asymptomatic. No clinical symptoms, but inflammation produced by caries, caries excavation, trauma.

Pulp necrosis. A clinical diagnostic category indicating death of the dental pulp. The pulp is nonresponsive to vitality testing.

Previously treated. A clinical diagnostic category indicating that the tooth has been endodontically treated and the canals are obturated with various filling materials other that intracanal medicaments.

Previously initiated therapy. A clinical diagnostic category indicating that the tooth has been previously treated by partial endodontic therapy (eg, pulpotomy or pulpectomy).

Apical (Periapical)

Normal apical tissues. Teeth with normal periradicular tissues that are not abnormally sensitive to percussion or palpation testing. The lamina dura surrounding the root is intact and the periodontal ligament space in uniform.

Symptomatic apical periodontitis. Inflammation, usually of the apical periodontium, producing clinical symptoms, including painful response to biting and percussion. It may or may not be associated with an apical radiolucent area.

Asymptomatic apical periodontitis. Inflammation and destruction of apical periodontium that is of pulpal origin, appears as an apical radiolucent area, and does not produce clinical symptoms.

Acute apical abscess. An inflammatory reaction to pulpal infection and necrosis characterized by pain onset, spontaneous pain, tenderness of the tooth to pressure, pus formation and swelling of associated tissues.

Chronic apical abscess. An inflammatory reaction to pulpal infection and necrosis characterized by gradual onset, little or no discomfort, and the intermittent discharge of pus through an associated sinus tract.

1.3 Pathogenesis of endodontic diseases

It is widely accepted that the most common cause of pulp and periapical diseases is the presence of bacteria within the involved tooth and the most common pathways of entry for these bacteria are via caries, cracks, fractures and open restoration margins. Other possible pathways for bacterial penetration are associated with periodontal disease and dental trauma (11).

After bacteria or their byproducts enter a tooth's pulp chamber, endodontic inflammation occurs. Acute endodontic inflammation generally manifests as a toothache, whereas chronic endodontic inflammation can remain asymptomatic for months or years.

The progression of endodontic diseases is the balance between invading bacteria/bacterial byproduct and host immune response (Figure 1-1).

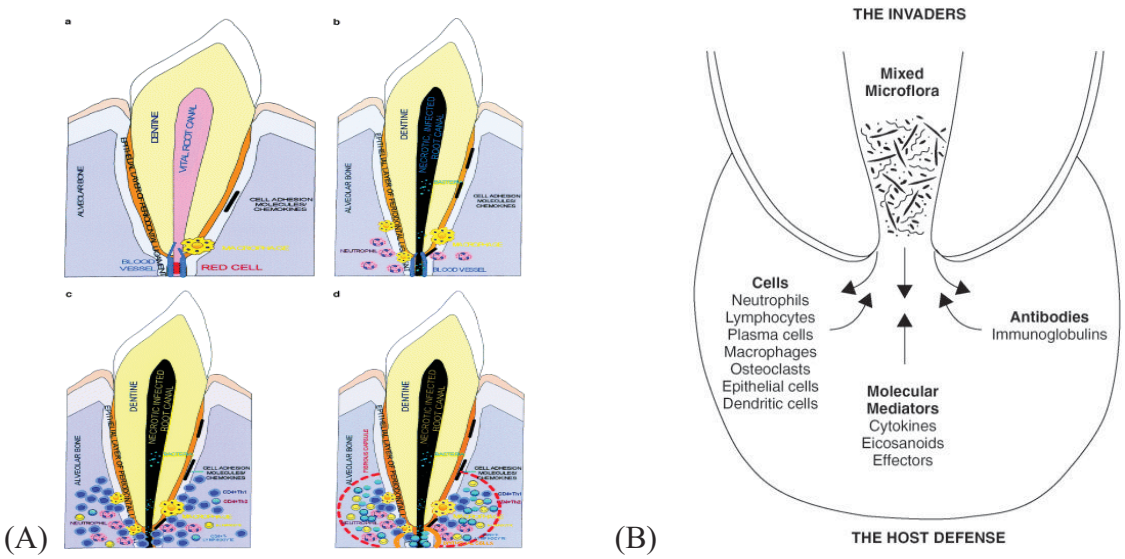

Figure 1-1 (A) Simplified schematic of progression of endodontic diseases from pulpal infection to periapical infection; **(B)** Simplified schematic of apical periodontitis, which is the body's defense response to the destruction of the tooth pulp and the hostile forei n occupation ' of the root canal. he microbial and host-defense forces clash and destroy much of the periapical tissues, causing the formation of different categories of apical periodontal lesions. (12)

1.3.1 The bacterial role in endodontic disease is well-established.

Endodontic disease is a polymicrobial, multistage inflammatory response initiated by the migration of opportunistic microorganisms from the oral cavity. This leads to an influx of inflammatory cells, resulting in pulpitis and periapical periodontitis. The significance of bacteria in the progression and perpetuation of pulpal and periradicular

inflammatory diseases is indisputable (13-14). Endodontic infections involve multiple bacteria. Two hundred different species have been identified within infected root canals, usually in combinations of 4-7 species (spp.) per canal (15-17). Of these, however, between 15 to 30 have been most frequently detected in infected root canals, including *Fusobacterium nucleatum*, *Streptococcus* spp., *Lactobacillus* spp. and *Actinomyces* spp. (16-18). Because the root canal environment and nutritional supply govern the dynamics of the microbial flora, the bacteria present in the root canal depend on the stage of the infection.

Primary intraradicular infection is dominated by anaerobic bacteria that includes both Gram –negative bacteria such as *Porphyromonas*, *Fusobacterium, Tannerella forsythia* (formerly *Bacteroides forsythus*), and Gram-positive bacteria such as *Pseudoramibacter alactolyticus, Streptococcus*, *Actinomyces* (19). After instrumentation/medication, Gram-negative bacteria are usually eliminated after treatment procedures (19). Gram-positive bacteria are more frequently present, including *Streptococci*, *Pavimonas micra*, *Actinomyces*, *Propionibacterium, lactobacilli*, *Enterococcus faecalis*, and *Olsenella uli*. Persistent or secondary intraradicular infection is characterized by persistence or appearance of apical periodontitis after treatment. The frequently detected bacteria in persistent infection include *Propionibacterium acnes, Propionibacterium propionicum, Actinomyces naeslundii, Actinomyces odontolyticus, Prevotella intermedia, Anaerococcus prevotii, Eggerthela lenta, E. faecalis, Gemella morbillorum, P. micra, P. alactolyticus, Streptococcus anginosus*, *Streptococcus mitis, F. nucleatum,* and *Candida albicans*. Extraradicular infection is characterized by microbial invasion of and proliferation in the inflamed periradicular tissues, and is derived from

intraradicular infection (20). Extraradicular infection can be dependent on or independent of intraradicular infection (19). Species reported by many studies include: *Actinomyces* species, *P. acnes, P. propionicum, Porphyromonas gingivalis, Prevotella intermedia, Prevotella oralis, P. micra,* and *F. nucleatum* (19).

1.3.2 Bacterial activation of pulpal immune responses contributes to endodontic pathogenesis.

Once bacteria invade into pulp tissue, a wide range of non-specific mediators of inflammation such as histamine, bradykinin, serotonin, interleukins (IL) and arachidonic acid metabolites are released. In addition, many neuropeptides (e.g. substance P and calcitonin gene-related peptide) are also involved. Besides non-specific inflammatory reactions, immunologic responses may also initiate and perpetuate pulp disease. In mild-to-moderate inflammation, cell-mediated immunity predominates. In severe inflammation, the appearance of B cells and plasma cells indicates local antibody production, therefore suggesting a predominance of humoral immunity. Bacterial substances may also trigger the complement system via antigen-antibody complexes, which become chemotactic for polymorphonuclear leukocytes. There is a distinct ratio difference between T-helper and T-suppressor lymphocytes in reversible and irreversible pulpits (21). Bacteria interact with immune cells via pattern recognition receptors such as Toll Like Receptors (TLRs) (Table 1) (22). Binding of bacterial adjuvants to these receptors activates nuclear factor-κB (NFκB), a central signaling pathway that leads to transcription of multiple proinflammatory molecules (23) All of these inflammation factors may contribute to disease progression.

Table 1 Pattern-recognition receptors and their ligands (22)

Pattern-recognition receptors	**Ligands**
TLR1	*Borrelia burgdorferi*, neisseria, lipoproteins (mycobacteria); triacyl lipopeptides (synthetic analogue)
TLR2	Trypanosomes, mycoplasma, borrelia, listeria, klebsiella, herpes simplex virus, zymosan (yeast), lipoteichoic acid and peptidoglycan (Gram+), lipoproteins (mycobacteria), atypical lipopolysaccharide ram , glycolipids, lipoarabinomannan, HSP 70 (endogenous ligand); di- and triacyl lipopeptides (synthetic analogue)
TLR3	Viral double-stranded RNA; Poly I:C (synthetic analogue)
TLR4	Plant product taxol, mycobacteria, respiratory syncytial virus, fibrinogen peptides, bacterial lipopolysaccharides ram , 0 endo enous ligand); lipopolysaccharide/lipid A mimetics (synthetic analogue); synthetic lipid A, E5564 (fully synthetic small molecule)
TLR5	Bacterial flagellins; discontinuous 13-amino-acid peptide (synthetic analogue)
TLR6	Zymosan (fungi), lipopeptides (mycoplasma), lipotechoic acid; diacyl lipopeptides (synthetic analogue)
TLR7	Single-stranded RNA, R-837 and R848; imidazole quinolines, i.e. Imiquimod, Resiquimod (fully synthetic small molecule); guanosine nucleotides, i.e. loxoribine (fully synthetic small molecule)
TLR8	Single-stranded RNA, R848; imidazole quinolines, i.e. Imiquimod (fully synthetic small molecule)
TLR9	Bacterial DNA, viral DNA, other DNA with low content of non-methylated CpG sequences; CpG oligonucleotides (synthetic analogue)
TLR10 (mouse)	Unknown
TLR11 (mouse)	Bacterial components from uropathogenic bacteria
TLR12 (mouse)	Unknown
TLR13 (mouse)	Unknown
NOD1	Bacterial peptidoglycans
NOD2	Bacterial peptidoglycans
Macrophage mannose receptors	Sulfated sugars, mannose-, fucose- and galactose-modified polysaccharides and proteins
Type 3 complement receptors	ymosan particles, β-glucan

1.3.3 Association between bacterial number and species and severity of endodontic diseases has been indicated.

The severity of disease clinically is defined as the size of periapical lesions and patients' symptoms, includin pain and swelling. In this project, the level of inflammatory factors produced within pulp tissue was used as the indication of the severity of irreversible pulpitis on a molecular level. A positive correlation exists between the number of bacteria in an infected root canal and the size of periradicular radiolucencies (24). There tends to be an association between different bacterial species and different clinical signs and symptoms. However, the association is not conclusive (16, 25). Whether other pathogens involved in endodontic pathogenic processes could affect the above mentioned association is not clear.

1.4 Viral involvement in endodontic diseases.

As early as 1965, viral involvement in endodontic disease had been investigated (26). Cohen & Shklar (26) in their mice study described that varying degrees of pulpal pathosis in noncarious teeth of mice injected at birth with polyoma virus and sacrificed at 6 to 12 months. In teeth with extensive carious involvement and carious exposures, the pulps were found to have undergone complete hemorrhagic necrosis in all cases. In the early 1990s, HIV was detected in pulp and periapical tissue is AIDS patient (27-28). Herpes viruses are prevalent in human. Whether the herpes viruses involved in endodontic diseases still needs investigation. The classification of herpes viruses is listed in Table 2.

Table 2 Classification of Human Herpes Viruses

Human herpes type	**Name**	**Target cell type**	**Latency**	**Transmission**
1	Herpes simplex-1 (HSV-1)	Mucoepithelia	Neuron	Close contact
2	Herpes simplex-2 (HSV-2)	Mucoepithelia	Neuron	Close contact usually sexual
3	Varicella Zoster virus (VSV)	Mucoepithelia	Neuron	Contact or respiratory route
4	Epstein-Barr Virus (EBV)	B lymphocyte, epithelia	B lymphocytes	Saliva
5	Cytomegalovirus (CMV)	Epithelia, monocytes, lymphocytes	Monocytes, lymphocytes and possibly others	Contact, blood transfusions, transplantation, congenital
6	Herpes lymphotropic virus	T lymphocytes and others	T lymphocytes and others	Contact, respiratory route
7	Human herpes virus-7 (HHV-7)	T lymphocytes and others	T lymphocytes and others	Unknown

1.4.1 EBV and CMV have emerged as putative pathogens for endodontic disease.

During the past decade, herpes viruses have emerged as putative periapical pathogens, particularly EBV and CMV (29-32). EBV is present more frequently in endodontic pathoses than in healthy controls (33), thus it may be that herpes viruses play a role in the development of symptomatic periapical lesions (31-32). In a study comparing the presence of CMV and EBV in samples from symptomatic versus asymptomatic periapical lesions, CMV was detected in 100% of the symptomatic and in 37% of the asymptomatic study lesions. In contrast, EBV was identified only in CMV-infected periapical lesions (32). Teeth with necrotic pulp and periapical lesions harbor herpes viruses in periapical granulomatous tissue. The occurrence of these viruses was

significantly greater when comparing large versus small periapical lesions, irrespective of whether or not the periapical lesions were symptomatic (30). Thus, herpes viruses, in cooperation with endodontic pathogenic bacteria, may play major roles in the pathogenesis of aggressive types of periapical pathosis in humans (30). Li *et. al.* (33)found the EBV DNA and RNA in significantly higher percentages (43.9 and 25.6 percent, respectively) in endodontic patients than in healthy patients (0 percent). They found HCMV DNA and RNA in measurable numbers in endodontic patients (15.9 and 29.3 percent, respectively) as well as in healthy patients (42.1 and 10.5 percent, respectively). The researchers found HSV DNA in low percentages of endodontic patients (13.4 percent), and they found VZV in only one endodontic patient. Chen *et.al.(34)* identified HCMV, EBV, herpes simplex virus-1 (HSV-1), and Varicella zoster virus (VZV) present in some patients presenting with acute apical abscesses and cellulitis of endodontic origin.

1.4.2 EBV and CMV infect immune cells and produce cytokines modulating inflammatory response.

EBV and CMV belong to the herpes virus family, and 60-100% adults are carriers of these viruses (35). EBV infects B-lymphocytes (36), while CMV infects monocytes/macrophages, T-lymphocytes, ductal epithelial cells of salivary glands, endothelial cells, fibroblasts and polymorphonuclear leukocytes, and establishes latent infection mainly in cells of the myeloid lineage (36) (Table 2).

