

A preliminary study investigating the survival of tetracycline resistant *Enterococcus faecalis* after root canal irrigation with high concentrations of tetracycline

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Abstract

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Aim To compare the ability of two *Enterococcus faecalis* strains to survive exposure to an irrigation solution containing a high concentration of tetracycline in the root canals of bovine teeth.

Methodology The root canals of twelve bovine incisor root sections were chemo-mechanically prepared using commercially available drills, sodium hypochlorite and ethylenediamine tetra-acetic acid. The root sections were divided into two groups and inoculated with either a tetracycline sensitive or resistant strain of *E. faecalis*. The strains are isogenic, however one contains a conjugative transposon related to Tn916 which confers resistance to tetracycline, and the other strain is sensitive to the antibiotic. After 26 days of incubation the root canals were irrigated using one of three solutions (sterile distilled water, 50%

ethanol or tetracycline at a concentration of 30 mg mL⁻¹). The roots were sampled by grinding dentine and canal contents and the debris collected were incubated in broth to assess growth.

Results Irrigation with sterile distilled water or 50% ethanol did not remove all of the cells present. The tetracycline containing solution was efficient in preventing any growth of sensitive *E. faecalis*, however the resistant strain was able to survive a 5 min exposure at 30 mg mL⁻¹.

Conclusions The presence of the Tn916-like conjugative transposon containing the tetracycline resistance gene *tet(M)* allowed an *E. faecalis* strain to survive irrigation using a solution containing an extremely high concentration of tetracycline in a root canal model.

Keywords: conjugative transposon, root canal irrigation, *tet(M)*, tetracycline, tetracycline resistance, Tn916.

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Introduction

Enterococcus faecalis is usually found in the human and animal gastrointestinal tract, causing no harm to the host; however they are responsible for a variety of human infections including bacteraemia, infective

endocarditis, urinary tract infections and endodontic infections (Mejare 1975, Lemoine & Hunter 1987, Boulanger *et al.* 1991, Jett *et al.* 1994, Pinheiro *et al.* 2004). *E. faecalis* can be isolated in relatively low numbers and at a relatively low frequency in primary endodontic infections, while it is isolated far more frequently and in greater numbers in cases of post-treatment endodontic disease (Molander *et al.* 1988, Peciulienė *et al.* 2000, Hancock *et al.* 2001). *E. faecalis* possesses certain characteristics and virulence factors that enable them to survive for long periods of time in

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the root canal. These include the production of proteolytic enzymes, aggregation substances and adhesins (Stuart *et al.* 2006). Additionally *E. faecalis* has the ability to survive long periods of starvation (Love 2001, Figdor *et al.* 2003), can form biofilms (Distel *et al.* 2002) and can invade and live within the dentinal tubules (Akpatá & Blechman 1982). *E. faecalis* is also able to acquire exogenous DNA, which may confer resistance to antimicrobials, such as tetracycline. Indeed the recently sequenced genome of *E. faecalis* V583 is composed of over 25% DNA which is believed to either be mobile or from foreign source (Paulsen *et al.* 2003).

The tetracycline resistance found in bacteria from root canals from patients at the Eastman Dental Hospital has been recently analysed. A *Neisseria* sp. which was able to transfer tetracycline resistance to a strain of *E. faecalis* has been isolated. This was the first time such an intergeneric transfer has been reported from a *Neisseria* sp. donor, furthermore it has been shown that the tetracycline resistance was mediated by the *tet(M)* gene and that this gene was present on a conjugative transposon related to Tn916 (Rossi-Fedele *et al.* 2006). The protein, Tet(M) confers tetracycline resistance by binding to the 30S subunit of the ribosome, removing and preventing the drug from binding (Connell *et al.* 2003). In addition to tetracycline, Tet(M) also confers resistance to minocycline and doxycycline (Chopra & Roberts 2001).

Tetracyclines are used in endodontics in a number of treatment regimes. Biopure Mixture of Tetracycline, Acid and Detergent (MTAD) (Dentsply Tulsa Dental, Tulsa, OK, USA) for example is a mixture of a tetracycline isomer (doxycycline hyclate) in a final concentration of 3% (according to manufacturers instructions; <http://www.tulsadental.com>), citric acid and a detergent (Tween 80) and is used as a final rinse for disinfection of the root canal system (Shabahang & Torabinejad 2003). MTAD has been shown to be effective against *E. faecalis* during *in vitro* experiments (Shabahang & Torabinejad 2003, Portenier *et al.* 2006) however, the concentration of the drug present in this medicament does not appear to have been tested on a defined resistant strain of *E. faecalis*. The generation of *E. faecalis* T1 (Rossi-Fedele *et al.* 2006) has given us the opportunity to determine, using isogenic strains of *E. faecalis*, if the presence of an orally derived Tn916-like conjugative transposon could allow the *E. faecalis* to survive the effects of high level tetracycline irrigation similar to levels that may be encountered during root canal treatment.

