Persistence of endodontic infection and Enterococcus Faecalis: Role of Horizontal Gene Transfer

Running title: ***Enterococcus faecalis*** **and horizontal gene transfer**

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Abstract

The endodontic literature states that a diversity of microorganisms is implicated in cause of root canal infection. There may be a possibility that the actual existence of a specific species is not as imperative as the presence of specific virulent strains of that organism. There are genetic modifications in the cell that furnish an organism with greater pathogenicity. Primary and persistent endodontic infections have difference in their micro-flora. Primary infections usually comprises of mostly anaerobic microbiota whereas multidrug resistant *Enterococcus faecalis* has been linked to persistent endodontic disease. Horizontal gene transfer is a mechanism that leads to a varied number of traits including acquired antibiotic resistance. Horizontal gene transfer takes place by three processes transduction, conjugation and transformation. The present review expatiates on the mechanism of horizontal gene transfer of acquired antibiotic resistance in *E. faecalis*.

Keywords: Enterococcus faecalis; horizontal gene transfer; persistent endodontic infection; pulpitis.

Introduction

The father of oral microbiology, W.D. Miller in 1890 claimed the association between bacteria and pulp. He was the first investigator to link the presence of bacteria with pulpal disease. In 1965 an experiment by Kakehashi et al verified that the microbiota were the etiology of pulpal and periradicular disease. Endodontic infections are polymicrobial and these microbes have abundant virulence factors, including bacterial capsules, fimbriae, lipopolysaccharides, enzymes, extracellular vesicles, fatty acids, polyamines, ammonia, and hydrogen sulfides (Cohen and Burns, 2002). The microorganisms in the root canal are largely non-motile and seldom leave root canal space and enter the periapical tissue. Nonetheless, they have the ability to grow out of the canal and penetrate the periapical tissue. These microorganisms may prompt secondary infection at another site in the body, or may even cause systemic complications (Chandra and Krishna 2010l; Provenzano et al., 2013).

The infected pulp is a repository of large number of antibiotic-resistant bacteria (Ready et al., 2006). The dental pulp is a connective tissue which is delicate in nature and interfused with blood vessels, lymphatics, myelinated and unmyelinated nerves, and undifferentiated connective tissue cells. This connective tissue complex is enveloped by hard tissue structure from all sides. The blood vessels supplying the pulp enters the tooth via the apical foramina, which results in cessation of the development of collateral blood supply to the inflamed part (Rajendran and Sivapathasundharam 2006). Thus it makes the usage of systemic antibiotics incompetent in the treatment of endodontic infections (Reynaud et al., 2006).

Enterococcus faecalis is a gram-positive cocci and a facultative anaerobe (Stuart et al., 2006). It is non-spore forming, fermentative micro-organism which is ovoid in shape. It occurs single, in

pairs or in short chains, it is mostly non-hemolytic and non-motile (Rocas et al., 2004). It is a normal inhabitant of the oral cavity. The concentration of this bacteria changes amongst patients in the initial endodontic treatment, in the middle of the treatment, patients receiving retreatment and patients with no history of endodontic treatment. In case of primary endodontic infection, *E. faecalis* is associated with asymptomatic chronic periradicular lesion. In case of failed root canal treatment, its prevalence is nine times more than the primary infection. Few cases have reported presence of only *E. faecalis* in the root filled teeth with periradicular lesion (Stuart et al., 2006).

The virulence factors of *E. faecalis* include cytoplasm, lytic enzymes like gelatinase & hyaluronidase, aggregation substance, pheromones and lipoteichoic acid. It adheres to host cells and even express protein that enable it to compete with other protein that enable it to compete with other bacterial cells. It has been observed that *E. faecalis* is more dependent on its survival traits than upon its virulence factors (Rocas et al., 2004). It has the capacity to maintain intracellular pH homeostasis through the action of proton pump which is pivotal in enhancing its survival potential during usage of intracanal medicaments (Tay et al., 2015).

E. faecalis can cause a monospecies infection. Various techniques have failed to eradicate the bacteria. Recently a study tested effects of passive ultrasonic irrigation on *E. faecalis* from root canal but failed to remove *E. faecalis* from the root canal system (Guerreiro-Tanomaru et al., 2015). Its molecules like serine proteases, gelatinase and collagen-binding protein helps it to bind to dentin. It has a small size which is helpful for it to reside in the dentinal tubules. While residing in dentinal tubules, it has the caliber to resist calcium hydroxide dressing for about 10 days (Rocas et al., 2004). It has been observed to invade the dentinal tubules faster than other microorganisms causing pulpal inflammation and was noted to survive in the canal for the

longest period of time. It has the capacity to survive without nutrition in obturated canal cavity and can reproduce when it comes in contact with human serum (Dammaschke et al., 2013).