Herpes viruses may establish latency or a productive (lytic) state of replication. In the viral productive cycle, the herpes virus genome is amplified 100- to 1000-fold by

the viral replication machinery (36). Herpes virus infections induce strong innate and adaptive antiviral immune responses. Lytic infection of cells with herpes viruses leads to an inhibition of the synthesis of cellular macromolecules. Herpes viruses evade the immune system through a complicated multifactorial mechanism either via NF- pathway or another undefined mechanism (36). EBV latent membrane protein-1 (LMP-1) has been shown to trigger cellular NF- and AP-1 activity (37) is essential for EBV-mediated B-cell immortalization. Herpes viruses interfere with innate and adaptive cellular and humoral immune responses by affecting cytokine production, the activation and silencing of natural killer cells, down-modulating antigen presentation in the major histocompatibility complex class I and II pathways, and impairing apoptosis (38). Cytokines produced in viral infection can significantly modulate the inflammatory response and include interleukin (IL)-1β, I -6, IL-1 , tumor necrosis factor α NF-α , interferon (INF)-α β, and INF- for CMV infection and I -1β, I -1 receptor antagonist (IL-1Ra), IL-6, IL-8, IL-18, TNF-α, INF-α β, INF- , for V infection (38).

1.5 Synergistic effects between virus and bacteria have been indicated in other infectious diseases.

Synergisms between virus and bacteria have been indicated in other infectious diseases such as respiratory tract infection (39) and otitis media (40). The increase evidence supports the concept that the increased susceptibility to bacterial infection of the virus-infected host is, at least in part, due to virus-induced alterations of the functional integrity of the host immune system (39). This may occur either directly by viral interaction with leukocytes or indirectly through virus-induced release of cellular

mediators or cytokines. In addition, cytokines may contribute to the development of pathological lesions and clinical symptoms, either alone or in concert with direct microbial cytopathic effects (39). The molecular and genetic basis for viral-bacterial synergy has been described in Bovine respiratory disease (BRD) (41).

A mouse model of synergy between influenza and pneumococcal in pneumonia has been established in mice (42). The study using this model suggests that influenza up-regulates the platelet-activating factor receptor and thereby potentiates pneumococcal adherence and invasion in the lung (42). This bacterium-virus synergy can be regulated by stress (43). Research in otitis media indicated that the etiopathogenesis of acute otitis media (AOM) involves viruses, bacteria, and a large network of other factors that affect each other in a time-dependent manner. Respiratory viruses play a crucial role in initiating the whole cascade of events that ultimately lead to the development of AOM. In the middle ear, viruses seem to interact with bacteria and enhance the local inflammatory process, which in turn may significantly impair the outcome of the disease (40).

1.6 EBV and CMV effects in endodontic disease may be indirect, via interactions with bacteria and the host

An interaction between viruses and bacteria during inflammation has been found (44). The purified major outer membrane component of Gram-negative bacteria, lipopolysaccharide (LPS), has been shown to be able to reactivate latent viral infection (44). Both short chain fatty acid-containing spent media and crude spent media containing LPS activate signal transduction pathways to allow for herpes virus reactivation. On the other hand, destruction of epithelial cells by herpes virus infection can facilitate the penetration of bacteria into connective tissue. Herpes viruses including

CMV inhibit the expression of macrophage surface receptors that recognize LPS and other components of the Gram-negative bacteria. Furthermore, herpes viruses destroy components of the major histocompatibility complex (MHC) pathway within macrophages and impair the immune defense in its ability to present antigen, silence natural killer cells, stop cell death, and divert antiviral cytokine responses (45-46). Therefore, herpes virus infections can promote bacterial colonization and can enhance the growth and pathogenicity of bacteria by disprupting several aspects of the host immune defense.

CMV and EBV infections may cause periapical pathosis by inducing cytokine and chemokine release from inflammatory or connective tissue cells, or by impairing local host defenses, resulting in heightened virulence of resident bacterial pathogens (32). The pathophysiologic mechanisms of severe periapical pathosis and the processes of periapical release of herpes virus-associated cytokines are intimately related. Viral-bacterial interactions are through both host cells, such as macrophages and leukocytes, and inflammatory molecules such as cytokines and chemokines.

1.7 The limitations of previous studies and hypothesis

Previous literature indicated that EBV, HCMV involved in endodontic diseases. However, majority of the studies have focused solely on the detection of viruses in a non-quantitative manner. There have been no mechanisms identified for the role of viral infection in endodontic disease. More importantly, the majority of the studies did not take into consideration of bacterial effect when establishing the role of virus in endodontic diseases.

In this study, we proposed the following models for bacterial and viral interactions and their involvement in endodontic diseases (Figure 1-2) and hypothesized that herpes viruses, EBV and/or HCMV contribute to endodontic disease via herpes virus–bacterium–host response interactions. In our *in vitro* study, we investigated whether *E. faecalis* and EBV can facilitate each other's patho enesis and whether *E. faecalis* re-activate latent EBV in lymphocytes via TLR2 mediate NF- pathway. In our *in vivo* study, we investigated whether EBV viral loads were associated with pulpal inflammation after adjusting for bacterial activity.

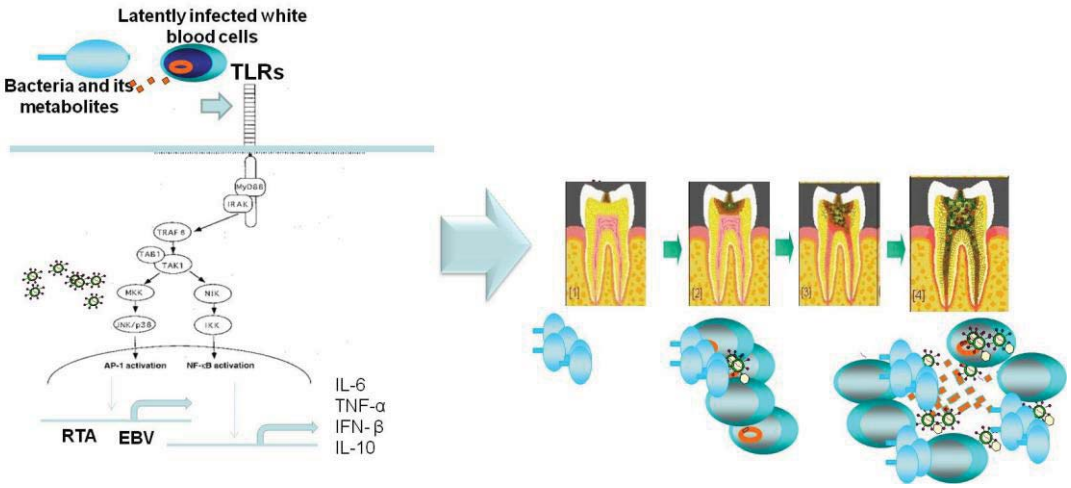

Figure 1-2 Hypothetical model of viral involvement in endodontic disease progression via viral-bacterial interaction. Healthy pulp becomes infected with bacteria. This results in the recruitment of inflammatory cells which potentially harbor latent viruses and re-activation of virus latently resident in the immune cells present in the pulp tissue. The presence of the bacteria and their associated components reactivates the virus via TLRs dependent NF- pathway within these cells resulting in viral replication, which influences the inflammatory milieu subsequently promoting disease.

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Chapter 2

Interaction between *Enterococcus faecalis* and latent Epstein-Barr virus

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Interaction between Enterococcus faecalis and latent Epstein-Barr virus

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ABSTRACT

Introduction: This study aimed to explore potential pathogenic interactions between Epstein-Barr virus (EBV) and the Gram-positive bacterium, *Enterococcus faecalis*.

Methods: EBV reactivation within latently infected cells was assessed following exposure to *E. faecalis* metabolic end products and cell wall by quantitative PCR of media for EBV virions and of cellular mRNA for viral gene expression. To determine potential mechanisms of EBV reactivation, the effect of *E. faecalis* mediated activation of latent EBV was evaluated when the cells were simultaneously exposed to bacterial products and specific pharmacologic inhibitors of signal transduction pathways. The growth and virulent gene expression of *E. faecalis* when exposed to lymphoid cells with and without latent EBV infection were evaluated using real-time PCR. To assess the role of viral and bacterial interaction on the host response, transcription of cellular inflammatory genes was determined using real-time RT-PCR.

Results: Lipoteichoic acid (LTA), a cell wall component of *E. faecalis*, can re-activate latent EBV. *E. faecalis* LTA -mediated induction of lytic EBV infection was significantly reduced by both a TLR2 antagonist and an inhibitor of the NF-kB/Ikb pathway. Interestingly, the growth of *E. faecalis* increased 5-fold with presence of EBV. The expression of *E. faecalis* virulence genes were significantly increased in presence of EBV. Proinflammatory cytokine expression was also significantly higher in the presence of EBV.

Conclusions: Collectively, our results show that *E. faecalis* and EBV may have synergistic effect during inflammatory process.

INTRODUCTION

Polymicrobial infections are present throughout the body and play a significant role in disease development. Viral-bacterial synergy in infectious diseases has been proposed (1-3). Epstein-Barr virus (EBV) infections are ubiquitous in the human population with 60-100% adults carrying the virus (3). Whether EBV-bacterial interactions play a role in infectious diseases requires further investigation.

EBV belongs to the herpes virus family and latently infects B-lymphocytes. The viral life cycle takes approximately 72 hours. Viral gene expression occurs in a temporally regulated manner, initiating with expression of the immediate early genes that encode regulatory proteins, followed by early gene expression that generally encodes enzymes for replicating viral DNA. Lytic infection of cells with the herpes viruses leads to an inhibition of the synthesis of cellular macromolecules. After viral replication has commenced, the late genes are expressed that encode structural components of the virion (4).

Herpes viruses may establish latency or can commence a productive (lytic) state of replication. In the viral productive cycle, the herpes virus genome is amplified 100- to 1000-fold by the viral replication machinery, resulting in a significant increase of virion production and release (4). Herpes virus infections often induce strong antiviral innate and adaptive immune responses (5). Herpes viruses interfere with innate and adaptive cellular and humoral immune response by affecting cytokine production, the activation and silencing of natural killer cells, down-modulating antigen presentation in the major histocompatibility complex class I and II pathways, and impairing apoptosis (6). Cytokines produced in viral infection can significantly modulate the inflammatory

response and include interleukin (IL)-1β, I -1 receptor antagonist (IL-1Ra), IL-6, IL-8, IL-18, TNF-α, INF-α β, INF- (6). EBV latent membrane protein-1 (LMP-1) triggers both cellular NF- and AP-1 activity and is essential for EBV-mediated B-cell immortalization (7). Herpes viruses evade the immune system through a complicated multifactorial mechanism either via NF- pathway or another undefined mechanism (4).

Enterococci now rank among the top three nosocomial bacterial pathogens(8). Two species are common commensal organisms in the intestines of humans: *Enterococcus faecalis* (90-95%) and *Enterococcus faecium* (5-10%). Enterococci can survive and adapt to adverse conditions such as heat, ethanol, hydrogen peroxide, acidity, and alkalinity. The virulence factors of *E. faecalis* and their functions have been studied extensively, including enterococcal surface protein (ESP) (9), LTA, aggregation substance (AS), surface adhesins (Adh), bacteriocins (Bact), binding substance (BS), collagen peptides (CP), cytolysin (Cyl), elastase (Elas), gelatinase (Gel), hyaluronidase (Hya), and hydrogen peroxide (H_2O_2) (10). The virulence factors of *E. faecalis* stimulate PMN leukocytes, lymphocytes, monocytes, and macrophages, causing inflammation (10). Bacteria interact with immune cells via Toll Like Receptors (TLRs) (11). Different TLRs bind to specific "molecular signatures" of different classes of micro-organisms or to individual features present on diverse commensals or pathogens; for example, TLR2, lipopeptide; TLR4, lipopolysaccharide; and TLR5, flagellin (11). It has been suggested that *E faecalis* binds to TLR2 via its cell wall component, LTA. Binding of bacteria to these receptors activates nuclear factor-κB (NFκB), a central signaling pathway that initiates transcription of multiple proinflammatory molecules (12).

Previous studies have determined a role for bacterial infection in reactivation of Kaposi's sarcoma-associated herpesvirus (KSHV) (13). The interaction between herpes virus and bacteria in periapical pathosis has been proposed. The mechanism of the interaction has not yet been described. Slots *et al.*(14) hypothesized that HCMV and EBV infections may cause periapical pathosis by inducing cytokine and chemokine release from inflammatory or connective tissue cells, or by impairing local host defenses, resulting in heightened virulence of resident bacterial pathogens. Host leukocytes are often latently infected with viral pathogens, during bacterial mediated endodontic disease it is feasible that the presence of leukocytes harboring virus may compound the disease process. It may be that the pathophysiologic mechanisms of severe periapical pathosis and the processes of periapical release of herpes virus-associated cytokines are intimately related.

Very few studies have investigated interactions between herpesviruses and Gram-positive bacteria (13, 15). In this study, we hypothesized that *E. faecalis* can re-activate latent EBV infection. We further hypothesized EBV infected cells can facilitate *E. faecalis* infection; and EBV-*E. faecalis* interaction may aggravate the inflammatory response. We investigated the mechanism of this reactivation during *E. faecalis* pathogenesis.

MATERIALS AND METHODS

Cell lines, cell culture, collection of E. faecalis spent medium and cell wall component extraction

B958 is a latently infected EBV lymphoma-derived cell line. DG75 is an EBV-negative B lymphoblastoid cell line. BL 41 with and without latent EBV are lymphoma-derived cell lines with and without latently infected EBV. Cell lines were maintained in 5% CO_2 at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and containing penicillin, streptomycin, sodium pyruvate, and L-glutamine (GIBCO-BRL, Gaithersburg, MD).

E. faecalis strains ER3/2s and ER5/1 were kind gifts from Dr. Christine Sedgley (University of Michigan, Ann Arbor, MI). *E. faecalis* strains were grown aerobically in Brain Heart Infusion (BHI) at 37°C. Spent medium was collected from cells that were harvested from overnight culture at a late exponential stage of growth via centrifugation of bacterial culture at 5,000 rpm for 10minutes. The supernatant was filtered through 0.2 M filter. he filtrate was added at a 1:20 dilution as spent medium for induction of re-activation of latent EBV/HCMV in immune cells. The crude extraction of cell wall component of *E. faecalis* was performed using lithium chloride method published previously (16).

Antibodies and chemicals

The goat polyclonal antibody against ß-actin was purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. The EBV antibody to Early D Antigen (EA-D) was obtained from ARUP laboratory (Salt Lake City, UT). *E. faecalis* LTA was purchased from Sigma (St. Louis, MO). The I phosphorylation inhibitor, Bay 11-7082 (Calbiochem, Gibbstown, NJ) was used to inhibit transcription factor NF-kB activity. The monoclonal antibody specific to TLR2, TLA2.1 (eBioscience, San Diego, CA) was

applied as the TLR2 antagonist. RQ1 DNase was obtained from Promega, Madison, WI. All reagents were prepared as recommended by their suppliers.

Induction and treatment

E. faecalis spent medium, the commercially available major cell wall component of *E. faecalis*, LTA, as well as the crude extraction of cell wall were used as induction material in experiment. The fresh bacterial growth medium and cell wash solution, distilled phosphate-buffered saline (PBS) were used as negative controls. Ten milliliters of fresh growth medium was added to the cells prior to treatments. Latently infected lymphoid cells (B95-8, DG75 and BL-41) were maintained at densities between 2.5 x 10^5 and 3.0 x 10^5 cells/ml. B95-8, BL41 cells and DG75 cell lines and induction materials were incubated for 12hrs or 48 hrs. For inhibition experiments, cells were pre-incubated in culture medium supplemented with TLR2 antagonist (TLA2.1) at 20 μg/ml or Bay 11 at 2uM for 1 h at RT. These cells were subsequently induced into the lytic cycle by exposure to 20 μg/ml LTA (17). The successful re-activation of latent virus was assessed via qPCR for virions released into the supernatant and by viral protein expression.