Materials and methods

The two isogenic strains of *E. faecalis* used throughout this study were *E. faecalis* JH2-2 which is tetracycline sensitive (Jacob & Hobbs 1974) and *E. faecalis* T1 which is a JH2-2 derivative containing a Tn916-like conjugative transposon conferring tetracycline resistance via *tet(M)* (Rossi-Fedele *et al.* 2006). The strains were grown in Brain Heart Infusion (BHI) broth or on BHI agar (Oxoid, Basingstoke, UK). Tetracycline (Sigma, Poole, UK) was used at a concentration of 10 µg mL⁻¹ in all media and at a concentration of 30 mg mL⁻¹ in the irrigant.

Bovine incisors were used throughout this study. The animals were less than 1 year old and slaughtered for commercial purpose in a Spanish slaughterhouse. The study exerted no influence on the animal's fate at any stage. The teeth were extracted and stored in 4% formal saline until the study was performed. The apical 5 mm and the crown were dissected and the remaining root was cut into 1 cm slices with a diamond disc (Abrasive Technology Inc, Westerville, OH, USA). Subsequently, the canal lumen was widened to a minimal diameter of 1.14 mm using the ParaPost[®] XPTM Endodontic post system drills (Coltene/Whaledent, Konstanz, Germany).

Finally the smear layer was removed by copious irrigation in an ethylenediamine tetra-acetic acid (EDTA) solution (Smear Clear; SybronEndo, Scafati, Italy) (4 min) and sodium hypochlorite (Teepol Bleach; Teepol, Orpington, UK) (4 min) using a 27 gauge Monoject syringe (Kendall; Tyco, Mansfield, MA, USA) in an up and down motion.

Three different irrigation solutions were used to irrigate the root canals: sterile distilled water, 50% ethanol (JM Loveridge, Southampton, UK) (diluted with sterile distilled water) and tetracycline at a concentration of 30 mg mL⁻¹ dissolved in 50% ethanol according to manufacturers instructions. This concentration of tetracycline was used as it is the same as the final concentration of doxycycline used in MTAD.

Twelve roots were placed individually in 10 mL of BHI broth and autoclaved. These were left to cool to room temperature and then incubated overnight at 37 °C to verify the sterility of the samples. The overnight incubation of the sectioned bovine root canals resulted in no growth in any of the samples indicating that all the root sections were sterile at the start of the experiment. Six of the broths containing the roots were inoculated with 100 µL of an overnight culture of *E. faecalis* JH2-2 (tetracycline sensitive) and six with 100 µL of an overnight culture of T1

(tetracycline resistant) and left for 26 days at 37 °C to allow for bacterial growth, infiltration of the dentine tubules and *E. faecalis* biofilm formation.

Next, each group of six was divided into three subgroups and roots were irrigated by placement of 1 mL of one of the three irrigation solutions in the canal, using a 27 gauge Monoject syringe 2 mm short of the root end, having previously sealed the apical aspect with autoclaved physiowax (RALamb Ltd, Eastbourne, UK); the solution was left in-situ for 5 min. Irrigation was completed by a final flush using a further 4 mL of the irrigation solution.

After removal of the apical seal to allow for the irrigation solution to drain, the coronal 5 mm portion of the specimen was sampled by grinding dentine and canal contents using ParaPost® XP™ Endodontic post system drills of diameters 1.25 and 1.40 mm, obtaining a dentine surface and deep dentine sample for each root.

Debris collected in the flutes of each drill was placed in 10 mL of BHI broth (without antibiotics) and incubated overnight at 37 °C to assess growth. A loop full (approximately 10 µL) of each broth (regardless of whether or not growth could be observed) was plated onto both antibiotic free and tetracycline containing (10 µg mL⁻¹) BHI agar plates and incubated overnight at 37 °C, to assess for purity.

To control for tetracycline carry-over (with the dentine debris) into the 10 mL BHI broths five additional sterile bovine teeth were irrigated with 30 mg mL⁻¹ tetracycline and sampled as described above. The dentine shavings from surface and deep samples for each tooth were transferred to an individual 10 mL BHI broth. These broths were immediately inoculated with 100 µL aliquots of a dilution of an overnight culture of *E. faecalis* JH2-2 containing approximately 100 viable cells (determined by plating an aliquot onto fresh antibiotic free BHI agar plates) and incubated overnight at 37 °C. Two additional broths (containing no debris) were inoculated with aliquots of *E. faecalis* JH2-2 and incubated as described above.