Genes and Resistance

In 2013 Rocas and Siqueira (2013) found that the most prevalent genes in endodontic infections which were encoded resistance to beta-lactams blaTEM, cfxA, tetracycline tetM, tetQ, tetW and erythromycin ermC The authors concluded that the most prevalent genes in dental abscesses were blaTEM (24%) and ermC (24%), while tetM (42%) and tetW (29%); these were prevalent in asymptomatic cases. The blaTEM gene was significantly associated with acute cases, whereas tetM was found to be more prevalent in asymptomatic secondary cases. Rocas and Siqueira (2012) assessed presence of 14 genes encoding resistance to beta-lactams, tetracycline and macrolides. The most prevalent genes observed were blaTEM, tetW, and ermC. Fusobacterium and Prevotella isolates were found to be positive for blaTEM, cfxA and tetM.

Bacterium and resistance

Nandakumar et al. in 2009 carried out metaproteomic analysis of root canal infections. They utilized reverse-phase nano-liquid chromatography-tandem mass spectrometry to identify bacterial proteins in seven cases, four primary, two persistent; and one was declared neither primary nor persistent infection. Proteins of cell wall or membrane origin, from endodontic bacteria including *E. faecalis*, *Enterococcus faecium*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Treponema denticola* were found in all the examined samples tested. The proteins were involved with adhesion, autolysins, proteases, virulence factors, conjugation and antibiotic resistance. These included glycosyltransferase, tight adherence protein G, collagenase,

a metalloprotease, a serine protease, an extracellular protease, and endopeptidases to name a few. (Provenzano et al., 2013).

Bacteria are acknowledged for their capability to adapt to varying environmental conditions (Provenzano et al., 2013). In natural habitats, they are recurrently exposed to diverse environmental fluxes which include severe nutrient limitation, fluctuations in pH and temperature, and changes in oxidative and osmotic tensions. For their survival, bacteria have rapid and adaptive responses, which are usually mediated by regulatory proteins which control transcription, translation, or other events in gene expression so that the physiological responses are suitable to the environmental changes (Chattoraj et al., 2011).

There are various mechanisms that help bacteria to persist and survive even under unfavorable condition. During nitrogen starvation, there is activation of Ntr gene system which enables bacteria to scavenge even the traces of ammonia and during high concentration of ammonia, the gene system gets uncoupled. When there is scarcity of molecular oxygen the Arc system may get activated. It is composed of arcA and arcB genes and in this system metabolic pathways are activated thus permitting use of alternate terminal electron acceptors for metabolism of respiration. This leads to shift from aerobic to anaerobic metabolism. During condition like limitation of glucose, the catabolic repressor system can be activated by cya and crp genes. This initiates synthesis of enzymes for utilization of various different organic carbon sources (Siqueira JF Jr 2001).

As well as adaptation of bacteria to new environment via signal transduction and specific transcriptional responses Bacteria can also acquire novel genes through horizontal gene transfer (HGT) (Itzek et al., 2011) allowing them to exploit new ecological niches. During the process of

evolution, bacteria acquired new traits eminently via HGT. It is the prime force for acquisition of new pathogenic properties, colonization niches and the metabolic adaptations. The significance and the influence of each mechanism of HGT is different as per the specie targeted (Nozawa et al., 2011). After a bacteria acquire resistance genes for its protection against various antimicrobial agents, they can use varied biochemical types of resistance mechanisms like antibiotic inactivation, target modification i.e. inhibition of protein synthesis, disturbance of nucleic acid synthesis, alteration in permeability and "bypass" metabolic pathway (Giedraitiene et al., 2011).