To determine the effect of EBV *on E. faecalis* infection, cell lines with and without latent EBV were incubated with *E. faecalis* at a starting concentration of 10^3 CFU/ml for 12 hrs. Bacterial cells were harvested by centrifugation of the culture at 8,000 rpm for 15 minutes. The cell pellets were stored at -80°C for assessment of quantities and virulent gene expressions.

Extracellular EBV virion DNA isolation and quantification

Cells were removed from the medium by centrifugation at 1,500 x *g* for 5 min. The supernatant was collected and centrifuged again for 30 min at 3,000 rpm. The cell-free culture medium was filtered through a 0.45-micrometer-pore-size filter distributed into Beckman Ultra Clear tubes, and viral particles were pelleted by centrifugation at 22,000 x *g* for 2 h at 4°C. The supernatant was removed, and viral pellets were resuspended in buffer containing 40 mM Tris-HCl (pH 7), 10 mM NaCl, 6 mM $MgCl_2$, and 10 mM $CaCl_2$. RQ1 DNase was added, followed by incubation at 37°C for 1 h to eliminate free DNA. DNase was inactivated by adding EDTA to 20 mM and heating to 80°C for 5 min. Viral DNA was isolated using a High Pure viral nucleic acid kit (Roche, Indianapolis, IN).

Viral DNA within each sample was quantified using quantitative PCR (QPCR) with the specific primers targeting W area of EBV, (5'-CCTGGTCATCCTTTGCCA-3' and 5'-TGCTTCGTTATAGCCGTAGT-3'), and SYBR Green PCR Kit (Roche). For each sample tested, the QPCR assay was performed with a final volume of 25µl reaction mixture, containing 100 ng of DNA. Amplification conditions were initial 2 min at 50°C to eliminate carryover contamination, denaturation at 95°C for 10 min, 40 cycles consisting of denaturation at 95°C for 15 s, annealing at 60°C for 1 min., and extension at 72°C for 30 s, and final extension at 72°C for 7 min. DNA from uninfected DG75 cells and reactions run in the absence of added DNA served as negative controls. To make viral quantities within each sample comparable, β-actin DNA within each sample was used as internal control. β-actin DNA was quantified using QPCR with specific primer (5'-GGAACCGCTCATTG CC-3' and '-ACCCACACTGTGCCCATCTA-3' .

Analyses of the data were performed with associated Prism 7000 software (Applied Biosystems). The uninduced EBV level was arbitrarily set at 1.

Cell lysis, bacterial lysis and Nucleic acid isolation

Cells were pellet from treated cell culture via centrifugation at 1,500 x *g* for 5 min. The cell was then resuspended in DPBS and split into two portions, 1/3 was used for DNA isolation using DNeasy kit (Qiagen, Carlsbad, CA, USA), 2/3 was used for RNA isolation using RNeasy kit (Qiagen) according to the procedure recommended by the manufacturer.

The bacterial cell pellet was resuspended in a lysis buffer consisting of 20mM Tris (pH 8.0), 2mM EDTA, 1.2% Triton X-100 in RNase free water supplied with Lysozyme (20mg/ml), and incubated at 37 °C for 30 minutes. The lysate was split into two portions, 1/3 was used for DNA isolation using DNeasy kit (Qiagen), 2/3 was used for RNA isolation using RNeasy kit (Qiagen) according to the procedure recommended by the manufacturer.

cDNA generation

RNA was reverse transcribed to cDNA using Superscript II Reverse Transcriptase (RT) and Random Hexamer pd$(N)_6$ according to the procedure recommended by the manufacturer. For each sample, a no-RT control was used. Absence of DNA contamination in the process was assured by end-point PCR for no-RT control with bacterial universal primers ’- TCCTACGGGAGGCAGCAGT -3’ and ’- GGACTACCAGGGTA TCTAATCCTGTT -3’ (18) designed based on a conserved

region of 16S rDNA. The PCR mix (25 µl) contained 1 µl template cDNA, 1 mM $MgCl_2$, 0.1 mM concentrations of each deoxynucleoside triphosphate, 0.4 µM concentrations of each primer, 0.5 U of *Taq* Polymerase, and TAQ buffer with final concentrations of 10 mM Tris-HCl (pH 8.3) and 10 mM KCl. Amplification conditions were initial denaturation at 94°C for 4 min, 40 cycles consisting of denaturation at 94°C for 30 s, annealing at 55°C for 30 s., and extension at 72°C for 30 s, and final extension at 72°C for 7 min..

Assessment of E. faecalis virulent gene expression

The expression levels of virulent genes in *E. faecalis* were evaluated using real-time PCR with the corresponding primers (listed in Table 1), which were designed based on the *E. faecalis* sequences using Primer3, and SYBR Green PCR Kit (Roche). The real-time PCR conditions were set at 50°C for 2 min and 95 °C for 10 min, followed by 40 cycles at 94°C for 15 s, annealing at 55°C for 30 s. All analyses were performed in triplicates and included the negative control of sterile H_2O.

Protein preparation and Western blot

For extraction of total cellular protein, cells were collected by centrifugation, washed once in PBS, resuspended in 200 µl ice cold extraction (RIPA) buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 1% Na deoxycholate, 25 µg/ml leupeptin, and 50 µl protease inhibitor cocktail [Sigma]), passed through a 25-gauge needle, and centrifuged. For nuclear protein extraction, cell pellets were washed as described above and resuspended in 500 µl ice

cold extraction buffer A (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 1.0 mM dithiothreitol) and centrifuged. Cells were then resuspended in 200 µl buffer A containing 0.1% NP-40 and 1x complete proteinase inhibitor, incubated on ice for 10 min, and centrifuged to pellet nuclei. Nuclear pellets were washed in buffer A, centrifuged, and resuspended in 50 µl of extraction buffer C (10 mM HEPES [pH 7.9], 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1.0 mM dithiothreitol, 25% glycerol), incubated on ice for 30 min with occasional vortexing, and centrifuged. Cell lysates were resuspended in SDS sample buffer, and 50 to 150 µg of proteins was loaded in lanes for SDS-polyacrylamide gel electrophoresis (10% gel was used). Western blot analysis was performed for the detection of proteins expressed in active EBV using antibodies specific for EBV EA-D protein following the procedure published previously (13). The blot was also reacted with antibodies to detect β-actin to confirm equal protein loading. The relative levels of expression of each cellular or viral protein were compared by quantitative densitometry.

RESULTS

Treatment of latent EBV infected cell lines with *E. faecalis* results in EBV reactivation

The cell wall components containing LTA among others had significant effect on the ability of latently infected cells (B958) to release virions into the medium, and resulting in a 6-fold increase in viron release. Compared to treatment with the medium alone (Figure 1A), LTA at 20 µg/ml resulted in a 17 fold increase in virus release. Interestingly, the spent medium of *E. faecalis* used in this study was comparable to the uninduced negative control and had minimal effect on induction of the EBV lytic cycle.

Lymphoma cell line, BL 41 containing EBV, had a 340-fold increase in virion quantity post LTA induction (Figure 2B). The ability of the major cell wall component of *E. faecalis*, LTA, to activate latent EBV infection was further confirmed by the Western blot detection of the early lytic antigen EAD (Early Antigen Diffuse) expression. Upon densitometry, it was determined that EAD expression was enhanced greater than 6-fold upon *E. faecalis* LTA treatment (Figure 1B). Additionally, viral genes from each stage of the temporal lytic cycle were assessed. Compared to uninduced cells, in cells with 12-hour post *E. faecalis* LTA treatment, immediate early transcripts encoding for RTA were increased 8-fold; early transcripts encoding EAD were increased 90-fold; and late gene transcripts encoded by BcRF1 increased 4x 10^3-fold (Figure 1C).

TLR2 antagonists and NF-kB inhibitor, Bay 11 diminished *E. faecalis* induced EBV replication.

E. faecalis LTA -mediated induction of lytic EBV infection was significantly reduced by treatment with both the TLR2 antagonist and an NF-kB/Ikb pathway inhibitor, Bay 11. In this set of experiments a 7-fold increase in virion production was detected upon *E. faecalis* LTA treatment. Pretreatment with the TLR 2.1 antibody resulted in diminished virion production to levels comparable to uninduced. Assessment of TLR2 antagonist treatment also significantly reduced expression of the EBV lytic protein EAD (Figure 1B). This indicated that *E. faecalis* activation of latent EBV may occur through TLR2 mediated NF- pathway, but may also involve other undefined mechanisms. Similar experiments were performed in BL41 cell line with latent EBV infection. BL41 cells treated with LTA demonstrated enhanced virion production that was significantly

reduced approximately 7-fold upon treatment with both the TLR2 antagonist, and the NF-kB/Ikb pathway inhibitor, Bay 11. However, these levels were not reduced to the level of uninduced upon pretreatment with the TLR 2.1 antibody and Bay 11 (Figure 2B),

EBV enhanced the growth and virulence of *E. faecalis*.

Co-culture of *E. faecalis* and lymphoid cells with or without EBV showed that more *E. faecalis* grew in the culture when EBV presented. The QPCR data are consistent with the density data (Figure 3). When comparing bacterial quantities by qPCR, a 4.7-fold difference was detected in bacterial load when bacteria were co-cultured with BL 41 cells containing latent EBV versus bacterial cells cultured with virus negative cells (Figure 3B). Furthermore, the presence of EBV, in co-culture resulted in detection of enhanced *E. faecalis* virulence-associated genes expression. Two distinct virulent EF strains, ER5/1 and ER3/1, were assayed post co-culture with BL 41 cell with or without EBV. Three different virulence-associated genes were assessed for each strain. For ER 5/1, v1-Ef0485, an aggregation substance gene, v2-gelE, a gelatinase gene and, v3-ace a collagen binding antigen were assessed. For ER 3/2s v1-Ef0485, an aggregation substance gene, v2-esp, a surface adhesion protein and, v3-ace a collagen binding antigen were assessed. For analysis, the co-culture containing BL41 no virus was set to one. In co-cultures containing EBV, 2-3 fold increases were detected in expression of all of the virulence-associated genes regardless of *E. faecalis* strain (Figure 3C).

***E. faecalis* and EBV increased inflammatory gene expressions**

Both LTA and crude cell wall extract resulted in an 8- and 7-fold increases, respectively, in IL-6 transcript expression as determined by real-time RT PCR (Figure 4A). At 12 hours post treatment of B958 cells with *E. faecalis* LTA, the expression of pro-inflammatory genes was assessed. Enhanced expression of all four genes assayed was detected in *E. faecalis* LTA treated virus containing cells compared to untreated B958 cells; A 38-fold increase in TNF-α mRN , a 20-fold increase in IL-6 mRNA, a 120-fold increase in IFN-β mRN , and a 60-fold increase in IL-10 mRNA were detected. All increases were statistically significant ($P < 0.001$). We next directly compared the expression of these inflammatory mediators in *E. faecalis* treated EBV positive and negative cell lines. The expression of the inflammatory mediators was significantly higher in the cell line with EBV infection treated with *E. faecalis* LTA compared to cell line without EBV infection treated with *E. faecalis* LTA; for TNF-α 3 folds higher, IFN-β 11 folds higher, IL-10 50 folds higher and IL-6 15 folds higher (Figure 4C).

DISCUSSION

Polymicrobial infections are present throughout the body and play a significant role in disease development. Viral-bacterial synergy in infectious diseases has been proposed (1-3). In this study we investigate the potential for interaction of the Gram-positive bacterium, *E. faecalis* and *EBV*, as both organisms that have been detected in severe persistent endodontic infection. The results from this study support our hypothesis that *E. faecalis* can re-activate latent EBV infection. The reactivation is most likely via TLR2 mediated NF-kB/Ikb pathway, although other yet undefined pathways may be involved as well. The data presented in this study support our hypothesis that EBV may

facilitate *E. faecalis* pathogenesis. It appears that the interactions of EBV and *E. faecalis* aggregate cellular inflammatory response.

Previous studies from our group have determined that metabolic end products of the Gram-negative anaerobic pathogens, *F. nucleatum* and *P. gingivalis* can reactivate KSHV lytic replication, resulting in an increase in the release of virions; while metabolic end products from Gram-positive organisms, *S. aureus* and *S. mutans*, did not result in activation of latent KSHV (13). In this study, metabolic end products from the Gram-positive bacterium, *E. faecalis* did not result in activation of the related gamma herpesvirus latent EBV. We demonstrate that *E. faecalis* LTA engagement of the TLR2 receptor (17, 19-20) is a potent inducer of EBV reactivation. Likewise, it has been shown that activation of TLR 7/8 results in reactivation of the related herpesvirus, KSHV (21). In our study, we demonstrate that *E. faecalis* LTA mediated reactivation of EBV most likely occurs though TLR2 mediated NF-kB/Ikb pathway. Mechanisms of bacteria mediated reactivation are distinct between these two gamma herpesviruses (22). Previous studies suggested cross-talk exists between different TLRs (23-24), some TLRs might share similar signal transduction pathways. Recent studies also determined that activation of TLRs may lead to activation of other transcriptional factors in addition to NF-kB (25-26). In our case, inhibition of TLR2 diminished the LTA mediated re-activation of latent EBV, but not to uninduced levels, suggesting that other pathways might be also involved. The data from different latently infected lymphoid cell lines suggests that the magnitude of *E. faecalis* activation of latent EBV infection and the activation pathways might be cell-type specific. There may be dependence on the types and density of cell surface receptors that bind *E. faecalis* cell wall components. Future

studies are necessary to validate mechanisms and delineate other potential signal transduction pathways involved in the process of re-activation.

Studies have not yet thoroughly investigated the profile and concentration of the components in the metabolic end produce and their relation to the effect of re-activation of latent herpesviruses. Some components in the metabolic end products of gram-positive bacteria might be able to re-activate latent herpesvirus when higher concentration used. Using current experimental conditions, it seems that the effect of gram-positive bacteria re-activation of latent herpesvirus is mainly through the interaction between bacteria and the host cells.

In our study, it was shown for the first time that the presence of EBV tended to promote both *E. faecalis* growth and enhances the expression of bacterial virulence factors. The mechanism of the interaction is unclear. EBV may condition the host cell media to facilitate *E. faecalis* growth. Alternatively, there may be indirect bacterial viral interactions via the cell inflammatory response or changes of local environment, or finally direct interaction between EBV and *E. faecalis*. It is known that virulence genes in *E. faecalis* including genes encoding enterococcal surface protein (*esp*), aggregation substance, clustered in a special regions of the genome termed pathogenicity island (PAI) (27). Genes responsible for regulation of PAI genes often locate in the same PAI (28). Recent study (29) showed AraC-type transcriptional regulators within PAI, which are usually functional in carbon metabolism or stress response, contributes to *E. faecalis* pathogenesis. Cellular metabolism is modulated by viral infection, suggesting the changes of local environment (30). There is the potential that metabolic products expressed in EBV infected cell culture supernatants might promote *E. faecalis* growth

and virulence. The effect of EBV on *E. faecalis* might attribute to inflammatory factors. It is known that EBV can increase cellular IL-10 secretion and expresses a viral homolog, vIL-10 (31). Interestingly, previous study has demonstrated that hIL-1β, si nificantly increased *Staphylococcus aureus* virulence (32). The mechanism by which hIL-1β e erts direct effects on *S. aureus* gene expression is not clear. However, the study showed the genes from the *S. aureus* PAI were modulated upon exposed to hIL-1β. here is a possibility that the increased IL-10 level of IL-10 or vIL-10 has direct effect on *E. faecalis*, increasing its virulence-associated gene expression levels. Alternatively, the EBV virions may interact directly with the bacteria. There is a precedent for this in a study that suggested a direct interaction between influenza A virus and the capsule of the bacterium *N. meningitidis* facilitated bacterial adhesion to cultured epithelial cells enhancing bacterial virulence (33). More research is warranted to 1) confirm that the phenomenon is not bacterial stain or species dependent; and 2) whether such effect is host cell –mediated or metabolic product related.

