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Prevalence of Tetracycline Resistance Genes in Oral Bacteria

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Tetracycline is a broad-spectrum antibiotic used in humans, animals, and aquaculture; therefore, many bacteria from different ecosystems are exposed to this antibiotic. In order to determine the genetic basis for resistance to tetracycline in bacteria from the oral cavity, saliva and dental plaque samples were obtained from 20 healthy adults who had not taken antibiotics during the previous 3 months. The samples were screened for the presence of bacteria resistant to tetracycline, and the tetracycline resistance genes in these isolates were identified by multiplex PCR and DNA sequencing. Tetracycline-resistant bacteria constituted an average of 11% of the total cultivable oral microflora. A representative 105 tetracycline-resistant isolates from the 20 samples were investigated; most of the isolates carried tetracycline resistance genes encoding a ribosomal protection protein. The most common *tet* gene identified was *tet*(M), which was found in 79% of all the isolates. The second most common gene identified was *tet*(W), which was found in 21% of all the isolates, followed by *tet*(O) and *tet*(Q) (10.5 and 9.5% of the isolates, respectively) and then *tet*(S) (2.8% of the isolates). Tetracycline resistance genes encoding an efflux protein were detected in 4.8% of all the tetracycline-resistant isolates; 2.8% of the isolates had *tet*(L) and 1% carried *tet*(A) and *tet*(K) each. The results have shown that a variety of tetracycline resistance genes are present in the oral microflora of healthy adults. This is the first report of *tet*(W) in oral bacteria and the first report to show that *tet*(O), *tet*(Q), *tet*(A), and *tet*(S) can be found in some oral species.

Tetracycline is a broad-spectrum antibiotic which is used in the treatment of bacterial infections in humans and animals, as well as protozoal infections in humans; it is also used as a growth promoter in animals and aquaculture and as an immunosuppressor in humans (8, 31). Tetracycline is commonly used in dental practice as a prophylactic agent and for treatment of oral infections (8). The wide use of tetracycline has had the effect of exposing commensal as well as pathogenic bacteria from different ecosystems to the drug, resulting in a major increase in the rate of tetracycline resistance among bacteria since this antibiotic was first used in the 1950s (8, 30). Resistance to tetracycline is commonly mediated by efflux of the drug or ribosomal protection; however, there has also been one example of a gene encoding tetracycline inactivation (9, 35). So far eight classes of genes encoding ribosome protection proteins have been described: *tet*(M), *tet*(O), *tet*B(P), *tet*(Q), *tet*(S), *tet*(W), *tet*(T), and *tet*(32). The most common of these is *tet*(M) (8, 31). One of the reasons for the success of this gene is the fact that it is commonly contained within conjugative transposons, which have an extraordinarily broad host range (8, 28, 35). The *tet*(Q) gene is also common and is contained within a conjugative transposon, and there is also some evidence that *tet*(W) and *tet*(32) are contained within conjugative chromosomal elements, although these are not yet well characterized (15, 17, 33). Thirteen genes encoding tetracycline efflux pumps have also been described: *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(J), *tet*(Y), *tet*(Z), *tet*(30), *tet*(K), and *tet*(L) (2, 8, 20, 30). Efflux genes are found in both gram-positive and gram-negative species. The efflux genes from gram-negative organisms are widely distributed and are associated with large plasmids, most of which are conjugative (8). Efflux genes [*tet*(K) and *tet*(L)] from gram-positive organisms are usually found on small transmissible plasmids (4). A novel *tet* gene, *tet*(34), which apparently is different from the three known groups, has recently been described (22).

tet(M) has been found in many different bacterial genera in the oral cavity (12); *tet*(O) and *tet*(Q) have been isolated from gram-positive and gram-negative oral species, respectively (21, 24, 25); *tet*(K) and *tet*(L) have been isolated from *Streptomyces* species (26); and several efflux genes have been isolated from gram-negative oral species (32). However, the prevalence of the newly discovered tetracycline resistance genes in the oral cavity has not been investigated. Furthermore, oral species from healthy adults have not been investigated for the presence of the different tetracycline resistance genes. Therefore, the aim of this work was to investigate the prevalence of tetracycline resistance genes in the oral microflora, a complex ecosystem containing many different genera and species of bacteria where there is ample opportunity for genetic exchange (11, 18). Oral bacteria also have the opportunity to come into contact with bacteria from other body sites (18). In this study we screened the tetracycline-resistant oral microflora for the presence of 12 different tetracycline resistance genes and show, for the first time, that *tet*(W) is common in oral bacteria and that *tet*(O), *tet*(Q), *tet*(A), and *tet*(S) were found for the first time in some oral species.