Antibiotic Resistance

Resistance can be explained as the competence of a microorganism to thrive in the presence of high concentrations of an antimicrobial agent or the regimen of antimicrobial medication (Kudiyirickal and Ivancakova 2008). Bacterial resistance to antibiotics can be categorized as intrinsic and acquired. Intrinsic resistance is characteristic of a particular bacterium and is dependent on inherent anatomy of the microorganism. It is resistance to an antibiotic that all members of a species were resistant to. Whereas acquired resistance refers to a heritable variation in the bacterial DNA (Mullany 2014). The modes by which acquired resistance befall is either by the accretion of exogenous genes by plasmids, conjugative transposons and phages or mutation of cellular genes, or the combination of all these mechanisms (Giedraitiene et al., 2011). "Horizontal gene transfer (HGT) involves the transmission of genetic material between distinct evolutionary lineages and can be an important source of biological innovation" (Savory et al., 2015). It was first observed in 1928, in an experiment by Frederick Griffith. He showed that there was transfer of virulence from virulent to non-virulent strains of Streptococcus pneumonia. He stated that this genetic information can be horizontally transferred between the

bacteria through a mechanism called transformation (Ravenhall et al., 2015). The principle mechanisms behind transmission of resistance gene in a bacterium are transfer by viral delivery, plasmid transfer, transfer by conjugative transposons and transfer of free DNA. These mechanisms include; transduction which is carried out by bacteriophages and (Mullany 2014; Giedraitiene et al., 2011). It can be generalized or specialized depending upon packaging of the replicated resistance genes (Garcez et al., 2010). The second to name is conjugation, which utilizes plasmids and conjugative transposons to bring about the transfer (Mullany 2014; Giedraitiene et al., 2011). It incorporates physical contact between donor and recipient cells to mediate transfer (Garcez et al., 2010). Lastly transformation, which entails the uptake of naked DNA from the surrounding environment and has the potency to transmit DNA to other microorganisms (Mullany 2014; Giedraitiene et al., 2011; Garcez et al., 2010).

Bacteriophage

Bacteriophage is counted as one of the mobile genetic elements that transmit virulence genes and disject them horizontally (McCarthy et al., 2012). It gets attached to the bacteria and then interjects the genetic material even including host bacterial DNA. Once the foreign DNA gets stabilized in the bacterium, it may initiate the production of new phage particles (Verraes et al., 2013).

Bacterial integrons

Bacterial integrons are a gene capture system that uses a specific recombination mechanism. They encode three primary elements in the 5´ conserved segment, these components enlist an enzyme integrase; these act as a specific recombination system to insert or to erase a new gene

cassette, at a specific recombination site and a promoter that initiates the gene transcription (Giedraitiene et al., 2011).

Plasmids

Plasmids are primary agents of horizontal gene transfer that cause acceleration of bacterial adaptation by transmitting ecologically important traits between strains and species.. They endorse their own transfer and even the transfer of other plasmids from one bacterium to another. They are also involved in genome transfer, for example plasmids like RP4 and F factor (Sorensen et al., 2005). There are various kinds of plasmids like a metabolic plasmid encoding a metabolic function; in the same way resistance plasmid is any plasmid that carries the antibiotic resistance genes (Bennett 2008; Lee et al., 2010).

Resistance transposons

Resistance transposons are the jumping gene system that assimilates a resistance gene within the element. They come in many forms, and all of these elements have the capability to transfer both intra- and inter-molecularly (Bennett 2008). They encode proteins required for their excision from the donor, formation of a conjugative bridge and transposition of gene into the strain receiving it. These elements promote the transfer of resistance genes between bacterial genomes. A resistant gene which confers selectable phenotype, it may be mobilized as a complex transposon. They can target multiple different integration sites (Juhas et al., 2009).

E. Faecalis **and Endodontic infection**

Endodontic infections can be subcategorized as primary and persistent. The primary infections most of the times occur due to anaerobic gram-negative organisms, whereas persistent infections

are caused by gram-positive facultative bacteria. Microbiota corresponding to the persistent root canal infections and antibiotic resistance include beta-lactamase positive Prevotella spp. relating to dentoalveolar infections and the multidrug resistance found in *E. faecalis*. *E. faecalis* has been linked to persistent endodontic disease (Jungermann et al., 2011).

E. faecalis is a facultative gram-positive bacterium that has been regarded as one of the most resistant species in endodontic infections. It's presence in root canal space has also been related to be the etiology of post-treatment disease after root canal treatment (Vivacqua-Gomes et al., 2005). A large number of authors have successively concluded *E. faecalis* to be treatment-resistant specie in endodontic infections (Jasni et al., 2010).

