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Incidence and behaviour of Tn*916*-like elements within tetracycline-resistant bacteria isolated from root canals

G. Rossi-Fedele[1], W. Scott[1], D. Spratt[2], K. Gulabivala[1], A. P. Roberts[2]
[1]Endodontic Unit and [2]Division of Microbial Diseases, Eastman Dental Institute, University College London, University of London, London, UK

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Introduction: Tetracycline resistance is commonly found in endodontic bacteria. One of the most common tetracycline-resistance genes is *tet*(M), which is often encoded on the broad-host-range conjugative transposon Tn*916*. This study aimed to determine whether *tet*(M) was present in bacteria isolated from endodontic patients at the Eastman Dental Institute and whether this gene was carried on the transferable conjugative transposon Tn*916*.
Methods: The cultivable microflora isolated from 15 endodontic patients was screened for resistance to tetracycline. Polymerase chain reactions for *tet*(M) and for unique regions of Tn*916* were carried out on the DNA of all tetracycline-resistant bacteria. Filter-mating experiments were used to see if transfer of any Tn*916*-like elements could occur.
Results: Eight out of 15 tetracycline-resistant bacteria isolated were shown to possess *tet*(M). Furthermore, four of these eight were shown to possess the Tn*916*-unique regions linked to the *tet*(M) gene. Transfer experiments demonstrated that a *Neisseria* sp. donor could transfer an extremely unstable Tn*916*-like element to *Enterococcus faecalis*.
Conclusions: The *tet*(M) gene is present in the majority of tetracycline-resistant bacteria isolated in this study and the conjugative transposon Tn*916* has been shown to be responsible for the support and transfer of this gene in some of the bacteria isolated.

Key words: conjugative transposon; endodontic bacteria; *tet*(M); tetracycline resistance; Tn*916*

Adam P. Roberts, Division of Microbial Diseases, Eastman Dental Institute, University College London, University of London, 256 Gray's Inn Road, London, WC1X 8LD, UK
Tel.: +44 (0) 207 915 1180/2385;
fax: +44 (0) 207 915 1127;
e-mail: aroberts@eastman.ucl.ac.uk
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Tetracycline resistance among bacteria that normally inhabit the oral cavity has been shown to be relatively common. Previous studies have shown that between 0 and 88% of the total cultivable oral flora from individuals are resistant to tetracycline (7, 8, 17, 26); however, the average values are around 11% (26). The most commonly detected gene encoding resistance to tetracycline in the cultivable oral microflora has repeatedly been shown to be *tet*(M) (7, 8, 26). In this study we wanted to investigate the proportion of the oral microflora associated with endodontic infections.

Endodontic infections usually involve complex mixed species communities of, predominantly, anaerobic bacteria (14). Various studies have analysed the types of bacteria present (2, 4, 6, 16) and some have concentrated on the antimicrobial susceptibility of these endodontic isolates (1, 6, 16). Some studies included an assessment of tetracycline resistance in the isolates. Manch-Citron et al. (10), using polymerase chain reactions (PCR), found the tetracycline-resistance genes *tet*(M), *tet*(O) and *tet*(Q) in samples derived from the subgingival plaque of patients undergoing tetracycline fibre therapy (10). Tetracycline resistance has been found to be relatively prevalent among enterococci isolated from the endodontic environment (2, 15). However, there is relatively little work published giving genetic support for the observed tetracycline resistance.

Tetracycline resistance can be conferred upon bacteria by *tet*(M), a gene encoding a ribosomal protection protein designated Tet(M). The *tet*(M) gene is often found within broad-host-range genetic elements known as conjugative transposons. The

most promiscuous *tet*(M)-containing conjugative transposon identified so far has been designated Tn*916*.