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TABLE 1. Description of primers used to detect each of the tetracycline resistance genes

Group and PCR[a]	*tet* genes	Amplicon size (bp)	Annealing temp (°C)	Reference
Group 1, multiplex PCR	*tet*(B)	659	55°C	20
	tet(C)	418	55°C	20
Group 2, multiplex PCR	*tet*(A)	210	55°C	20
	tet(E)	278	55°C	20
Group 3, multiplex PCR	*tet*(K)	169	55°C	20
	tet(M)	406	55°C	20
	tet(O)	515	55°C	20
	tet(S)	667	55°C	20
Individual PCR	*tet*(L)	267	55°C	20
Individual PCR	*tet*(Q)	904	55°C	20
Individual PCR	*tet*(T)	169	46°C	1
Individual PCR	*tet*(W)	168	64°C	1

[a] A multiplex PCR was used for groups 1, 2, and 3; and an individual PCR was used for *tet*(Q), *tet*(T), and *tet*(W).

MATERIALS AND METHODS

Sampling. Saliva and plaque samples were obtained from 20 healthy adults attending the Orthodontic Dentistry Clinic at the Eastman Dental Hospital. All the individuals were medically healthy and not suffering from any oral diseases. Furthermore, the individuals had not received any antibiotics during the previous 3 months. A saliva sample (2 ml) was collected in a sterile container. Plaque samples were collected with a sterile swab, and subgingival plaque samples were collected from four different sites with sterile paper points; both samples were pooled into 4 ml of Ringer's solution and mixed with the saliva sample.

Culture. The bacteria were resuspended, and serial 10-fold dilutions were made in Todd-Hewitt broth (Oxoid). They were held in this medium for the briefest possible time to ensure that the ratios of the different bacteria remained unchanged. Each sample from each individual was plated on duplicate blood agar plates (with 5% horse blood) containing tetracycline-HCl (Sigma) at a concentration of 8 μg/ml (19) as well as antibiotic-free medium to determine the total viable count. One set of plates was incubated anaerobically in an anaerobic chamber (10% CO_2, 10% H_2, 80% N_2) for 7 days at 37°C, and the other was incubated in 5% CO_2–air for 2 days at 37°C. Resistant isolates were enumerated according to their morphologies, and one representative colony of each colony type was subcultured for further identification. The MIC for each isolate was determined by an agar dilution assay on Iso-Sensitest agar plates (Oxoid) with an inoculum of 10^4 CFU/spot. The plates were read after 24-h incubation at 37°C aerobically or anaerobically for 72 h, according to the atmospheric requirement of the isolate. The MIC was defined as the concentration that prevented growth. All of the isolates were stored in a Microbank cryovial (Pro-Lab Diagnostics) at −70°C.

Detection of tetracycline resistance determinants. All of the isolates were tested for the presence of tetracycline resistance genes by PCR. A multiplex PCR assay (Tables 1 and 2) was used by the method described by Ng et al. (20). Individual PCRs were performed for *tet*(Q), *tet*(W), *tet*(L), and *tet*(T); the primers and PCR conditions used were those described by Aminov et al. (1). All PCRs were performed with DNA isolated by use of the Puregen kit (Gentra system; Flowgen). Some of the PCR results were confirmed, when required, by DNA sequencing. DNA sequencing was carried out with the Big Dye Terminator ready reaction mixture (PE Biosystems, Warrington, United Kingdom) and analyzed on a 310 genetic analyzer (PE Biosystems).

Tet(W) was further analyzed and compared with the *tet*(W) gene from *Butyrivibrio fibrisolvens* by using primers specific for the whole gene (Fig. 1, primers AF and AR,) as well as primers specific for the whole gene and the downstream region (Fig. 1, primers AF and CR). Both sets of primers were provided by C. M. Melville (primer AF, 5′-TTG GGG CTG TAA AGG GAG GAC-3′; primer AR, 5′-CAT CGG TGC TCC ATA AC-3′; primer CR, 5′-GTG TTG CTG CAA TAA CAC CAT C-3′) (R. Brunel and K. P. Scott, unpublished data).