In 1982, Fabricius et al. (1982) evaluated the ability of 11 bacterial strains to induce periapical reactions in varied combinations. It was observed that *E. faecalis* survived for 6 months in all of nine inoculated root canals in monkeys as pure cultures, though it could induce a mild inflammatory periapical response. Potential virulence factors of *E. faecalis* that might promote adaptation and survival in diverse habitats subsume Enterococcus surface protein and aggregation substance, pheromones, and lipoteichoic acid and factors that enable secretion of toxins for e.g. cytolysin (Sedgley et al., 2005; Stuart et al., 2006). It has also been observed that the sequenced genome of *E. faecalis* V583 comprises of more than 25% DNA which is either mobile or belongs to a foreign source (Rossi-Fedele and Roberts 2007).

E. faecalis even possesses certain characteristics and virulence factors that enable them to survive for long periods of time in the root canal including aggregation substances and adhesins. Moreover, *E. faecalis* has the ability to survive long periods of starvation, formation of biofilms and invading and dwelling in the dentinal tubules (Rossi-Fedele and Roberts 2007). Ace is a

proteinaceous adhesin of *E. faecalis* that helps in bacterial adhesion to collagen. It contains a trench-shaped binding site that furnishes the triple- helical collagen molecule. Ace is produced by the bacteria under condition of stress and host factors including collagen and serum induce its production (Kayaoglu et al., 2008). Hubble et al (2003) stated that along with ace, serine protease also plays a pivotal role in bonding *E. faecalis* to dentin. Sedgley (2007) evaluated survival of *E. faecalis* strains with and without gelatinase producing ability and concluded that strains which produce gelatinase can thrive long-term in root canal space. It can be surmised that adhesins produced by *E. faecalis* aids in its resistance to antimicrobials.

E. faecalis **and Horizontal Gene Transfer (Table – 1)**

Tetracycline

Another crucial etiology behind high incidence of antibiotic resistance in *E. faecalis* is horizontal gene transfer, the mechanism observed in this bacterium is conjugation utilizing transposons (Rossi-Fedele et al., 2006). A study by Rossi-Fedele and Roberts (2006) compared the capability of two *E. faecalis* strains to withstand exposure to an irrigation solution containing a high concentration of tetracycline endodontic therapy and concluded that the presence of the Tn916-like conjugative transposon containing the tetracycline resistance gene tet(M) helped *E. faecalis* to thrive even in high concentration of tetracycline in irrigating solution. Literature suggests that eight of fifteen tetracycline-resistant bacteria isolated possessed the tet(M) gene and were resistant to tetracycline irrigation in an in-vitro tooth model. Four of these eight genes tested contained the conjugative transposon Tn916 that has been associated to the tet(M) gene (Jungermann et al., 2011).

The tet(M) gene is often found on conjugative transposons falling in the Tn916 family (Roberts et al., 2006). It has been observed in 42 genera and is mostly found on conjugative transposons of the Tn916/Tn1545 family (Ready et al., 2006). Tet(M) confers tetracycline resistance due to its bond with the 30S subunit of the ribosome, elimination and prevention of the binding of drug, thus leading to tetracycline resistance (Roberts 1989; Tritton 1977). Along with tetracycline, tet(M) gene even confers resistance to minocycline and doxycycline (Rossi-Fedele and Roberts 2007). Tn916 was first discovered in the late 1970s when tetracycline resistance was transferred from a resistant to a susceptible strain of *E. faecalis* in the absence of a detectable plasmid (Franke and Clewell 1981).

Christie et al in 1978 observed that Tn925 can transfers between *E. faecalis* cells in the absence of plasmid DNA and was seen to transpose to various sites on pAD1 plasmid. Tn925 along with Tn916-like elements, enlisting Tn916, Tn918, and Tn919, insert at high frequencies within a location of pADl that result in hyper-expression of the hemolysin gene. The hemolysin genes of pADl and other plasmids related to it have been noted to act as virulence factors. The element could be transferred at a range of frequencies when Tn925 was examined in relation to *E. faecalis*. It was also observed that Tn925::Tn917 composite transposon could function as a conjugal delivery system for Tn917 into a number of gram-positive bacteria (Christie et al., 1987). In a study Tomich et al., (1980) mentioned that pAMα1 is a non-conjugative plasmid which furnishes tetracycline resistance. It can be transferred by pAMγ1 conjugative plasmid.