Conjugative transposons of the Tn*916* family are mobile genetic elements that usually reside in the chromosome of the host bacteria and possess all of the necessary genetic information to undergo intracellular transposition and intercellular conjugation to a new host cell. The recipient cell does not need to be the same species or even genus of bacterium for successful transfer of Tn*916* to occur. Indeed, Tn*916* has been identified in, or introduced into, over 50 different species (including more than 30 different genera) of bacteria (18). In this study we wanted to assess the prevalence of conjugative transposons of the Tn*916*/Tn*1545* family with respect to any tetracycline resistance observed in clinical endodontic isolates taken at the Eastman Endodontic unit. Furthermore, because an endodontic infection is a relatively 'closed' system in terms of bacterial population dynamics we wanted to determine whether any of the Tn*916*-like elements discovered could transfer to other bacteria under laboratory conditions.

Materials and methods

Patient selection

Ethical approval for this project was obtained from the University College Hospitals joint research and ethics committee (study ref: 2/E019). Samples were recovered from the root canals of teeth associated with radiographically detectable periapical lesions without evidence of previous root canal treatment or where the previous root canal treatment was judged to have failed using the criteria of European Society of Endodontology (28).

Procedure for clinical sampling of bacteria from root canals

After rubber-dam isolation, the tooth, the rubber-dam clamp and the surrounding rubber dam were decontaminated with 30% hydrogen peroxide (Sigma, Poole, UK) for 1 min, then swabbed with 10% povidone-iodine (Seton Healthcare Group, Oldham, UK) for 1 min. The iodine was inactivated by swabbing with a sterile 5% sodium thiosulphate solution until discoloration of the iodine had occurred.

Access to the pulp chamber was achieved by de-roofing with sterile tungsten carbide burs in a hand piece. As soon as the pulp chamber was opened, the decontamination procedure was repeated as above, care being taken to ensure that no chemicals entered the pulp chamber. A sterile, size 10 K-Flex file (Dentsply, Maillefer, Ballaigues, Switzerland) was used to file the canal walls to release organic particles and biofilm; the file was attached to an apex locator (AFA^{TM}, SybronEndo UK, Peterborough, UK) to permit the operator to locate the canal terminus. Sterile phosphate-buffered saline was used to moisten dry canals before filing.

The canal contents were sampled using three size 15 white paper points (Roeko, Langenau, Germany) per canal. Each paper point was introduced separately and left in the canal for 30 s. Sterile scissors were used to cut off the moist portion of the paper point into a 1.5-ml Eppendorf tube (Starstedt, Leicester UK) containing 1 ml reduced transport fluid, all three paper points were placed in the same Eppendorf tube, which was then capped immediately. The reduced transport fluid (100-ml batches) was prepared in advance in the laboratory and stored at 4°C; 5 ml reduced transport fluid was then activated on the day of clinical sampling.

Bacterial cultivation

All samples were transported to the laboratory for cultivation within 15 min of being obtained on the clinic. The paper point samples from the root canals were vortexed for 30 s and a dilution series was prepared from each sample to reduce the bacterial concentration. Fifty microlitres of each of the four dilutions (10^0–10^{-3}) of the paper point samples were plated on to Isosensitest blood agar plates (Oxoid, Basingstoke, UK), both with and without tetracycline (4 μg/ml), and incubated both anaerobically and aerobically for 2–4 days. Single colonies were subcultured by plating out on to fresh plates. All tetracycline-resistant pure cultures were subsequently subcultured into broth containing tetracycline (4 μg/ml) and grown overnight at 37°C. Glycerol stocks were made and molecular analysis undertaken. Minimum inhibitory concentrations (MIC) were determined for all of the organisms containing *tet*(M) using agar with tetracycline concentrations of 8, 16, 32, 64, 128 and 256 μg/ml. The MIC was taken as the concentration that completely inhibited bacterial growth after 24 h of growth in the appropriate environment (anaerobic or aerobic).