The phenomenon of viral bacteria synergy, particularly in respiratory disease, has been documented since early last century (34). In our study, an enhanced inflammatory response was observed when both viral and bacterial pathogens were co-present, consistent with previous studies on viral bacterial synergy in respiratory disease (35) . The release of exogenous agents (e.g. bacterial and viral products) and the subsequent induction of endogenous mediators (e.g. cytokines and chemokines) during the inflammatory response, contribute to the viral bacterial synergy (35). The various inflammatory factors may interact with each other, may regulate viral- bacterial virulence, or modulate host cellular inflammatory responses.

The mechanism by which herpesviruses bacteria interact with each other has been proposed in previous literature (3). Base on our data in this study and previous literature, we proposed the model of hepersviral-bacterial synergy (Figure 5). Briefly, bacteria infecting the healthy pulp result in activation of latent virus within immune cells presented in the pulp. The inflammation caused by bacteria infection may help recruit inflammatory cells which potentially harbor latent viruses. The presence of the bacteria and their associated components reactivates the virus within these cells resulting in viral replication, which influences the inflammatory milieu subsequently promoting disease.

In summary, the data presented in this study demonstrated interactions between *E. faecalis* and EBV, and suggested that there is an additive effect between *E. faecalis* and EBV with regard to their contributions to the proinflammatory process. More studies are needed to investigate the mechanisms of these interactions.

Table 1 Primers sequences for real-time PCR detection of selected virulent genes expression in *E. faecalis*

Virulent factors	Gene	rimer equences '--3'	
		Forward	Reverse
Aggregation Substance	*ef0485*	GTGATTGGGACGCTGTAGGT	GCGCACTAGATACAGGCACA
Surface Adhesin	*esp*	TTTAGCAATTGAGCGACGTG	CGATTTTATCCGCTGCTTTC
Gelatinase	*gel*	GGGGCAATACAGGGAAAAAT	TCCTTCCCCAGTTTCCTTTT
Collagen binding antigen	*ace*	ATGGCTACCACGAAAAATC	ATTCGGTTGCGAACTATTGG

Figure legends

Figure 1. Treatment of B958 cells with *E. faecalis* LTA results in EBV reactivation (A). *E. faecalis* induces EBV virion release. The levels of EBV virion after the cells with latent EBV infection (B958) treated with spent media and cell wall components of *E. faecalis* were quantified as described in Material and Methods. Fold increases were calculated by comparison of the EBV virion quantity from cell culture treated with inducers over uninduced culture (the cell without treatment). The virion level was normali ed usin cell numbers indicated by cellular β-actin levels. **(B). Increased viral protein (EA-D) expression induced by cell *E. faecalis* LTA was inhibitable with TLR2 antagonist.** B958 cells were treated with *E. faecalis* LTA, *E. faecalis* LTA plus TLR2 antagonist. Cellular protein was isolated and immunoblotted for EBV EA-D and β-actin as described in Materials and Methods section**. (C). Increased viral gene expression was induced by *E. faecalis* LTA.** Viral genes expression levels were assessed post 12 hrs treatment of *E. faecalis* LTA compared to the one without treatment (uninduced). RNA was isolated, cDNA was generated and real-time PCR was performed as described in Materials and Methods section. The viral gene expression levels within uninduced samples were set as 1. The difference was expressed in fold. ⋆Statistically significant ($P<0.05$).

Figure 2. TLR2 antagonist and Bay 11 decrease *E. faecalis* induced EBV replication. The latent EBV infected cell cultures were pretreated with TLR2 antagonist or Bay 11 and then treated with *E. faecalis* LTAfor 1 hrs. he virion and β -actin levels were quantified using QPCR. he virion levels were normali ed usin cellular β-actin levels.

Shown data were the mean of fold increased of virion compared to the one without LTA treatment (uninduced). LTA, *E. faecalis* LTA treatment only; ihTLR2+LTA, cell treated with TLR2 antagonist for 1hr and then treated with *E. faecalis* LTA; Bay11+LTA, cell treated with Bay11 for 1hr and then treated with *E. faecalis* LTA. **(A)** B958 cell line; **(B)** BL41 cell line with latent EBV infection. ⋆ Statistically significant ($P<0.05$).

Figure 3 EBV enhanced the growth and virulence of *E. faecalis*. (A) The bacterial cell density after incubation with cell lines (BL41) with or without latent EBV. Overnight culture of *E. faecalis* was diluted to 1:100 and then incubated with BL-41 w/ or w/o EBV for 12 hrs. Cell density was determined by measuring the optical density at 600nm. **(B) The bacterial quantities after incubation with cell lines (BL41) with or without latent EBV.** The bacterial DNA from the culture treated as above were isolated and quantified using CR. acterial quantities were normali ed usin β-actin. **(C) The increases of virulent gene expression levels in *E. feacalis* with presence of EBV compared to the one without presence of EBV.** Clinical isolates of *E. feacalis* were incubated with cell line (BL41) with or without latent EBV for 12 hrs. The gene expression level was normalized using bacterial cell 16S rRNA. The expression levels of virulent genes and 16S rRNA were quantified using real-time PCR. The expression levels of virulent genes within individual *E. faecalis* strain co-cultured with BL41 without latent EBV infection were set as 1 (BL41-EBV+*E. faecalis*). V1: *Ef0485*; V2: *gelE* for *E. faecalis* 5/1 or *esp* for *E. faecalis* 3/2s; V3: *ace*. ⋆ Statistically significant ($P<0.05$).

Figure 4 *E. faecalis* and EBV increased inflammatory gene expressions. (A) The IL-6 gene expression in B958 cell line treated with *E. faecalis* LTA. The B958 cell line was treated with *E. facealis* spent medium, crude cell wall extractions and LTA for 48

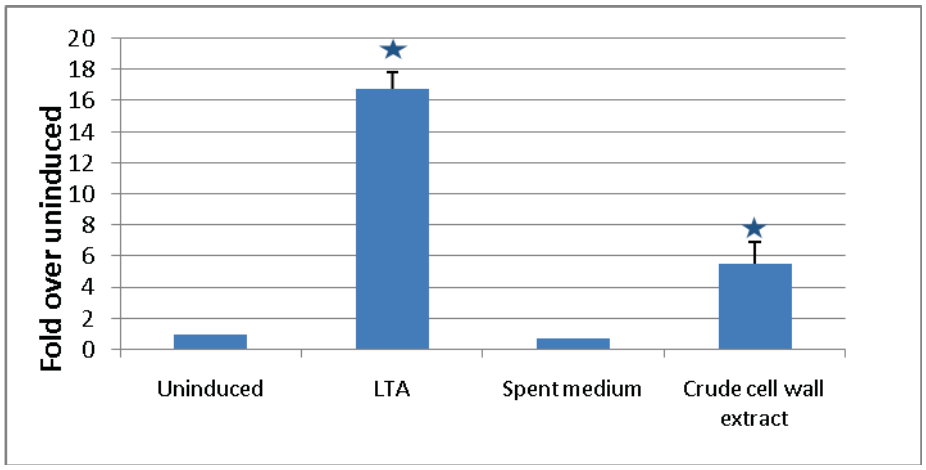
hrs. Cellular RNA was isolated and IL-6 gene expression levels were evaluated using real-time RT-PCR. The expression level within uninduced sample was set as 1. The difference was expressed in folds increase of IL-6 gene expression levels in B958 cells with treatments compared to the one without treatment (uninduced). Similar results were obtained for both *E. facealis* strains. **(B). The inflammatory genes expressions in B958 cell line treated with *E. faecalis* LTA.** B958 cells were treated with *E. facealis* LTA for 12 hrs. Cellular RNA was isolated. TNF-α, IFN-β, I -10 and IL-6 gene expression levels were evaluated using real-time RT-PCR. The expression level within uninduced sample was set as 1. The difference was expressed in folds increase of gene expression levels in B958 cells with treatments compared to the one without treatment (uninduced). Similar results were obtained for both *E. facealis* strains. **(C). The inflammatory genes expressions in cells with and without latent EBV infection after treated with *E. faecalis* LTA.** Cells with latent EBV infection (B958) and without latent EBV infection (DG-75) cells were treated with *E. facealis* LTA for 12 hrs. Cellular RNA was isolated. TNF-α, IFN-β, I -10 and IL-6 gene expression levels were evaluated using real-time RT-PCR. The expression level within uninduced sample was set as 1. The difference was expressed in folds increase of gene expression levels in cells with treatments compared to the one without treatment (uninduced). Similar results were obtained for both *E. facealis* strains. * Statistically significant ($P<0.05$).

Figure 5. Hypothetical model of viral involvement in endodontic disease progression via viral-bacterial interaction. Once the healthy pulp becomes infected with bacteria, this results in the recruitment of inflammatory cells which potentially harbor latent viruses. The presence of the bacteria and their associated components reactivates the

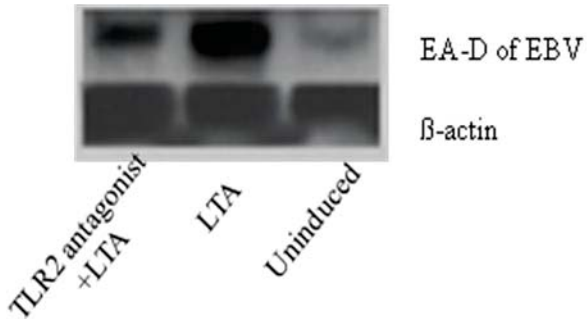
virus within these cells resulting in viral replication, which influences the inflammatory milieu subsequently promoting disease.

Figure 1.

(A)


Fold over uninduced
20
18
16
14
12
10
8
6
4
2
0
Uninduced
LTA
Spent medium
Crude cell wall extract

(B)


EA-D of EBV
ß-actin
TLR2 antagonist +LTA
LTA
Uninduced

(C)

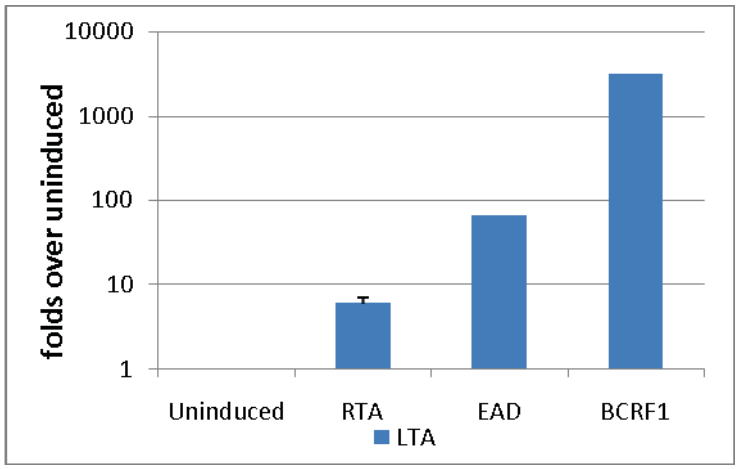
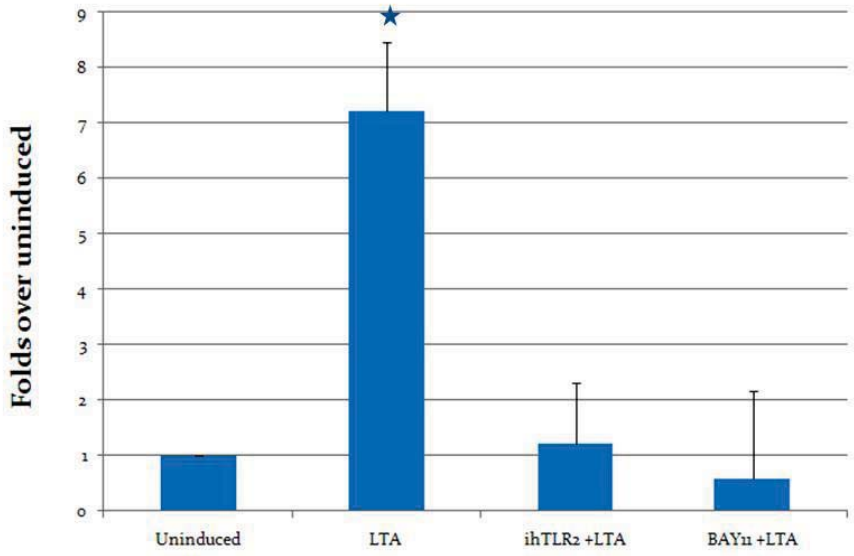

folds over uninduced
10000
1000
100
10
1
Uninduced
RTA
EAD
BCRF1
LTA

Figure 2.