In *E. faecalis*, the tet(S) gene is the second gene which has also shown to be culpable for causing tetracycline resistance (Roberts et al., 2006). The tet(S) gene acts by ribosomal protection, it shows 79% amino acid identity with tet(M), 72% with tet(O) and approximately 40% with tet(Q). It was first time identified in a Listeria monocytogenes strain on pIP811, a conjugative

plasmid (Charpentier et al., 1994). In a study by Francois et al (1997) there was transmission of tet(S) gene from *E. faecalis* BM4242 to *E. faecalis* strains JH2-2 and OGlRF in the presence of 55 kb conjugative plasmid pIP825 (Francois et al., 1997). Later tet(S) was found on plasmid pK214 from *Lactococcus lactis* and in the chromosome of *E. faecalis*. It was observed that tet(S) holds the same relative position as tet(M) in Tn916-related element. Thus it can be claimed that Tn916 involved in the dissemination of tet(M), as well as dissemination of tet(S) (Lancaster et al., 2004).

As mentioned in literature tet(O), gene is also able to transmit through conjugation into E faecalis. The presence of tet(O) gene has been observed in 11 genera including four Gram-negative and seven Gram-positive genera. In case of *Campylobacter jejuni* the tet(O) gene has been related to plasmids in relation to horizontal transfer. Wherein *E. faecalis*, tet(O) has been correlated with conjugation transposons for carrying out gene transfer. It has been observed that there is characterization of functional conjugative transposons that carry a tet(O) gene associated to an efflux mef(A) gene. The mentioned transposons are transmitted by conjugation to *E. faecalis*. This new conjugative element may allow wider dissemination of this particular gene in the future (Roberts 2005; Giovanetti et al., 2003; Brenciani et al., 2004).

Macrolides and Aminoglycosides

When the total cultivable flora of the oral cavity was assayed for resistance to erythromycin and the mef and ermB genes were the most commonly found genes. On an average, 7% of the cultivable microflora was found to be resistant to erythromycin and it possessed at least one erythromycin resistance gene. In many oral isolates the ermB gene was observed to be present on Tn916/Tn1545-like conjugative transposons, it also harbored tet(M) and aphA-3 conferring

tetracycline and kanamycin resistance (Roberts and Mullany 2010). Tn917 transposon also causes erythromycin resistance in *E. faecalis* (Franke and Clewell 1981). Lu et al (2007) examined erythromycin resistance in strains of *E. faecalis*, *E. faecium*, *E. hirae* and *Staphylococcus aureus*. It was observed that against erythromycin of Tn1545 and Tn917 conjugative transposons were found in the ermB gene. The study concluded that the presence of ermB gene along with Tn1545 and Tn917 transposons can be associated with transmission of antibiotic resistance via the mentioned transposons amongst strains and species of same and different genera.

Tomich et al., (1979) stated that *E. faecalis* plasmid pAD2 is responsible for resistance against erythromycin, streptomycin and kanamycin. They claimed it to be a MLS phenotype i.e. solo determinant possessing resistance against macrolides, lincosamides, and streptogramin B-type antibiotics. Cointegrate of pAD1 and pAD2 was found to be a conjugative factor and also possessed resistance against erythromycin, streptomycin and kanamycin. Cointegrate was stable during successive transmission to new recipients.

Glycopeptide Antibiotics

Six types of glycopeptide resistance genes have been observed in enterococci which are differentiated on grounds of the sequence of the structural gene for the resistance ligase including vanA, vanB, vanC, vanD, vanE, and vanG (Dahlen et al., 2000; Pinheiro et al., 2004; Sedgley et al., 2004).

The mechanism of HGT causes resistance to tetracycline, aminoglycosides, macrolides and glycopeptide antibiotics in *E. faecalis* thus making it more vulnerable to the treatment. This aggrandizes the prevalence of *E. faecalis* persistent endodontic infections.

Conclusion

Incidence of persistent endodontic infection is on the rise in clinical scenario. This article explains conjugation, the process in which the transposons deliver the required protein from the donor to recipient organisms, as the mechanism behind antibiotic resistance in *E. faecalis*. It makes the treatment arduous and the tooth vulnerable to reinfection. Thus it can be concluded that clinical and experimental studies are warranted to counteract the multi-drug resistance of *E. faecalis*.

Acknowledgement

The authors would like to thank Mrs. Parul Jain for helping in arranging literature. It was a self-funded research and authors declare that there was no conflict of interest.

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