Molecular analysis

Genomic DNA extractions for all bacteria were performed using the PureGene Gram-Positive and Yeast DNA Isolation Kit (Gentra Systems, Minneapolis, MN; supplied by Flowgen, UK). PCRs were performed using *Taq* DNA polymerase in the buffer supplied by the manufacturer (Promega, Southampton, UK). The typical PCR programme was as follows: 94°C for 4 min followed by 25–30 cycles of 94°C for 30 s, 50–60°C for 90 s and 72°C for 1–3 min, followed by a final incubation at 72°C for 5 min and a rapid thermal ramp; samples were then held at 4°C until analysis. PCRs were carried out specifically to detect an internal region of the *tet*(M) gene using previously published primers specific for *tet*(M) (13), the 16S rRNA gene (9) and PCRs for a region upstream of *tet*(M) (using primers RT1 and RT2) and the integrase gene which is unique to Tn*916* (using the primers intxis1 and intxis2) (19). Additional PCRs were performed for the joint of the circular form using the primers REO and LEO (27) that read out from each end of Tn*916*, therefore a product would only be amplified if the ends come together and ligate as they do following excision from the genome. The positions of each primer on Tn*916* are shown in Roberts et al. (19). PCR products were visualized on a 0.8–1.0% agarose gel depending on the size of the expected PCR product. 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).

Filter-mating studies

Filter-mating studies were carried out as previously described (21). Transconjugant bacteria were selected on brain-heart infusion (BHI) agar plates containing 5% defibrinated horse blood, 25 μg/ml rifampicin and 10 μg/ml tetracycline. All putative transconjugants were identified by partial amplification and comparative sequencing of the 16S rRNA gene to ensure that no spontaneous mutations conferring rifampicin resistance had occurred in the donor.

Stability assays

Assays to determine the stability of Tn*916*-like elements were carried out by growing a single colony in an overnight culture and plating out on to fresh plates. Following this, 10 well-isolated colonies were subcultured into 10 ml BHI broth. The genomic DNA was extracted from these

broths and assayed, by PCR, for the presence of various regions of Tn*916*. This experiment was carried out in the presence and in the absence of tetracycline.

BioInformatics

All sequence data were edited using CHROMAS 1.45 software (http://www.technelysium.com.au/chromas.html) and analysed using 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.

Results

Fifteen patients were sampled at clinic, for each of these patients only one tooth was sampled. A history of the patients' previous exposure to antibiotics was ascertained, this information revealed that all 15 patients had taken antibiotics within the 6 months before sampling; none of the patients had taken systematic tetracycline. However, all 15 patients had been prescribed amoxicillin by their general dental practitioner as part of the treatment for their endodontic problem.

Properties of bacteria isolated

A total of 94 morphologically distinct bacteria were isolated: an average of 6.3 isolates per patient. There were two patients from whom no bacteria were isolated and five patients from whom no tetracycline-resistant isolates were cultured. From the remaining eight patients, 15 tetracycline-resistant strains were isolated, which represented 15.9% of the total number of bacteria cultivated. These were labelled as 1.1, 1.2 etc., the first number denoting the patient number and the second number denoting the bacterial isolate obtained from that patient. Of these 15 tetracycline-resistant strains, eight were found by PCR to be positive for the *tet*(M) gene (Table 1); four of these were shown by PCR to be positive for the Tn*916* integrase gene and the region amplified by RT1 and RT2. Furthermore two of these were positive for the joint of the circular form of Tn*916*, indicating that these elements were excising and circularizing in the population of cells harvested for the DNA preparation. [The MIC values for all the strains containing *tet*(M) were also determined]. These results, together with the bacterial identifications that were determined by partial 16S rRNA gene sequencing, are summarized in Table 1.