A)


Folds over uninduced
9
8
7
6
5
4
3
2
1
0
Uninduced
LTA
ihTLR2 +LTA
BAY11 +LTA

B)

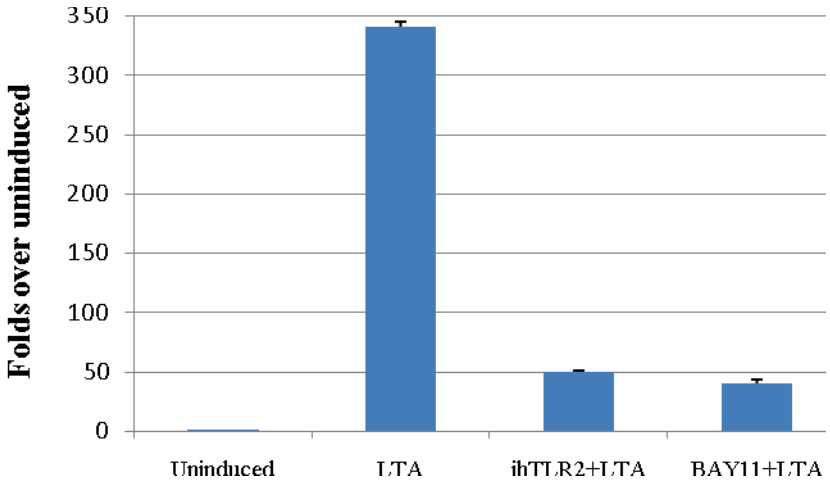
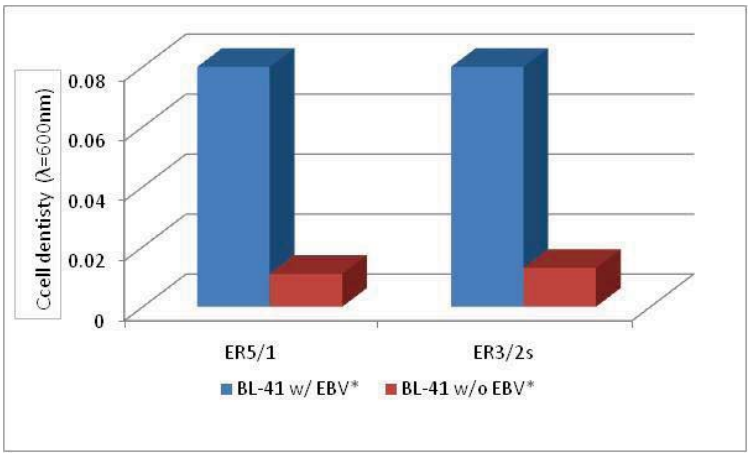
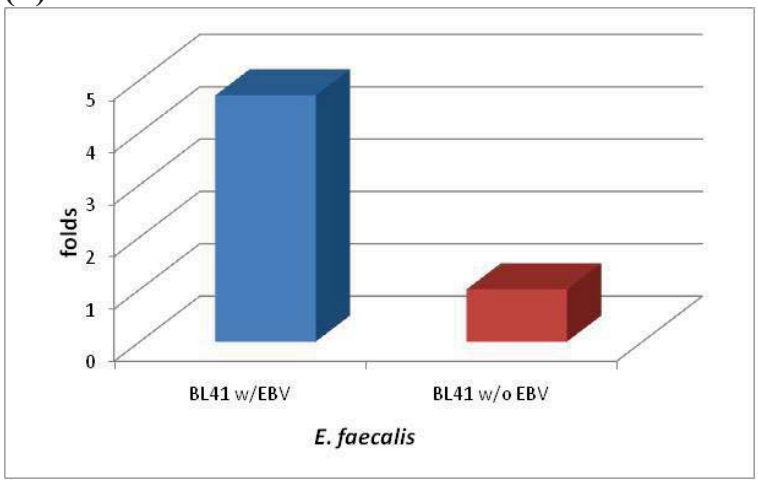

Folds over uninduced
350
300
250
200
150
100
50
0
Uninduced
LTA
ihTLR2+LTA
BAY11+LTA

Figure 3

(A)


Ccell dentisty (λ=600nm)
0.08
0.06
0.04
0.02
0
ER5/1
ER3/2s
BL-41 w/ EBV*
BL-41 w/o EBV*

(B)


folds
5
4
3
2
1
0
BL41 w/EBV
BL41 w/o EBV
E. faecalis

(C)

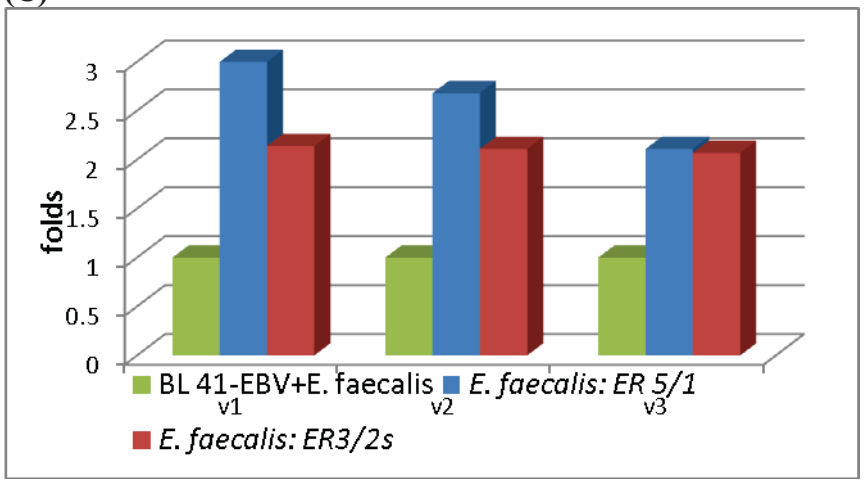
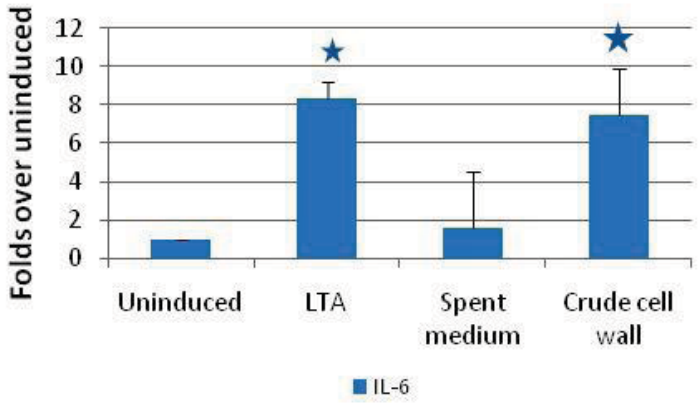
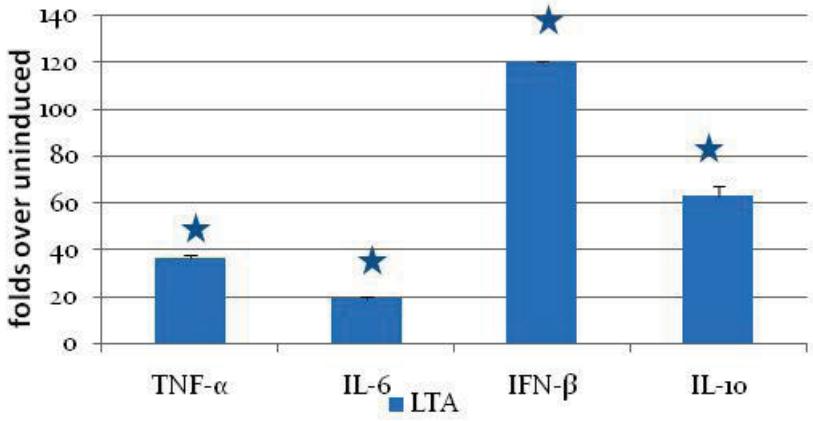

folds
3
2.5
2
1.5
1
0.5
0
BL 41-EBV+E. faecalis
E. faecalis: ER 5/1
E. faecalis: ER3/2s
v1
v2
v3

Figure 4

(A)


Folds over uninduced
12
10
8
6
4
2
0
Uninduced
LTA
Spent medium
Crude cell wall
IL-6

(B).


folds over uninduced
140
120
100
80
60
40
20
0
TNF-α
IL-6
IFN-β
IL-10
LTA

(C)

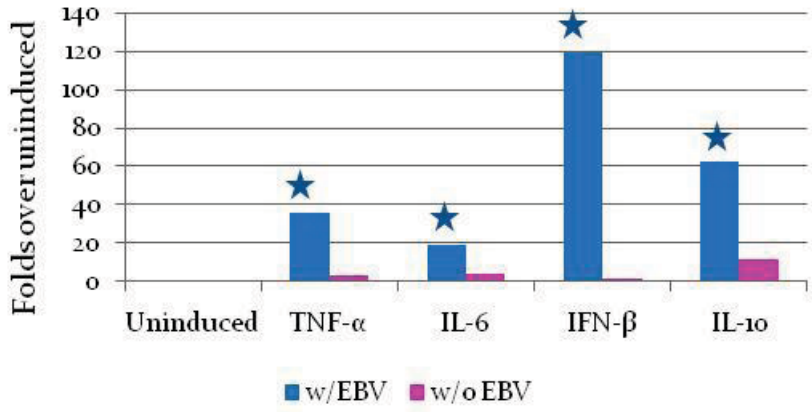
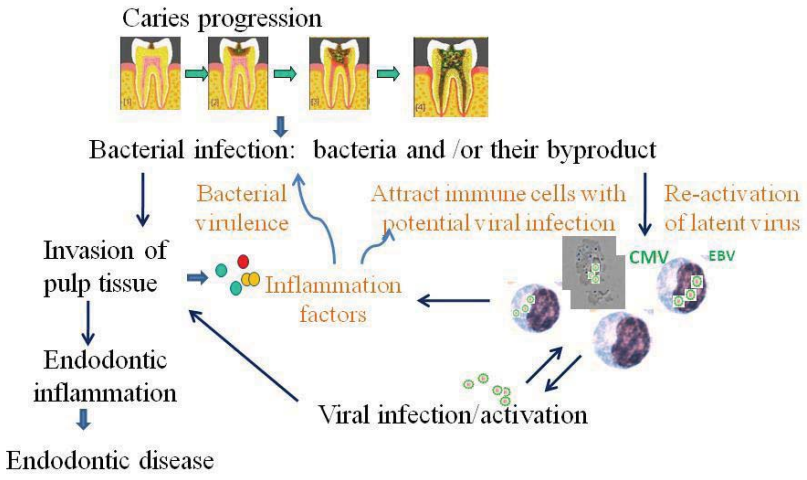

Folds over uninduced
140
120
100
80
60
40
20
0
Unimduced
TNF-α
IL-6
IFN-β
IL-10
w/EBV
w/o EBV

Figure 5


Caries progression
Bacterial infection: bacteria and /or their byproduct
Bacterial virulence
Attract immune cells with potential viral infection
Re-activation of latent virus
Invasion of pulp tissue
Inflammation factors
CMV
EBV
Endodontic inflammation
Viral infection/activation
Endodontic disease

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Chapter 3

Viral infection and activation of immune responses within acute and chronic endodontic infection: a case-control study

Will be submitted to *Journal of Endodontics* for publication

Viral infection and activation of immune responses within acute and chronic endodontic infection: a case-control study

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Short title: Detection of Viral and bacterial gene products in inflamed and healthy pulp tissue

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ABSTRACT

Introduction: We have previously shown *in vitro* that bacterial metabolites enhance Epstein-Barr virus (EBV) replication. In this study, we hypothesized that individuals with symptomatic endodontic disease would have elevated bacterial/viral replication and inflammation compared to that of asymptomatic individuals.

Methods: Twenty pulp tissue samples were collected from patients diagnosed with irreversible pulpits, 10 with symptoms of self-reported severe pain (>7 on 10-point pain scale, Symptomatic) and 10 without self-reported pain (Asymptomatic), respectively. As controls, ten pulp tissue samples were collected from extracted healthy 3rd molars, which had no fracture, caries, or periodontal disease. Additionally, two periapical tissue samples were collected during apicoectomy from patients with persistent periapical infection after root canal treatment. Total DNA and RNA were extracted from pulp tissue using DNeasy and RNeasy kit (Qiagen), respectively. Quantification of total bacteria, *Streptococcus sp.*, *Lactobacillus sp.*, *Fusobacterium sp.*, *Actinomyces sp.*, *E. faecalis*, EBV and Human Cytomegalovirus (HCMV) were assessed using quantitative PCR (qPCR) for consensus or organism specific DNA. Total bacterial RNA was used to indicate transcriptional activity and was evaluated using quantitative reverse-transcriptase PCR (RT-PCR). The expression of inflammatory genes including IL-6, IL-10, TNF-α, TNF-β and IFN-β was evaluated using quantitative reverse-transcriptase PCR.

Results: A greater than 5-fold increase was detected in bacterial DNA levels from pulp tissue of symptomatic patients compared to controls (P=0.004). These differences were not detected between asymptomatic patients and the control (P= 0.275). Likewise, total bacterial activity (RNA) was 5-fold higher in symptomatic patients than the

asymptomatic (P=0.027). *Lactobacillus sp.*, *Fusobacterium sp.* and *Actinomyces sp.* were present in inflamed pulp tissue, but not in healthy pulp tissue. Among the samples with *Streptococcus sp.,* asymptomatic pulp tissues had 30 fold higher levels, and symptomatic pulp tissues had 5 folds higher levels detected compared to controls (P=0.001, P=0.003 respectively). Interestingly, while EBV was widely detected, CMV was detected only in periapical tissues. Greater than a 30-fold increase in EBV DNA was detected in pulp tissue from symptomatic individuals compared to controls (P<0.001). Further, a 30-fold difference was shown in EBV detection in pulp tissue from symptomatic compared to asymptomatic (P=0.028) patients. The quantity of EBV in the pulp tissue from asymptomatic patients was comparable to the control (P=0.403). Real-time RT-PCR analysis of inflammatory gene expression in pulp tissues detected expression in disease but not within healthy controls. The IL-6 gene was expressed at levels 11-fold higher in symptomatic pulp than in asymptomatic pulp (P=0.026). Regression analysis indicated that inflammatory gene expression levels were associated with bacterial transcription and with EBV viral load.

Conclusion: The results suggest a significant association between bacteria, EBV and inflammation in symptomatic disease within the pulp tissue. CMV is more tightly associated with chronic infection than acute infection. These results support a paradigm shift in which elevated bacterial/viral replication and inflammation are associated with endodontic infection.

Keywords: EBV, CMV, bacteria, inflamed pulp tissue, quantitative-PCR

INTRODUCTION

Endodontic disease is a polymicrobial, multistage inflammatory response initiated by the migration of opportunistic microorganisms from the oral cavity. This leads to an influx of inflammatory cells, resulting in pulpitis and periapical periodontitis. The significance of bacteria in the progression and perpetuation of pulpal and periradicular inflammatory diseases is indisputable (1-2). Endodontic infections involve multiple bacteria. Two hundred different species have been identified within infected root canals, usually in combinations of 4-7 species (spp.) per canal (3-5). Of these, however, between 15 to 30 bacterial species have been most frequently detected in infected root canals (4-6). Among them, *Fusobacterium nucleatum*, *Streptococcus* spp., *Lactobacillus* spp. and *Actinomyces* spp. are the most commonly detected bacteria (4-6). A positive correlation exists between the number of bacteria in an infected root canal and the size of periradicular radiolucencies (7). There tends to be an association between different bacterial species and different clinical signs and symptoms, however, this association is not conclusive (4, 8). Whether other pathogens involved in endodontic pathogenic processes could affect the above mentioned association is not clear.

Once bacteria and/or their by-products invade pulp tissue, non-specific inflammatory reactions and immunologic responses are excited. Bacteria and their by-products interact with immune cells via toll-like receptors (TLRs) (9). Binding of bacterial adjuvant to these receptors activates Nuclear Factor-κB (NFκB), a central signaling pathway that leads to transcription of multiple proinflammatory molecules (10). All of these inflammatory factors may contribute to disease progression.

During the past decade, viruses have emerged as putative periapical pathogens, particularly Epstein Barr Virus (EBV) and human Cytomegalovirus (CMV) (11-14). EBV is present more frequently in endodontic pathosis than in healthy controls (8), thus may play a role in the development of symptomatic periapical lesions (12-13). EBV and CMV belong to the herpes virus family, and 60-100% of adults are carriers of these viruses (15). Herpes viruses may establish latency or a productive (lytic) state of replication. In the viral productive cycle, the herpes virus genome is amplified 100- to 1000-fold by the viral replication machinery, producing a significant increase of virions. (16). EBV infects B-lymphocytes and establishes latency (16), while CMV infects monocytes/macrophages, T-lymphocytes, ductal epithelial cells of salivary glands, endothelial cells, fibroblasts and polymorphonuclear leukocytes, and establishes latent infection mainly in cells of the myeloid lineage (16). Herpes virus infections induce strong antiviral innate and adaptive immune responses. They evade the immune system through a complicated multifactorial mechanism either via NF- pathway undefined mechanism (16). Herpes viruses interfere with innate and adaptive cellular and humoral immune response by affecting cytokine production, the activation and silencing of natural killer cells, down-modulating antigen presentation in the major histocompatibility complex class I and II pathways, and impairing apoptosis (17). Cytokines produced in viral infection can significantly modulate the inflammatory response and include interleukin (IL)-1β, I -1 receptor antagonist (IL-1Ra), IL-6, IL-8, IL-18, TNF-α, INF-α β, INF- (17).