TABLE 2. PCR primers targeting *tet* genes

Primer	Class targeted	Sequence	Reference
TetA-FW	*tet*(A)	GCT ACA TCC TGC TTG CCT TC	20
TetA-RV	*tet*(A)	CAT AGA TCG CCG TGA AGA GG	20
TetB-FW	*tet*(B)	TTG GTT AGG GGC AAG TTT TG	20
TetB-RV	*tet*(B)	GTA ATG GGC CAA TAA CAC CG	20
TetC-FW	*tet*(C)	CTT GAG AGC CTT CAA CCC AG	20
TetC-RV	*tet*(C)	ATG GTC GTC ATC TAC CTG CC	20
TetE-FW	*tet*(E)	AAA CCA CAT CCT CCA TAC GC	20
TetE-RV	*tet*(E)	AAA TAG GCC ACA ACC GTC AG	20
TetK-FW	*tet*(K)	TCG ATA GGA ACA GCA GTA	20
TetK-RV	*tet*(K)	CAG CAG ATC CTA CTC CTT	20
TetL-FW	*tet*(L)	TCG TTA GCG TGC TGT CAT TC	20
TetL-RV	*tet*(L)	GTA TCC CAC CAA TGT AGC CG	20
TetM-FW	*tet*(M)	GTG GAC AAA GGT ACA ACG AG	20
TetM-RV	*tet*(M)	CGG TAA AGT TCG TCA CAC AC	20
TetO-FW	*tet*(O)	AAC TTA GGC ATT CTG GCT CAC	20
TetO-RV	*tet*(O)	TCC CAC TGT TCC ATA TCG TCA	20
TetS-FW	*tet*(S)	CAT AGA CAA GCC GTT GAC C	20
TetS-RV	*tet*(S)	ATG TTT TTG GAA CGC CAG AG	20
TetQ-FW	*tet*(Q)	TTA TAC TTC CTC CGG CAT CG	20
TetQ-RV	*tet*(Q)	ATC GGT TCG AGA ATG TCC AC	20
TetT-FW	*tet*(T)	AAGGTTTATTATATAAAAGTG	1
TetT-RV	*tet*(T)	AGGTGTATCTATGATATTTAC	1
TetW-FW	*tet*(W)	GAGAGCCTGCTATATGCCAGC	1
TetW-RV	*tet*(W)	GGGCGTATCCACAATGTTAAC	1

Bacterial identification. Preliminary characterization of all isolates was performed and consisted of Gram stain, catalase, and oxidase tests. Additionally, a number of strains were further characterized and in most cases were identified to the species level by partial 16S rRNA gene sequencing (14) and analysis by use of the Ribosomal Database Project (16) and BLAST databases.

RESULTS AND DISCUSSION

On average, 11% of the cultivable microflora from the 20 samples was found to be tetracycline resistant. A representative set of 105 tetracycline-resistant bacteria was isolated from the 20 samples for further study. Most of the isolates carried tetracycline resistance genes encoding a ribosomal protection protein. The most common *tet* gene identified was *tet*(M), which was found in 79% of all the tetracycline-resistant isolates. Sixty percent of the *tet*(M) genes were found on their own, and 19% were found with one or more different *tet* genes (Table 3). The second most common gene identified was *tet*(W), which was found in 21% of all the isolates, followed by *tet*(O) and *tet*(Q) (10.5 and 9.5% of the isolates, respectively) and then *tet*(S) (2.8% of the isolates). Tetracycline resistance genes encoding an efflux protein were detected in 4.8% of all the tetracycline-resistant isolates; 2.8% of the isolates had *tet*(L), and 1% of the isolates carried *tet*(A) and *tet*(K) each (Table 3).