Transfer and stability studies

Transfer studies were only carried out on those isolates that were shown to possess both *tet*(M) and the integrase gene from Tn*916*. Of the four different Tn*916*-positive hosts only one demonstrated transfer by transferring the Tn*916* element present to the *Enterococcus faecalis* recipient. Three filters out of 25 resulted in transconjugants containing the Tn*916*-like element. The identity of this donor was a *Neisseria* sp. (strain 3.2). PCR was used to check that transfer of both the *tet*(M) gene and the integrase gene had occurred. The identity of the transconjugants was determined to be identical to the recipient used in these experiments by amplifying and sequencing the 16S rRNA gene of the putative transconjugants. Neither the integrase gene nor *tet*(M) could be amplified from the recipient before filter-mating. The stability of this Tn*916*-like element in the *Neisseria* sp. (strain 3.2) donor was low (see below) and was therefore investigated further. After one overnight subculture, a dilution was plated onto fresh plates and grown overnight to obtain well-isolated colonies. Ten colonies from each plate were subcultured into broth and this culture was used to extract genomic DNA. These experiments were carried out with and without tetracycline present in both the broth and the plates. The experiment carried out with tetracycline present showed that all 10 were *tet*(M) positive, however none were *int* positive; however, the necessary controls on *Bacillus subtilis*::Tn*916* strain BS34A (20) were positive. The experiment performed without tetracycline resulted in nine out of 10 being *tet*(M) positive, and two out of 10 being *int* positive. The *int*-positive samples in this experiment were also *tet*(M)-positive (results not shown).

Table 1. Identification and properties of *tet*(M)-containing bacteria isolated during this study

Strain no.	Closest match[1]	*tet*(M)	Integrase	RT1/RT2	Joint of CI	Transfer	MIC (μg/ml)
Endodontic isolates							
3.1	*Streptococcus mitis gr.*	+	+	+	−	ND	32
3.2	*Neisseria* sp.	+	+	+	+	+	128
7.1	*Streptococcus mitis gr.*	+	+	+	−	ND	64
7.2	*Streptococcus mitis gr.*	+	+	+	+	ND	64
8.1	*Streptococcus mitis gr.*	+	−	−	−	NT	32
8.2	*Neisseria* sp.	+	−	−	−	NT	64
12.1	*Mycobacterium* sp.	+	−	−	−	NT	16
13.1	*Amycolatopsis* sp.	+	−	−	−	NT	32
Transconjugants							
T1	*E. faecalis* JH2-2	+	+	+	+	NT	64
T2	*E. faecalis* JH2-2	+	+	+	+	NT	64
T3	*E. faecalis* JH2-2	+	+	+	+	NT	64
T4	*E. faecalis* JH2-2	−	−	−	−	NT	32

CI, circular intermediate molecule; NT, not tested; ND, not detected.
[1]As determined by partial comparative 16S rRNA gene sequencing. Therefore the data represents the closest homologue of the partial 16S rRNA sequence and not necessarily the identity of the bacteria isolated.

Discussion

The putative bacterial identities are inferred from homology searches of the 16S rRNA sequence derived from our bacterial isolates. Of the 15 tetracycline-resistant strains isolated from our subjects, seven were shown to be streptococci. Of these seven, four were shown to contain the *tet*(M) gene and three of these contained other regions unique to Tn*916*. This is in agreement with an earlier study looking at the effect of tetracycline on the streptococcal microbiota of periodontal pockets (3). In that study, the authors reported that out of 121 tetracycline-resistant streptococcal isolates only 23 harboured plasmids; this strongly suggested that the tetracycline resistance in the majority of these strains was not plasmid mediated (3).