Few studies have investigated the role of herpes virus in endodontic diseases (8, 12-13, 18), particularly in irreversible pulpitis. The limitations of these studies was that

bacteria was not viewed as a potential confounding factor and effect modifiers were not taken into consideration when establishing the associatoin between virus and endodontic disease. In this exploratory study, we investigated the levels and activity of total bacteria and herpes viruses (EBV and CMV) within both inflamed and healthy pulp tissue. Previous literature has been shown that IL-1 activity is greater in symptomatic than in asymptomatic pulps (19). In this study, we hypothesized that inflammatory mediators would be present at higher levels in symptomatic pulp tissue than the asymptomatic; and that quantitatively lower inflammatory mediators correlate with lower bacterial and viral levels present within the pulp and with diminished symptoms of pain.

MATERIALS AND METHODS

Study design and Collection of Specimens

A case-control study was performed (IRB#08-0229, UNC). One case group was defined as patients diagnosed clinically with irreversible pulpitis and seeking endodontic treatment. This case group was further divided into 2 different subgroups, patients with self-reported symptom of severe pain (>7 on 10 point pain scale) (Symptomatic group) and patients without symptoms of pain (asymptomatic, <1 on 10 point pain scale) (Asymptomatic group). All patients diagnosed with irreversible pulpitis had caries pulpal exposure in the study. Another case group consisted of patients who had previous root canal treatment and persistent periapical infection, and thus apicoectomy was indicated for treatment (Chronic Periapical group). Two patients recruited in this group had no symptoms of pain or swelling. The control group consisted of patients with healthy pulp

tissue (extracted third molars), and were matched with cases for age. Teeth with caries, prior restoration, or cracking were not eligible. Subjects over the age of 12 seeking endodontic treatment for irreversible pulpitis or have 3rd molar extracted were eligible for inclusion in the study. The age of all subjects recruited in the study was from 25 to 45 years old. The gender of the subjects in symptomatic, asymptomatic and control groups were comparable. Exclusion criteria included: 1) patients that were immune compromised, had diabetes or were currently taking antibiotics or other medications potentially influencing the immune response; 2) teeth with periodontal disease (probing depth >4mm, with periodontal bone loss) or vertical root fracture; and 3) immature teeth with open apices.

Specimens used in the study were collected from patients seeking dental care at the University of North Carolina, School of Dentistry. Selected teeth were rubber-dam isolated and disinfected with 2% Chlorohexidine. The teeth were disinfected with 2% Chlorohexidine again after caries removal but prior to pulpal exposure. The access was then performed using a sterilized round bur. For the control group, the extracted tooth was placed in 10% formalin. Then the tooth was disinfected with 2% Chlorohexidine and accessed using a sterilized round bur on the same day of extraction. The pulpal tissue was subsequently extirpated using sterilized barbed broach. The pulp tissue was then stored in RNA-later (Qiagen, Carlsbad, CA, USA) at -80 °C until further processing.

Extraction of Nucleic Acids from Specimens, cDNA Generation

All tissue specimens were minced with sterile scalpels. Approximately 1/3 of each total specimen was used for DNA isolation using a DNeasy tissue kit (Qiagen), and 2/3 was used for RNA isolation using RNeasy kit (Qiagen) according to manufacturer instructions.

RNA was then reverse transcribed to cDNA using Superscript II Reverse Transcriptase (RT,) and Random Hexamer $pd(N)_6$ according to manufacturer instructions (Invitrogen, Carlsbad, CA, USA). For each sample, a no-RT control was generated. The absence of bacterial DNA contamination in the process was assured by end-point PCR of the no-RT control using bacterial universal primers '-TCCTACGGGAGGCAGCAGT -3' and '- GGACTACCAGGGTA TCTAATCCTGTT -3' desi ned based on a conservative region of 16S rDNA. The PCR mix (25 µl) contained 1 µl template cDNA, 1 mM $MgCl_2$, 0.1 mM concentration of each deoxynucleoside triphosphate, 0.4 µM concentration of each primer, 0.5 U of *Taq* Polymerase, and TAQ buffer with final concentrations of 10 mM Tris-HCl (pH 8.3) and 10 mM KCl. Amplification conditions were as follows initial denaturation at 94°C for 4 min, 40 cycles consisting of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 7 min.

Assessment of Bacterial and Viral DNA loads of Bacterial RNA load and of Inflammatory Gene Expression Levels

The quantities of EBV, HCMV, total bacteria, *Streptococcus sp.*, *Lactobacillus sp.*, *Actinomyces sp.*, and *Fusobacterium sp.* were evaluated using quantitative PCR

(qPCR) with 1µl DNA isolated from the sample as the template, the corresponding primers (listed in Table 1) and SYBR Green PCR Kit (Roche, Indianapolis, IN, USA). The real-time QPCR conditions were set at 50°C for 2 min and 95 °C for 10 min, followed by 40 cycles at 94°C for 15 s, finished by annealing at 55°C for 30 s. The total bacterial transcriptional activity was quantified using qPCR with 1µl cDNA generated as the template. The expression levels of inflammatory genes, IL-6, IL-10, TNF-α, NF-β, IFN- β, were evaluated using real-time PCR with the corresponding primers (listed in Table 1) and SYBR Green PCR Kit (Roche). The QPCR conditions were as described above. All analysis was performed in triplicate and included the negative control consisted of sterile H_2O.

Statistical Analysis

The differences between bacterial and viral loads and inflammatory gene expression levels between groups were analyzed using ANOVA for multi-groups comparison and t-test for 2 group comparison. Regression analysis was performed to investigate the association between the inflammatory gene expression levels and bacterial quantity, activity and viral quantities (SPSS 17.0, Chicago, IL).

RESULTS

The bacterial profiles within the pulp tissue were unique in acute or chronic infected pulp tissue as compared to controls.

The prevalence of total bacteria, *Streptococcus sp*., *Lactobacillus sp*., *Actinomyces sp*., *Fusobacterium sp.* and *E. faecalis* within the tissue samples from 4 groups in the study were listed in Table 2. No *E. faecalis* was detected in the pulp tissue samples from all three groups (Table 2) but was consistently detected in 2/2 periapical tissue samples. In control group, among 5 bacterial species investigated, only *Streptococcus sp.*, was detected in 9 out of 10 samples; the other 4 were below the level of detection (Table 2). Alternatively, all 5 bacterial species were detected in the pulp tissue from both symptomatic and asymptomatic patients and in periapical tissue. However, *Fusobacterium sp*. and *Lactobacillus sp.* presented in 30% and 50% samples from asymptomatic patients, respectively; compared to 100% in symptomatic patients (Table 2). In periapical tissue, with the exception of *Actinomyces sp.* that presented in half of the samples, all other 4 bacterial species presented 100% (Table 2). The total bacterial DNA levels in pulp tissue were statistically significantly higher (5-fold) in pulp tissue from symptomatic patients than the control (P=0.004) but not the asymptomatic patients (P= 0.275) (Figure 1A). No statistical differences regarding total bacterial DNA in pulp tissue were detected between asymptomatic patients and control (P=0.407) either (Figure 1A). Among the samples with *Streptococcus sp.,* more bacteria was detected in diseased pulp from asymptomatic (30-fold) and symptomatic (5-fold) patients than in the controls, respectively (P=0.001, P=0.003 respectively) (Figure 1B). Further, statistically significant higher levels of *Streptococcus sp.* DNA was present in pulp tissue from asymptomatic compared symptomatic patients (P=0.048) (Figure 1B). No statistical significant differences between symptomatic and asymptomatic groups were found in *Lactobacillus sp.* DNA load (P=0.822), *Actinomyces sp.* DNA load (P=0.757), or

Fusobacterium sp. DNA load (P=0.403).

The total bacterial RNA analysis showed 5- fold more bacterial transcripts present in pulp tissue from symptomatic patients than the asymptomatic patients (P=0.027) (Figure 1C), even though there was no statistical difference in total bacterial DNA levels between two groups (P=0.407) (Figure 1A). Analysis of bacterial mRNA determined that total bacteria mRNA in samples from the control group was below the level of detection, indicating the bacterial activity was minimal if indeed present.

Significantly higher loads of EBV were detected within the pulp tissue from patients diagnosed with irreversible pulpitis and symptoms of severe pain.

In this study, HCMV was not detected in symptomatic, asymptomatic or controls while EBV was detected in each of these groups (Table 2). However, HCMV was detected in two samples collected from periapical lesions during apicoectomy (Table 2). Greater than 30-fold higher EBV levels were detected in pulp tissue from symptomatic individuals compared to the controls (P<0.001). In addition, statistically higher levels of EBV were detected in pulp tissue from symptomatic than asymptomatic (P=0.028) patients (Figure 2). Interestingly, EBV levels in the pulp tissue from asymptomatic patients were comparable to controls (P=0.403) (Figure 2).

Inflammatory gene expressions levels were significantly higher in symptomatic individuals

Gene expression of TNF-α, NF-β, I -6, IL-10 and IFN-β was consistently detected in the symptomatic pulp tissue, but not in the controls (Figure 3A). Expression

levels within the asymptomatic group were varied (data not shown). There was a significant positive correlation between the TNF-α, NF-β, I -6 and IL-10 (Table 3). Gene expression of IFN-β was negatively correlated with that of TNF-α, NF-β, I -6 and IL-10 (Table 3). Because of its high correlation with inflammatory genes expression, only IL-6 was selected to perform further analysis. The IL-6 gene was expressed at levels 11-fold higher in pulp tissue from symptomatic patients than in asymptomatic patients (P=0.026) (Figure 3B). Furthermore, regression analysis showed that expression of IL-6 was significantly associated with bacterial transcriptional activity (P= 0.045) and EBV viral load (P= 0.025) (Figure 4). Even after adjusting for bacterial gene expression, IL-6 expression was still significantly associated with EBV viral load (P=0.022), indicating that EBV contributes to the pulp's inflammatory response. After interaction between EBV viral load and total bacterial transcriptional activity was introduced to the regression model, IL-6 expression levels were significantly associated with EBV viral load (P=0.019), total bacterial transcriptional activities (P= 0.015) and their interaction was significant (P=0.014). The mean (Standard deviation) age were 31.4 (5.74), 33.2 (7.16) and 33.1 (4.75) for Symptomatic, Asymptomatic and Control group, respectively. There were 6 male, 5 male and 5 male in Symptomatic, Asymptomatic and Control group, respectively. There were not statistically significant differences in both age and gender among groups (P>0.05). Therefore, the age and gender were not included in the regression analysis.

DISCUSSION

In this study, we sought to determine the role that bacterial and viral pathogenesis play in the progression of endodontic disease. This study determined that 1) there was a significantly higher amount of total bacteria within the pulp tissue from the subjects s diagnosed with irreversible pulpitis than in the control groups; 2) that total bacterial transcriptional activity was statistically significantly higher in symptomatic pulp tissue than the asymptomatic; 3) that EBV but not CMV was detected in the pulp tissue from both case and control groups,; 4) that significantly higher EBV loads were detected in symptomatic pulp tissue compared to asymptomatic and the control groups; 5) that IL-6 expression is significantly higher in symptomatic pulp than in asymptomatic pulp; and 6) that IL-6 expression levels were significantly associated with both bacterial transcription and higher EBV viral load. The results from this study support our hypothesis that inflammatory mediators enjoy higher expression in symptomatic pulp, which might be associated with higher bacteria activity and EBV loads.

In this study, we used the pulp tissue from extracted 3rd molar as the control. All these 3rd molars were extracted due to mal-position or orthodontic needs. All these teeth were completely formed with closed apex. The pulps from these teeth should be the same as the pulp tissue from other healthy tooth. However, all these 3rd molars were extracted prior to have pulp tissue extirpated. The nucleic acid in these teeth has larger chances to be damaged. The teeth were preserved in RNA- later solution and accessed almost immediately after extraction (within 2 hours of extraction) to preserve nucleic acid and make it as much comparable to case groups as possible.

In this study, we employed the qPCR technique to estimate bacterial quantities. However, PCR detects all active, inactive and dead cells. The major limitation of using

quantification of DNA as an estimate of total bacterial cell numbers in this case is that it cannot differentiate between living and dead cells. Bacteria might exist in three natural status: living/active, dormant/inactive and dead. Determination of whether a bacterial cell is active or inactive has been a controversial issue during the last two decades (20-21). Three methods have been used to assess metabolic activity: 1) measurement of the incorporation of radioactive-labeled metabolic precursors, such as thymidine or leucine (22); 2) use of a redox-sensitive compound that fluoresces when reduced by an active electron transport system (23) and 3) using the amount of nucleic acids, particularly RNA within a cell as a proxy for metabolic activity (24). As the radioactive-labeled precursors and redox-sensitive compound cannot be applied *in-vivo*, detection of total amount of ribonucleic acid is the only *in-vivo* option. In this study, the total bacterial DNA from both living and dead bacterial cells was used to estimate the total number of bacteria. However, in order to reflect bacteria that were transcriptional active within the pulp tissue, quantitative RT-PCR (q-RT-PCR) to obtain the total amount of bacterial mRNA. The results showed more total bacterial RNA was detected in symptomatic than asymptomatic pulp, despite the lack of difference in total bacterial DNA levels. This suggests that bacteria in symptomatic pulp are more metabolically active than in asymptomatic pulp tissue.

Distinct bacterial profiles were detected within the groups that were studied. Bacteria was ubiquitous, and was detected in symptomatic, asymptomatic and in 100% of the control subjects. *Streptococcus sp.* Was detected in 90% of the controls. This is most likely due to the salivary bacterial contamination during the sampling process. *Streptococcus sp.* is relatively more prevalent in saliva than other bacteria (*Lactobacillus*

sp., *Fusobacterium sp.*, *Actinomyces sp.* and *E. faecalis*) selected in this study, which might explain why *Streptococcus sp.* rather than other selected bacteria was detected in majority of the controls (REF). In addition, mRNA analysis showed the total bacterial mRNA level was below the level of detection for all samples in control group, suggesting that the contaminating bacteria are not transcriptional active.