Tet(M) was the most common tetracycline resistance gene

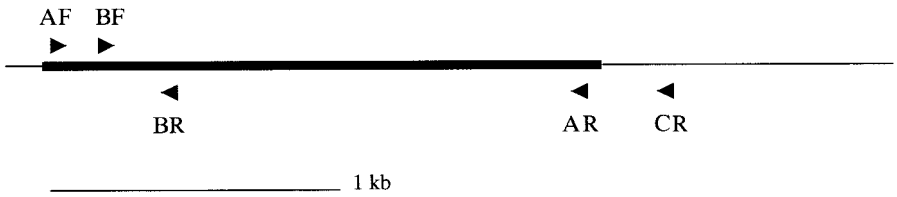

FIG. 1. Diagram showing the *tet*(W) gene. The thick line represents *tet*(W) DNA, and the thin line represents flanking sequences. The binding sites of the primers used in the PCR experiments are shown as arrows, with the direction of priming shown by the point of the arrow. The scale (in kilobases) is also shown.

isolated; it was isolated from every sample. Among the tetracycline-resistant bacteria from this group of 20 individuals, between 14.8 and 100% (mean, 81.1%; standard deviation [SD], 22.7%) harbored the *tet*(M) gene. The wide distribution of *tet*(M) probably reflects the fact that it is contained within broad-host-range conjugative transposons which have previously been found to be common in oral bacteria (29).

This is the first report of the isolation of the *tet*(W) gene from the human oral cavity, and this gene was found to be the second most common tetracycline resistance gene in the cultivable bacteria. Among the tetracycline-resistant bacteria from this group of 20 individuals, between 0.0 and 71.5% (mean, 16%; SD, 20%) harbored the *tet*(W) gene. This study has shown for the first time that the *tet*(W) gene is present in *Veillonella*, *Prevotella*, *Streptococcus*, *Staphylococcus*, *Streptomyces*, *Lactobacillus*, and *Neisseria* spp. (Table 4). This gene was originally identified from the bovine rumen anaerobe *Butyrivibrio fibrisolvens* and subsequently in human fecal anaerobes, pigs, and recently, a facultative anaerobe, *Arcanobacterium pyogenes* (3, 5, 33). In *B. fibrisolvens*, *tet*(W) is contained within a large conjugative transposon, Tn*B1230* (3), which is capable of high-frequency conjugative transfer among *B. fibrisolvens* species. In *A. pyogenes*, *tet*(W) was associated with a *mob* gene, which is not found in Tn*B1230*, and was capable of conjugative transfer at low frequency (5), indicating that at least two different genetic elements are responsible for the spread of this resistance gene. However, the genetic elements carrying *tet*(W) have not been fully characterized.

The *tet*(W) genes found in this study were compared with the original *tet*(W) gene from *B. fibrisolvens*. Of 22 isolates tested, the DNA sequence of the PCR product produced by primers BF and BR was almost identical to that of the PCR product of the original *tet*(W) gene (Fig. 1). However, only 12 isolates produced a PCR product with primers AF and AR as well as primers AF and CR, and all of the isolates gave a PCR product when primers AF and BR were used. These data indicate that some of the *tet*(W) genes are different from that originally found in *B. fibrisolvens.* It is possible that these variants are a new subgroup of *tet*(W) or a new *tet* gene with a high degree of homology to *tet*(W) when primers AF and BR are used (Fig. 1). It is also possible that the *tet*(W) gene in these samples may be closely related to the newly discovered *A. pyogenes tet*(W) gene (5).

Between 0.0 and 50% (mean, 8.3%; SD, 3.6%) of the tetracycline-resistant bacteria in the 20 samples harbored the *tet*(Q) gene. *Tet*(Q) was mainly found in gram-negative organisms (Table 3). *Tet*(Q) has been reported to be common in gram-negative oral bacteria that are associated with periodontal destruction (13, 24). However, this study has shown for the first time that *tet*(Q) is present in commensal *Neisseria* spp. (Table 4). Previous work has shown that the *tet*(Q) gene is frequently associated with conjugative transposons in *Bacteroides* and *Prevotella* (15, 23, 34). These elements were found to have a broad host range and may be responsible for the wide dissemination of the *tet*(Q) gene in oral bacteria.

The *tet*(O) gene was the fourth most commonly isolated gene in this study (Table 3). Between 0.0 and 87% (mean, 10.4%; SD, 22.5%) of the tetracycline-resistant bacteria from the 20 individuals harbored the *tet*(O) gene. The *tet*(O) gene has been found in different species (8), but it has been isolated only from gram-positive species (*Lactobacillus*, *Enterococcus*, *Staphylococcus*, and *Streptococcus* spp.) from the oral and respiratory tracts (32). However, in this study *tet*(O) was found for the first time in one *Neisseria* sp. (Table 4).