To compare the susceptibility of planktonically growing cells to this concentration of tetracycline 10 mL of an overnight culture (containing approximately 1.4¹⁰ cells mL⁻¹) of each strain of *E. faecalis* were spun down and resuspended in 5 mL of tetracycline (30 mg mL⁻¹) dissolved in 50% ethanol. These cells were incubated at room temperature for 5 min, re-pelleted and resuspended in fresh BHI broth.

Genomic DNA extraction was performed using the PureGene Gram-Positive and Yeast DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA supplied by

Flowgen, Nottingham, UK). PCRs were performed using *Taq* DNA polymerase in the buffer supplied by the manufacturer (Promega, Southampton, UK). The PCR programme was as follows: 94 °C for 4 min followed by 30 cycles of 94 °C for 30 s, 50 °C for 90 s and 72 °C for 60 s, followed by a final incubation at 72 °C for 5 min and a final thermal ramp to 4 °C where the samples were held until analysis. PCR products were cleaned for sequencing using a PCR purification kit (Qiagen, Crawley, UK). Sequencing was carried out using Big Dye Ready Reaction Mix V.3.1 and analysed on a 310 genetic analyser (Applied Biosystems, Foster City, CA, USA). All sequence data were edited using CHROMAS 1.45 (<http://technelysium.com.au/chromas.html>) and DNAMAN 5.2.2 software (Lynnon Biosoft, Quebec, Canada). The 16S sequence homology was determined using the ribosomal database project II (<http://rdp.cme.msu.edu/>) and BLAST® (<http://www.ncbi.nlm.nih.gov/BLAST/>) tools. The identity of all the bacteria was confirmed, by partial sequencing of the PCR amplified 16S rRNA gene.

Cultures of each isolate were made up to McFarland standard 1 and a multipoint inoculator used to inoculate plates containing various concentrations of tetracycline (8, 16, 32, 64 and 128 µg mL⁻¹). The MIC was taken as the concentration that completely inhibited bacterial growth in an aerobic environment after 24 h of incubation.

Results

The results of growth of *E. faecalis* following irrigation with various solutions in the bovine root model are shown in Table 1. All broths that contained growth showed heavy growth. All of these cultures were shown to be pure upon subculturing to a fresh plate. JH2-2 containing cultures grew on the antibiotic free plates but not on plates containing tetracycline. The T1 cultures grew on both antibiotic free and tetracycline containing plates. The identity of the bacteria in all of the cultures was confirmed as *E. faecalis* by partial sequencing of the 16S rRNA gene.

The assays designed to detect carry-over of tetracycline with the dentine debris showed no growth in the 10 test samples (five containing surface debris and five containing deep debris) and heavy growth in the two control samples (containing no debris).

The test for the susceptibility of planktonically growing cells of each strain of *E. faecalis* showed no growth of either strain after incubation in the tetracycline solution for 5 min.

Irrigant	Growth of <i>E. faecalis</i> JH2-2 ^a				Growth of <i>E. faecalis</i> T1			
	replicate 1		replicate 2		replicate 1		replicate 2	
	Surface	Deep	Surface	Deep	Surface	Deep	Surface	Deep
Water	+	+	+	-	+	+	+	+
50% ethanol	+	+	+	+	+	+	+	+
Tetracycline	-	-	-	-	+	+	+	-

^a+, growth; -, no growth.

All resistant strains showed the same MIC; 32 µg mL⁻¹, while sensitive strains showed no growth at any of the tetracycline concentrations tested.

Discussion

These experiments aimed to assess the survival of two isogenic *E. faecalis* strains that differ only in the presence of a Tn916-like conjugative transposon in strain T1. The bovine root model used here is a variation of the dentine block model suggested previously (Haapasalo & Ørstavik 1987), although in this investigation it was decided not to remove the cementum to avoid bacterial invasion from the external surface through dentinal tubules, and to allow the use of the root as an irrigation reservoir, in order to obtain a dynamic that resembles more closely clinical conditions.

The distilled water irrigation was used to evaluate the flushing action during the procedures, while 50% ethanol is used in laboratories to dissolve tetracycline and might have an antimicrobial effect. The results show that the irrigation with sterile distilled water did not remove all of the *E. faecalis* present. One of the deep samples showed no growth (Table 1) and this is most likely due to the lack of penetration of the *E. faecalis* into the dentinal tubules of this tooth. The cognate surface sample resulted in growth of *E. faecalis* following irrigation with the sterile distilled water demonstrating that the irrigation had not cleared all of the viable cells from the area that was drilled. The second irrigant (50% ethanol, diluted in sterile distilled water) was included as a control because the tetracycline would be dissolved in this solution. All samples resulted in growth of *E. faecalis* following irrigation with 50% ethanol demonstrating that this solution was unable to eradicate viable *E. faecalis* cells. The third irrigant (tetracycline dissolved in 50% ethanol) was efficient in preventing any growth of the sensitive *E. faecalis* in both the surface and deep samples; however the resistant strain was able to survive in both surface samples and showed positive growth in one of the deep