In this study, we did not detect *E. faecalis* in any samples, consistent with previous literature (25), confirming that *E. faecalis* is most likely not associated with primary endodontic infection. It is not surprising that *Streptococci sp.* was detected in all samples. Previous literature has shown that *Streptococci sp.* is one of the most common species in the root canal system in both primary (26) and permanent teeth (27). Interestingly, more *Streptococci sp.* was detected in asymptomatic pulp than in symptomatic pulp. Previous studies have determined that the presence of endotoxin is associated with pulpal pain and inflammation (28). *Streptococci sp.* is not a potent endotoxin producer, which might partially explain why it is associated with asymptomatic disease. *Fusobacterium* sp. was more prevalent in symptomatic pulp than asymptomatic pulp, consistent a previous *in vitro* study that showed that fusobacterial LPS up-regulated the secretion of the inflammatory cytokines IL-6, TNF-a by macrophage-like cells (29). Interestingly, total bacterial loads, EBV loads, and individual bacterial loads were more variable in asymptomatic pulp than in symptomatic pulp. This may partially explain why there were no statistically significant differences in *Fusobacterium sp*, *Actinomyces sp*. and *Lactobacillus sp*. between symptomatic and asymptomatic pulp tissue. Further study with larger sample size is warranted to draw a more definitive conclusion.

In this study, we determined that EBV loads were significantly higher in symptomatic pulp than asymptomatic pulp. The results showed, for the first time, that within endodontic disease a expression of the pro-inflammatory gene IL-6 was associated with EBV viral load even after adjusting bacterial activity. Importantly, these data suggest that EBV contributes to pulpal inflammation, and subsequently to patients' symptoms. In this study, EBV was detected in the pulp tissue from both case and control groups, but not CMV. The results appear to be slightly different from the study by Li *et. al*. (8), which also employed PCR to detect EBV. They detected EBV in 43.9% samples of the endodontic diseases, 0% in healthy control (extracted teeth). However, the different primer sets may have different detection sensitivity. The primers of qPCR for the detection of EBV was designed based on the sequence of WD repeats of EBV gene, which, because of the targeting of the repeats, may be more sensitive than the primers used in other literature (8). Interestingly, we also detected EBV in the control group. As EBV is latent within lymphocytes of the peripheral circulation, we may have detected lymphocytes trafficking through the pulp tissue.

Higher bacterial quantity and activity, along with the higher load of EBV contributed to the higher inflammatory activity in the pulp tissue. These result indicated inflammation activity is higher in symptomatic patients than asymptomatic patients. Various pro-inflammatory gene expression levels were assessed. Significant positive correlation between the TNF-α, NF-β, I -6 and IL-10 were observed. It was shown that the gene expression of IFN--β was negatively correlated with that of TNF-α, NF-β, I -6 and IL-10. The inflammatory response is regulated by a network of regulatory cytokines produced by Th1- and Th2-type lymphocytes along with other chemotactic molecules

(30). IFN--β belongs to the Th1 cytokine produced by Th1-type lymphocytes. IFN--β is important for immunity to certain viruses, bacteria, fungi, and parasites. The outgrowth of EBV-infected B-cells is first controlled by IFN--β (31). A previous study had shown that endodontic pathogens, such as *Fusobacterium sp*., did not induce much production of IFN--β (32). Collectively, it suggests the high expression of IFN--β might be due to acute EBV infection within the pulp tissue.

In conclusion, this study, for the first time, showed the possible association between inflammatory activity within the pulpal tissue during irreversible pulpitis and bacterial activity and EBV loads. Further study is warranted to investigate the mechanism and draw a more definitive conclusion.

Table 1 Primers sequences for QPCR quantification of selected bacterial species

Bacterial species	Primer name	equences '--3'	Reference
Total bacteria	U16S1020F	TTAAACTCAAAGGAATTGACGG	Sedgley et.al., 2006
	U16S1190R	CTCACGRCACGAGCTGACGAC	
Fusobacterium sp.	FusoF	AAGCGCGTCTAGGTGGTTATGT	Martin et.al., 2002
	FusoR	TGTAGTTCCGCTTACCTCTCCAG	
Streptococcus sp.	StrepF	AGATGGACCTGCGTTGT	Rudney et al., 2003
	StrepR	GCTGCCTCCCGTAGGAGTCT	
Lactobacilli sp.	LactoF	TGGAAACAGRTGCTAATACCG	Byun et.al., 2004
	LactoR	GTCCATTGTGGAAGATTCCC	
Actinomyces sp.	ActF	GGCKTGCGGTGGGTACGGGC	Xia, 2004
	Uni341	CTGCTGCCTCCCGTAGG	Paster, 1998
E. faecalis	E16S72F	CCGAGTGCTTGCACTCAATTGG	Sedgley et.al., 2006
	E16S210R	CTCTTATGCCATGCGGCATAAAC	

Table 2. Prevalence of selected bacteria and herpesviruse within specimens

	Symptomatic (10*)	**Asymptomatic (10*)**	**Periapical (2*)**	**Control (10*)**
Total Bacteria	10/10 (+++)	10/10 (++)	2/2 (+)	10/10 (++)
Streptococcus sp.	10/10 (+++)	10/10 (++++)	2/2 (++)	9/10 (++)
Lactobacillus sp.	10/10(+++)	5/10 (+++)	0/2	0/10
Actinomyces sp.	10/10(++)	10/10 (+++)	1/2 (++)	0/10
Fusobacterium sp.	10/10(+++)	3/10 (++)	2/2 (+)	0/10
E. faecalis	0/10	0/10	2/2 (++)	0/10
EBV	10/10(+++)	10/10(++)	2/2 (+)	10/10 (+)
CMV	0/10	0%	2/2 (+++)	0/10

Twenty inflamed pulp tissue samples were collected from patients diagnosed with irreversible pulpits, 10 with symptom of severe self-reported pain (>7 on 10-point scale of pain) and 10 without symptom of pain. Two periapical tissue samples were collected during apicoectomy from two asymptomatic patients with persistent periapical infection. Ten healthy pulp tissues were collected from extracted 3rd molars. Total DNA was extracted from pulp tissue using DNeasy kit (Qiagen). The quantities of total bacteria, *Streptococcus sp.*, *Lactobacillus sp.*, *Fusobacterium sp.*, *Actinomyces sp.*, *E. faecalis*, EBV and HCMV were assessed using quantitative PCR (qPCR). Shown in table was the number of samples with the presence of bacteria as the nominator, the total number of samples as the denominator. * Total number of samples. + indicate the level of quantities of bacterial or virus can be detected; ++ indicate more than 10 fold than +; +++ indicates more than 50 fold than +; ++++ indicates more than 100 fold than+.

Table 3. Correlation analysis of inflammatory genes expression level

Correlation analysis	IFN- β	IL-10	IL-6	TNF- α	TNF- β
IFN- β	1.00	-0.91	-0.90	-0.96	-0.94
Il-10		1.00	0.94	0.93	0.97
IL-6			1.00	0.93	0.95
TNF- α				1.00	0.95
TNF- β					1.00

Ten inflamed pulp tissue samples were collected patients diagnosed with irreversible pulpits. Total RNA were extracted from pulp tissue using RNeasy kit (Qiagen). The expression of inflammatory genes including IL-6, IL-10, TNF-α, NF-β and IFN- were evaluated using real-time RT- CR. he e pression levels were normali ed usin β-actin gene expression level. The Pearson correlation analysis was performed. Significant positive correlations ($P < 0.05$) were subsequently identified between IL-6, IL-10, TNF-α and TNF-β; while the negative correlations between IFN- β and IL-6, IL-10, TNF- α, TNF-β were observed. Shown in table were the Pearson Correlation Coefficients.

Figure legends

Figure 1. Comparison of bacterial DNA and RNA. Twenty inflamed pulp tissue samples were collected from patients diagnosed with irreversible pulpits, 10 with symptom of severe self-reported pain (>7 on 10-point scale of pain, **Symptomatic**) and 10 without symptom of pain (**Asymptomatic**). Two periapical tissue samples were collected during apicoectomy from two asymptomatic patients with persistent periapical infection (**Periapical**). Ten healthy pulp tissues were collected from extracted 3rd molars (**Control**). Total DNA, RNA was extracted from pulp tissue using DNeasy and RNeasy kit (Qiagen). The quantities of total bacteria, *Streptococcus sp.*, assessed using quantitative qPCR. The bacterial DNA and RNA quantities were normalized using cell number as indicated by cellular β-actin levels. **(A) Comparison of total bacterial DNA.** Folds were calculated by comparison of the total bacterial DNA within the pulp tissue from the case group (symptomatic and asymptomatic) to the control groups (set as 1). Statistically significant more bacterial DNA present in pulp tissue from symptomatic patients than the control (P=0.004). No statistical differences of total bacterial DNA were detected between symptomatic patients and asymptomatic patients (P= 0.275) or between asymptomatic patients and control (P=0.407**).** **(B) Comparison of *Streptococcus sp.* DNA.** Folds were calculated by comparison of the Streptococcus sp. DNA within the pulp tissue from the case group (symptomatic and asymptomatic) to the control groups (set as 1). Statistically significant more Streptococcus sp. DNA present in pulp tissue from symptomatic (P=0.003) and asymptomatic (P=0.001) patients than the control, respectively. Statistically significant higher load of Streptococcus sp. DNA

present in pulp tissue from asymptomatic than symptomatic patients (P=0.048). **(C) Comparison of total bacterial RNA.** Folds were calculated by comparison of the bacterial RNA within the pulp tissue from the symptomatic patients and asymptomatic patients to the lowest detection levels (set as 1). Bacterial RNA within the pulp tissue from control group and periapical tissues were below detection level. They were set as 1 arbitrarily. Statistically significant more bacterial RNA present in pulp tissue from symptomatic patients than the asymptomatic patients (P=0.027).

Figure 2. Comparison of EBV quantities. Twenty inflamed pulp tissue samples were collected from patients diagnosed with irreversible pulpits, 10 with symptom of severe self-reported pain (>7 on 10-point scale of pain, **Symptomatic**) and 10 without symptom of pain (**Asymptomatic**). Two periapical tissue samples were collected during apicoectomy from two asymptomatic patients with persistent periapical infection (**Periapical**). Ten healthy pulp tissues were collected from extracted 3rd molars (**Control**). Total DNA was extracted from pulp tissue using DNeasy and RNeasy kit (Qiagen). The quantity of EBV was assessed using qPCR. The EBV quantities were normali ed usin cell number as indicated by cellular β-actin levels. Folds were calculated by comparison of the EBV quantity within the pulp tissue from the symptomatic and asymptomatic patients to the control groups (set as 1). Statistically higher load of EBV present in pulp tissue from symptomatic than asymptomatic (P=0.028) patients and the control (P<0.001). No statistically difference of EBV loads in pulp tissues from asymptomatic patients and the control (P=0.403)

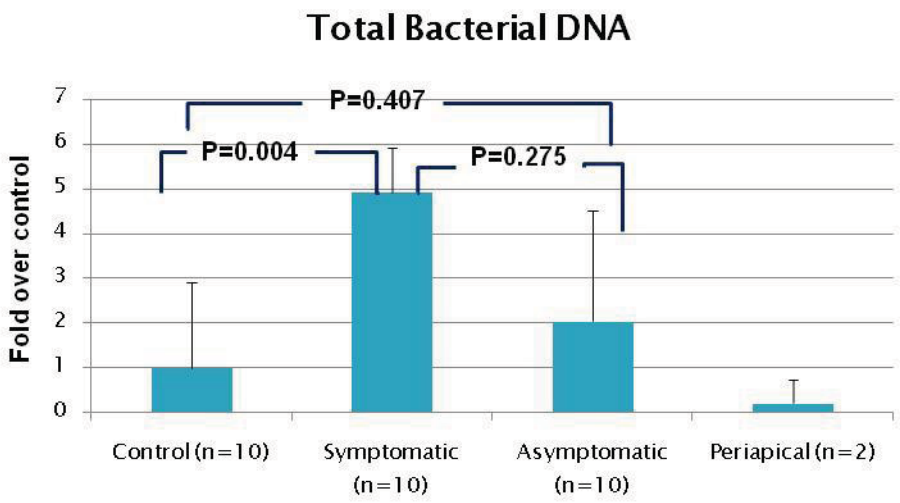
Figure 3 (A) Selected inflammatory gene expression analysis in inflamed and healthy pulp. Ten inflamed pulp tissue samples were collected from patients diagnosed as irreversible pulpits and extracted 3rd molars, respectively. Total RNA were extracted from pulp tissue using RNeasy kit (Qiagen). RT-PCR analysis of selected inflammatory gene IL-6, IL-10 and TNF-α showed that inflammatory enes e pressed in inflamed pulp tissue but not within healthy pulp tissue samples. he e pression of cellular β-actin was used as control for RT-PCR analysis. **(B) Comparison of IL-6 expression levels** Twenty inflamed pulp tissue samples were collected from patients diagnosed with irreversible pulpits, 10 with symptom of severe self-reported pain (>7 on 10-point scale of pain, **Symptomatic**) and 10 without symptom of pain (**Asymptomatic**). Two periapical tissue samples were collected during apicoectomy from two asymptomatic patients with persistent periapical infection (**Periapical**). Ten healthy pulp tissues were collected from extracted 3rd molars (**Control**). Total DNA, RNA was extracted from pulp tissue using DNeasy and RNeasy kit (Qiagen). Total RNA were extracted from pulp tissue using RNeasy kit (Qiagen). IL-6 gene expression level in the pulp tissue was assessed using real-time RT- CR analysis. he e pression of cellular β-actin was used as control for RT-PCR analysis. The expression of IL-6 gene was significant higher in pulp tissue from symptomatic patients than in asymptomatic patients (P=0.026).

Figure 4. Scatter plot with regression line. Regression analysis and scatter plot were performed using SPSS 17.0. **(A) Regression analysis between IL-6 expression levels and total bacterial activities** Dependent variable is IL-6 expression level. Independent variable was total bacterial RNA as the indicator for bacterial activity. Significant

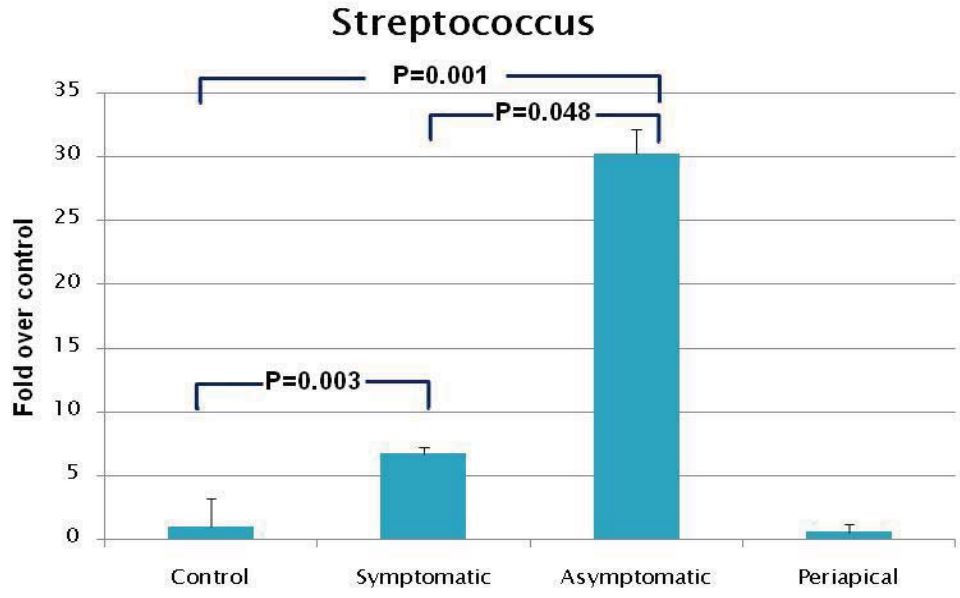
association between IL-6 expression level and total bacterial RNA activity was found (P=0.045). **(B) Regression analysis between IL-6 expression levels and EBV quantities.** Dependent variable is IL-6 expression level. Independent variable was EBV quantity. Significant association between IL-6 expression level and EBV quantity was shown (P=0.025).