TABLE 3. Distribution of tetracycline resistance genes among cultivable oral bacteria

Identity of organism	No. of isolates from which tetracycline resistance genes were isolated							No. of isolates with more than one tetracycline resistance gene[a]
	Total	*tet*(M)	*tet*(W)	*tet*(Q)	*tet*(O)	*tet*(S)	Others[a]	
Streptococus spp.	37	32	5	1	6			5 (1 M/W/Q, 4M/W)
Gram-positive organisms other than *Streptococcus* spp.	32	23	8		3		2 L	5 (1 L/M/W, 2 M/W, 1 K/Q, 1 W/L)
Neisseria spp.	15	14	6	4	1			7 (3 M/W, 3 M/Q, 1 O/W)
Gram-negative organisms other than *Neisseria* spp.	7	4		2			1 L	
Gram-negative anaerobes	14	10	3	4		3	2 (1 A, 1 K)	5 (2 M/S/W, 1 M/W/Q, 1 M/S/A, 1 M/W)

[a] The letters refer to the tetracycline resistance gene class.

TABLE 4. Tetracycline resistance genes isolated for the first time from oral species

Isolate	Genus	*tet* gene(s) isolated for the first time from a particular geneus	Other *tet* gene(s) found in the same isolate
T22-3	*Streptomyces*	*tet*(W)	*tet*(M)
T22-5	*Lactobacillus*	*tet*(W)	
T23-5	*Actinomyces*	*tet*(W)	*tet*(M)
T23-8	*Veillonella*	*tet*(W)	*tet*(M)
T25-2	*Streptococcus*	*tet*(W)	*tet*(M)
T25-4	*Neisseria*	*tet*(W)	*tet*(M)
T27-5	*Staphylococcus*	*tet*(W)	*tet*(M), *tet*(L)
T32-2	*Neisseria*	*tet*(W), *tet*(O)	
T32-4	*Streptococcus*	*tet*(W)	*tet*(M)
T32-8	*Veillonella*	*tet*(W), *tet*(S)	*tet*(M)
T34-1	*Neisseria*	*tet*(Q)	*tet*(M)
T34-4	*Bacillus*	*tet*(W)	*tet*(L)
T35-7	*Veillonella*	*tet*(A), *tet*(S)	*tet*(M)
T37-7	*Veillonella*	*tet*(S), *tet*(W)	*tet*(M)
T38-2	*Prevotella*	*tet*(W)	*tet*(M), *tet*(Q)

Of the 105 tetracycline-resistant isolates, the *tet*(S) gene was found in only 3 different gram-negative anaerobes from three different samples; it is the first time that *tet*(S) has been isolated from gram-negative anaerobes (Table 3). *Tet*(S) was first detected in *Listeria monocytogenes* BM4210, where it is carried by self-transferable plasmids (6). It has also been found in *Enterococcus faecalis*, where it is integrated into the chromosomal DNA (7, 10), and in *Lactococcus* spp., where it is located on a conjugative plasmid (27).

Tet(A), *tet*(S), and *tet*(W) were isolated from *Veillonella* spp. for the first time (Table 4). Previously, the only *tet* genes isolated from this organism were *tet*(L), *tet*(M), and *tet*(Q) (8). This shows that *Veillonella* spp. may be an important reservoir for different tetracycline resistance genes.

We found that the carriage of more than one tetracycline resistance gene was common (Table 3). However, this did not have any effect on the MIC, indicating that a need for an increased level of tetracycline resistance is not the selective pressure for carriage of more than one gene. Rather, this could be because some of the *tet* genes are contained within conjugative transposons [i.e., *tet*(M), *tet*(Q), and *tet*(W); furthermore, *tet*(S) may also be on a conjugative transposon]. The possession of one conjugative transposon is not a barrier to that same cell being able to receive other related or unrelated conjugative transposons (8, 18). It has also been shown that some bacteria (*Neisseria*, *Haemophilus*, and *Streptococcus* spp.) are naturally competent, which could help with the further dissemination of the tetracycline resistance genes (32).

In conclusion this study has shown that the bacteria in the oral cavity contain a variety of tetracycline resistance genes, indicating that oral bacteria have access to and/or are a reservoir of resistance genes. In future it will be important to determine the genetic supports for these resistance genes in order to understand how they disseminate.

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