The MIC of the eight bacterial isolates containing *tet*(M) was shown to be as high as 128 μg/ml. This, again, agrees with an earlier study by Williams et al. (29) who also showed that *Streptococcus* spp., *Veillonella* spp. and *Neisseria* spp. isolated from the subgingival microbiota of periodontal patients were consistently resistant to high levels (up to 128 μg/ml) of tetracycline. The transfer studies demonstrated that transfer of tetracycline resistance could occur from only *Neisseria* sp. (strain 3.2). Transconjugants containing

the Tn*916*-like element were only detected from three of the 25 filter-matings that were carried out. One transconjugant was kept from each mating for further study to avoid working on colonies derived from siblings. Interestingly, we did obtain another putative transconjugant that did not contain the *tet*(M) gene or the integrase gene from Tn*916*. This prompted us to re-check the donor *Neisseria* sp. (strain 3.2) which we found had also lost the *tet*(M) gene. However, the *Neisseria* sp. was still resistant to tetracycline, which has led us to believe that there is a second transferable, but as yet uncharacterized, tetracycline-resistance-encoding element present in this donor (no spontaneous tetracycline-resistant mutants were detected on the recipient control plates). The genetic basis for this resistance is currently under further investigation. Interestingly the MIC for the transconjugants containing *tet*(M) was higher than that of the transconjugant containing the unknown resistance mechanism. Additionally the MIC of the *Neisseria* sp. 3.2 was higher (128 μg/ml) than the MIC of all of the transconjugants, suggesting that there may indeed be multiple resistance genes present in this strain (Table 1).

Tetracycline resistance is relatively common among *Neisseria* species, especially in the gonococci. Tetracycline-resistant *Neisseria gonorrhoeae* and *Neisseria meningitidis* usually possess a 25.2-MDa plasmid encoding the *tet*(M) gene (11, 22). This plasmid is highly related to a 24.5-MDa plasmid from *N. gonorrhoeae* and it is believed that this smaller plasmid acquired the *tet*(M) gene from a *Streptococcus* species and subsequently spread among *N. meningitides*, *Kingella dentrificans* and *Eikenella corrodens* (5, 11). On the other hand, this plasmid was not found in commensal isolates of *Neisseria* spp. and it has been shown that the *tet*(M) gene in these strains is located to the chromosome (5, 23). In addition, deletions of Tn*916*-like transposons have been implicated as responsible for *tet*(M)-mediated tetracycline resistance in pathogenic *Neisseria* spp. (25). The authors report two distinct types of Tn*916* insertion into *N. gonorrhoeae* and *N. meningitides*. Class I insertions occur when the entire transposon is inserted and is stably maintained and class II insertions occur where Tn*916* has undergone deletions, leaving *tet*(M) in the *Neisseria* spp. chromosome. It was also shown that class II insertions are present on the 25.2-MDa plasmid, indicating that conjugative transposons of the Tn*916* family are responsible for the spread of the *tet*(M) gene into the 24.5-MDa plasmid and subsequent dispersal among the pathogenic *Neisseria* spp. This paper represents the first report, to our knowledge, of a *Neisseria* sp. strain acting as a donor for a Tn*916*-like conjugative transposon and further extends the number of species shown to participate in intergeneric gene transfer of these elements. In long-standing endodontic infections, the root canal is a relatively closed habitat where bacterial flora is selected depending upon ecological and environmental factors (24), and the different bacteria are often organized in tightly packed biofilm-like structures (12).

These factors, long-term cohabitation, close proximity between bacterial cells and isolation from both the oral environment and the immune system, seem to provide the ideal conditions for genetic transfer. The infected root canal space may be a potential reservoir for tetracycline resistance and an ideal place for its dissemination to other bacteria.

From the same patient we were able to isolate another (streptococcal) strain (3.1) that was also shown to contain a Tn*916*-like element containing *tet*(M). However, we could not demonstrate tetracycline resistance transfer from this strain.

The data presented in this study confirm previous work that showed *tet*(M) to be common among bacteria isolated from root canals. In our study it was present in the majority of tetracycline-resistant isolates and in bacteria from 33% of patients tested. In addition, we have discovered a tetracycline-resistant *Neisseria* sp. capable of transferring an unstable Tn*916*-like element to *E. faecalis*, representing the first time such a transfer has been recorded. The instability of the element may well explain why we only obtained transconjugants from three of the 25 filters and more work is needed to define the genetic basis for the tetracycline resistance observed in this strain.

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