Figure 1

(A)


Total Bacterial DNA
Fold over control
7
6
5
4
3
2
1
0
P=0.407
P=0.004
P=0.275
Control (n=10)
Symptomatic (n=10)
Asymptomatic (n=10)
Periapical (n=2)

(B)


Streptococcus
Fold over control
35
30
25
20
15
10
5
0
P=0.001
P=0.048
P=0.003
Control
Symptomatic
Asymptomatic
Periapical

(C)

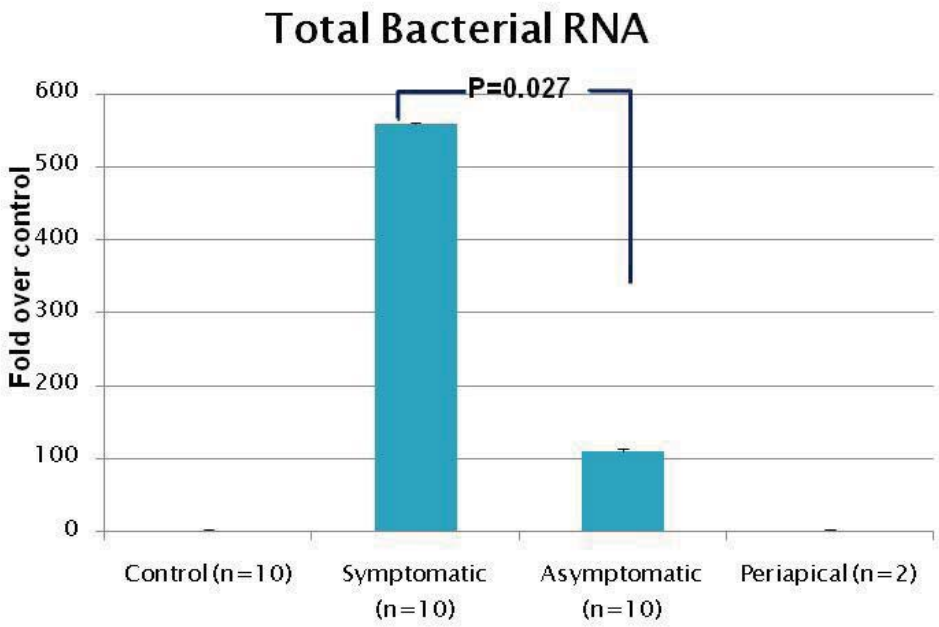

Total Bacterial RNA
P=0.027
Fold over control
600
500
400
300
200
100
0
Control (n=10)
Symptomatic (n=10)
Asymptomatic (n=10)
Periapical (n=2)

Figure 2

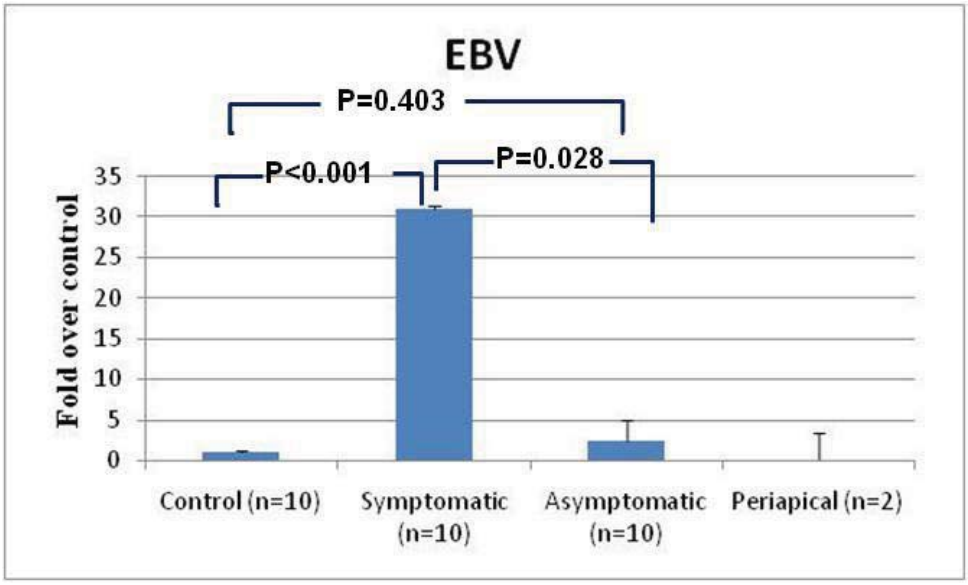
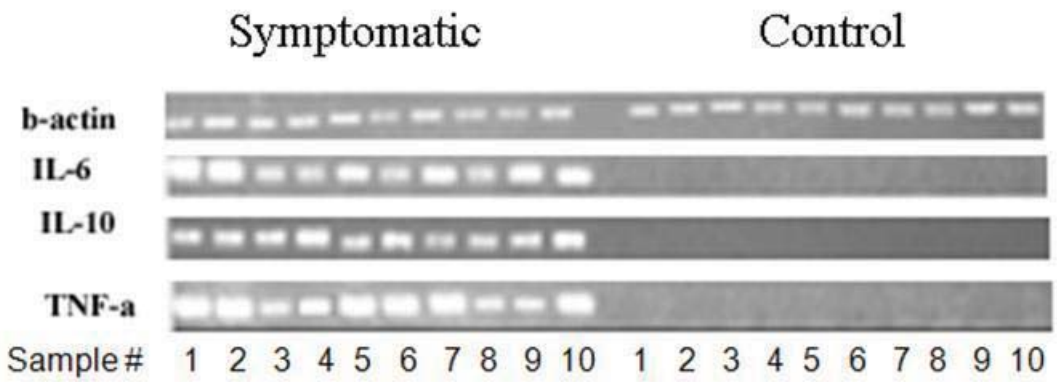

EBV
P=0.403
P<0.001
P=0.028
Fold over control
35
30
25
20
15
10
5
0
Control (n=10)
Symptomatic (n=10)
Asymptomatic (n=10)
Periapical (n=2)

Figure 3

(A)


Symptomatic
Control
b-actin
IL-6
IL-10
TNF-a
Sample # 1 2 3 4 5 6 7 8 9 10 1 2 3 4 5 6 7 8 9 10

(B)

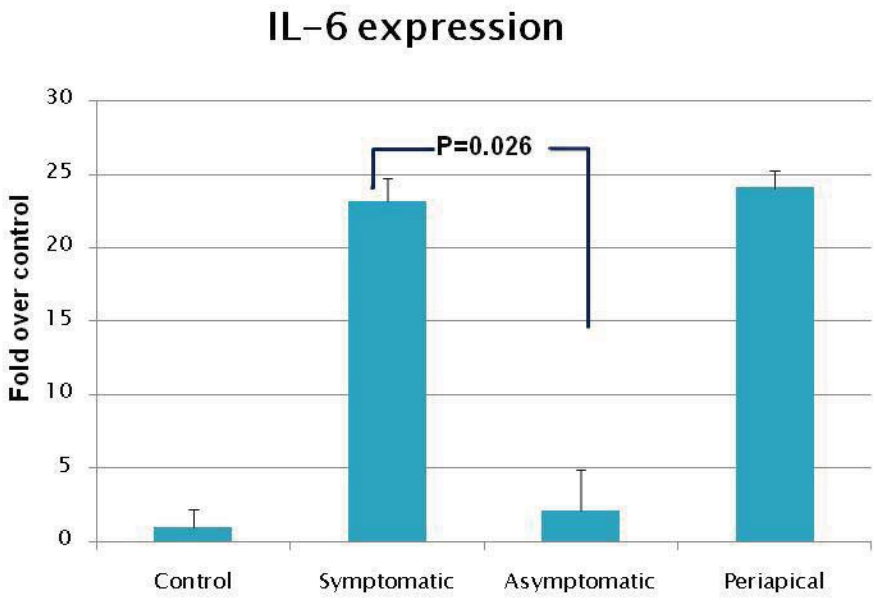
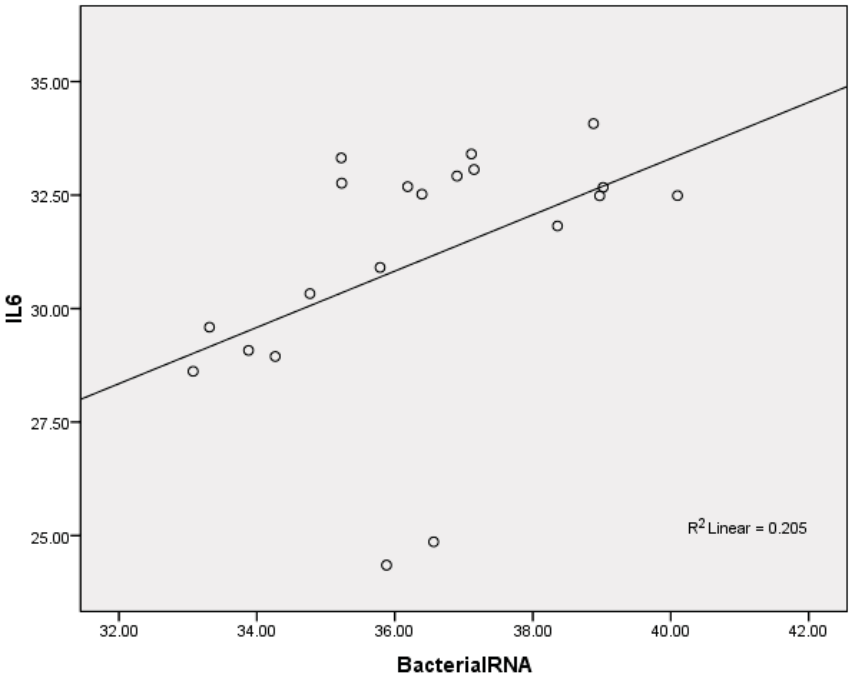
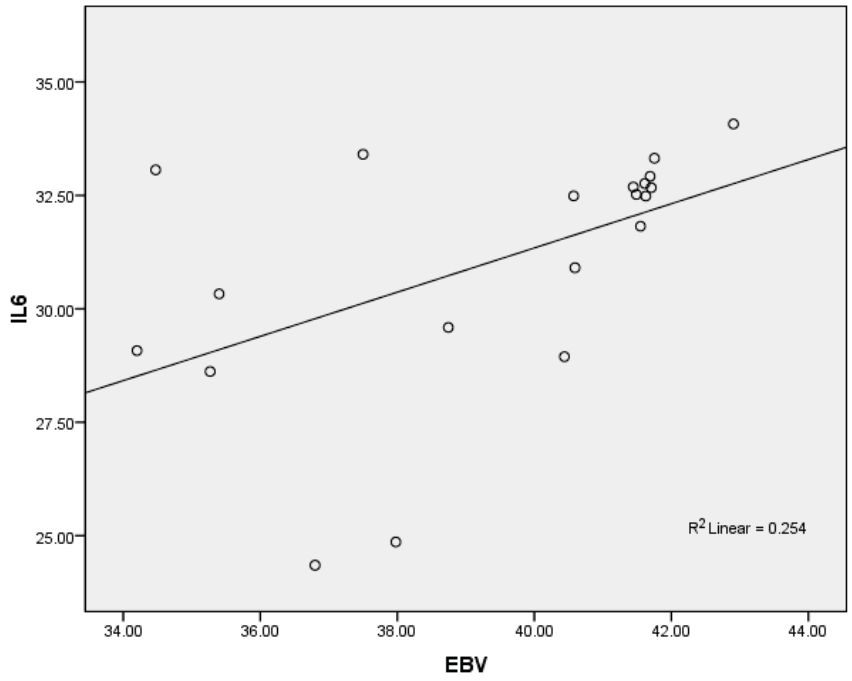

IL-6 expression
Fold over control
30
25
20
15
10
5
0
P=0.026
Control
Symptomatic
Asymptomatic
Periapical

Figure 4

(A)


35.00
32.50
30.00
27.50
25.00
IL6
R^2 Linear = 0.205
32.00
34.00
36.00
38.00
40.00
42.00
BacterialRNA

(B)


35.00
32.50
30.00
27.50
25.00
IL6
R^2 Linear = 0.254
34.00
36.00
38.00
40.00
42.00
44.00
EBV

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Chapter 4

Summary and the opportunities for future research

This dissertation provides both *in vitro* and *in vivo* evidences that interaction between bacterial and viral interaction aggravates inflammatory response. In *in vitro* study, *E. faecalis*, a gram-positive species, was employed to investigate bacterial and virus, EBV, interaction. We showed that a cell wall component of *E. faecalis*, LTA, can significantly re-activate latent EBV in a lymphocyte cell line. The effect of *E. faecalis* on EBV was via TLRs mediated NF- I b pathway. When latent EBV presented in the cells, the growth and virulent gene expressions within *E. faecalis* had significantly increased. In addition, the inflammatory response is significantly higher with the co-infection of latent EBV and *E. faecalis*. Collectively, our results show that *E. faecalis* and EBV interactions might aggravate inflammation *in vitro*. Further research is warranted in order to delineate the detailed cellular signal transduction pathway of *E. faecalis* re-activation of latently infected EBV. In our study, we showed that *E. faecalis* LTA -mediated induction of lytic EBV infection is significantly reduced by the TLR2 antagonist and inhibitor of the NF- I b pathway, yet not fully diminished, su estin other possible mechanism mi ht exist and there might be cross-talks between these different mechanisms. The mechanism by which EBV influence *E. faecalis* growth and virulence requires further investigated. Very limited research has been done to investigate the direct interaction between virus and bacteria. This is a new yet exciting research field that needs a lot of attention and investigation. The current model regarding viral bacterial interaction proposes the interaction is indirectly through the host. Our results showed the cellular inflammatory response significantly increased when co-infected with both *E. faecalis* and EBV. It's nown that *E. faecalis* is associated with chronic intestinal infection. An

animal study is necessary to investigate whether *E. faecalis* and EBV synergy exist in vivo and contribute to the pathogenesis.

Our in vivo study showed, for the first time, that the pro-inflammatory genes such as IL-6 were associated with EBV viral load even after adjusting for bacterial activity, suggesting the possibility that EBV contributes to pulpal inflammation and consequently patient symptoms. The results indicated that inflammatory mediators had higher expression in symptomatic pulp, which might be associated with higher bacteria activity and EBV loads. The bacterial profile seems different in symptomatic and asymptomatic. For example, significantly more streptococci were present in asymptomatic pulp than symptomatic pulp. The results support a paradigm shift in which elevated bacterial/viral replication and inflammation are associated with endodontic infection. The players (bacteria/viral species) change over the course of infection. Studies with larger sample size are certainly needed to draw more definitive conclusions.

In summary, this thesis offers *in vivo* and *in vitro* evidence for viral-bacterial synergy in inflammatory response. The mechanism of the interaction between herpes virus, EBV and the Gram-positive bacterium, *E. faecalis* was explored. More research was warranted in this field.