Table 1 Growth of *Enterococcus faecalis* following irrigation with various irrigants

samples (Table 1). Again the growth in the surface sample and absence in the cognate deep sample can be explained by lack of dentinal tubule penetration at that depth in those roots. An important consideration is the carry-over of tetracycline with the debris collected in the drill flute. Tetracycline has been shown to bind very well to dentine (Bjorvatn *et al.* 1985) and carry-over of the antibiotic with dentine shavings has been demonstrated and alluded to in previous studies (Shabahang & Torabinejad 2003, Portenier *et al.* 2006). Therefore, a control experiment was undertaken to determine if any carried-over tetracycline would be present in sufficient concentrations to inhibit the sensitive strain. All of the 10 test samples (five surface and five deep) showed inhibition of growth when compared with the controls indicating that the negative results for the growth of the sensitive strain JH2-2 after tetracycline irrigation may be explained by either the complete killing during the irrigation of the tooth, by the inhibition of growth in the BHI broth by the tetracycline carried over with the dentine shavings or by a combination of both of these factors. Therefore, there is a possibility that the sensitive strain did survive the 5 min irrigation period within the tooth and was prevented from growing in the broth by the carried-over tetracycline. However, this distinction is not possible using the current model. The fact that there is enough carry-over of the antibiotic to inhibit the growth of the sensitive strain does not affect the results of the resistant T1 strain as these grew well in the BHI broths. Furthermore, tetracycline containing medications are likely to be active after the irrigation stage in clinical situations due to the strong affinity between tetracycline and calcium containing enamel and dentine (Bjorvatn *et al.* 1985). Additionally no attempt is made to neutralize the antimicrobial effect following the irrigation regime in the clinical setting.

Another consideration is that while it can be said that the killing or inhibition of growth was 100% in all the negative samples, theoretically only a single cell needs to survive to result in heavy growth in broth after the drill debris have been incubated for 18 h at

37 °C. Therefore these results are not quantitative, however a similar situation exists clinically where it might be preferable that all bacteria are cleared, considering that the success in treatment of chronic apical periodontitis depends on the control of the microbial infection present in the root canal system (Dahlén & Haapasalo 1998).

The MIC of *E. faecalis* T1 when grown in plates is 32 µg mL⁻¹, while in the root model they survived irrigation with a tetracycline concentration of 30 mg mL⁻¹, almost 1000 times more than the concentration present in plates. There are certain factors that may go some way to explain this extraordinary survival; interactions between the dentine and the tetracycline may have reduced the overall concentration of the drug in contact with the cells. It has previously been demonstrated that the presence of dentine caused a delay in *E. faecalis* killing *in vitro* when MTAD and 0.2% chlorhexidine was used (Portenier *et al.* 2006). Additionally, it is likely that the majority of the cells were present as a biofilm on and within the tooth. Biofilm growing cells are often up to 1000 times more resistant to antimicrobial agents than planktonically growing counterparts (Walker *et al.* 2004). In an additional experiment during this study it was also demonstrated that planktonically growing cells of either strain were killed efficiently by incubation in 30 mg mL⁻¹ of tetracycline for 5 min. The metabolic activity of the cells may also have an effect on the susceptibility of the cell to tetracycline as those in a metabolically inactive growth phase (as the *E. faecalis* are likely to be after 26 days incubation) are less susceptible to antimicrobials (Mah & O'Toole 2001). Additionally, access to the dentinal tubules by the drug may not have been possible (Shabahang & Torabinejad 2003) therefore some of the bacterial cells may have failed to come into contact with the tetracycline or the exposure time of these cells may have been reduced. However, all of these factors apply to both strains of *E. faecalis* tested in these experiments, the only difference being the presence of an orally derived Tn916-like conjugative transposon. Therefore, it is likely that one or more of these above factors reduced the level of tetracycline and possibly reduced the exposure time to a level where the T1 strain could survive for 5 min due to the presence of the *tet*(M) gene on the conjugative transposon. If this happened in the clinical setting it is possible that treatment failure could follow. It also demonstrates the point that resistance mechanisms which may appear to confer a relatively low MIC on a bacterium in the laboratory may make a

fundamental difference in the outcome of treatment regimes.

Conclusions

Under the conditions of this preliminary study, it can be concluded that the presence of a tetracycline resistance encoding conjugative transposon in *E. faecalis* enabled it to survive root canal irrigation with tetracycline at a concentration of 30 mg mL⁻¹.

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