J Clin Periodontol 2006; 33: 743–748 doi: 10.1111/j.1600-051X.2006.00975.x

Journal of Clinical Periodontology

Proportion of antibiotic resistance in subgingival plaque samples from Mexican subjects

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Almaguer-Flores A, Moreno-Borjas JY, Salgado-Martinez A, Sanchez-Reyes MA, Alcantara-Maruri E, Ximenez-Fyvie LA. Proportion of antibiotic resistance in subgingival plaque samples from Mexican subjects. J Clin Periodontol 2006; 33: 743–748. doi: 10.1111/j.1600-051X.2006.00975.x.

Abstract

Aim: To determine the proportion of bacteria resistant to amoxicillin and doxycycline in subgingival plaque samples from Mexican subjects.

Materials and Methods: Two subgingival plaque samples were taken from 20 Mexican subjects. Samples were dispersed, diluted and plated on non-antibiotic agar plates and on plates containing 0.5, 1, 2, 4, 8 and 16 μg/ml of either amoxicillin or doxycycline. The proportion of resistant bacteria was calculated based on the total number of colony-forming units present in the non-antibiotic containing plates.

Results: On average, 0.4–13.4% and 0.9–20.4% of the total cultivable subgingival microbiota was resistant to the concentrations tested of amoxicillin and doxycycline, respectively. The differences between antibiotics were statistically significant for the 0.5, 2 and 4 μg/ml concentrations ($p < 0.05$, Wilcoxon's test).

Conclusions: Our findings revealed that a relatively small proportion of the total cultivable subgingival microbiota from Mexican subjects was resistant to amoxicillin and doxycycline.

Key words: antibiotic resistance; Mexican subjects; periodontal disease; subgingival plaque

Accepted for publication 29 May 2006

The use of systemically administered antibiotics as adjuncts to conventional or surgical therapy is common in periodontal clinical practice (Ramberg et al. 2001, Herrera et al. 2002, Haffajee et al. 2003a). A number of studies have focused on the clinical results achieved with the usage of antimicrobial agents as adjuncts to scaling and root planing and surgical procedures (Haffajee et al. 1995, Carvalho et al. 2004). For the most part, the results of such studies have suggested that the use of systemic antibiotics can improve clinical parameters such as pocket depth, attachment level, suppuration and bleeding on probing. However, there is growing concern about the indiscriminate use of antibiotics in periodontal therapy and of its role in the development of antimicrobial resistant bacteria (Palmer et al. 2000, Addy & Martin 2003). A number of studies have suggested that the incidence of bacterial resistance worldwide has increased significantly in recent years and that the overuse of antibiotics in clinical practice and by individuals living in countries with limited or no control over antibiotic usage, as well as by certain practices in agriculture and aquaculture have increased the potential of bacteria to develop antimicrobial resistance (Livermore 2002, Roberts 2002).

It has been reported that microbial resistance patterns may differ significantly in populations around the world (Pacini et al. 1997, Poulet et al. 1999, van Winkelhoff et al. 2000, Ready et al. 2002, Handal et al. 2003, Rodrigues et al. 2004). This is thought to be due, in part, to varying strategies of antibiotic usage and control (Baquero et al. 1991, Cullmann 1996, Pradier et al. 1997). Thus, the study of antibiotic resistance by subgingival microbial species from individuals in different locations may provide a better understanding of the potential effectiveness of antibiotics for the treatment of periodontal diseases in specific populations. In Mexico, the use of systemic antibiotics is poorly controlled and self-medication is a common practice by a significant proportion of the population (Calva et al. 1993, Leyva 1999). Amoxicillin and doxycycline are two of the most frequently used antibiotics for the treatment of periodontal infections in Mexico. However, no studies have been published in which their use in the treatment of periodontal diseases or the antimicrobial resistance patterns of subgingival species to such antibiotics have been evaluated in Mexican subjects. The purpose of the present study was to determine the proportion of bacteria resistant to amoxicillin and doxycycline in subgingival plaque samples from Mexican subjects.

Materials and Methods

Subject population

The present study received approval from the ethics committee for human studies of the Division of Postgraduate Studies and Research of the School of Dentistry of the National University of Mexico (UNAM). All subjects were asked to sign informed-consent forms, with which they acknowledged their willingness to participate.

Twenty randomly selected subjects were included in the study. Subjects were recruited from the population of individuals seeking consults and/or treatment at the clinics of the Division of Postgraduate Studies and Research of the School of Dentistry of UNAM in Mexico city from January to August 2003. Every subject who fit the entry criteria was included in the study. None of the subjects had received any form of periodontal therapy in the past other than professional supragingival plaque removal, had at least 20 natural teeth (excluding third molars) and were 28 years of age or more. All subjects were born and lived in Mexico and were of Mexican descent, i.e. both of their parents and at least two of their grandparents were born and lived in Mexico. Exclusion criteria included pregnancy, lactation, systemic antibiotic therapy in the previous 3 months and other systemic conditions such as diabetes, HIV/AIDS or autoimmune diseases. A summary of the characteristics of the subject population is provided in Table 1.

Sample collection

After drying and isolating with cotton rolls, supragingival plaque was removed from the sampled sites and subgingival plaque samples were taken with individual sterile Gracey curettes (Hu-Friedy, Chicago, IL, USA). Two individual samples of subgingival plaque were obtained from the distobuccal sites of two molars in each subject ($n = 40$ samples).

Assessment of antibiotic resistance

Samples were placed in individual tubes containing 5 ml of pre-reduced anaerobically sterilized Ringer's solution supplemented with 0.5 mg/ml L-cysteine (Sigma-Aldrich, St. Louis, MO, USA) and 0.0001% resazurin (Sigma-Aldrich), and sonicated for 10 s under a constant nitrogen flow. Five 10-fold serial dilutions were made and 100 μl of each dilution were plated on two sets of agar plates containing *Mycoplasma* agar base (BBL, Becton-Dickinson, Sparks, MD, USA) with 5% defibrinated sheep blood, 5 μg/ml hemin (Sigma-Aldrich), 0.3 μg/ml menadione (Sigma-Aldrich) and 10 μg/ml *N*-acetyl muramic acid (Sigma-Aldrich), supplemented or not with amoxicillin (Sigma-Aldrich) or doxycycline (Sigma-Aldrich). The antibiotics were tested at six different concentrations (0.5, 1, 2, 4, 8 and 16 μg/ml). Plates were incubated for 7 days at 35°C under anaerobic conditions (80% N_2, 10% CO_2 and 10% H_2). The time between the collection of samples and the incubation of plates did not exceed 40 min. Colony-forming units (CFUs) were visually counted on both the antibiotic-containing and non-antibiotic media in order to determine the proportion of antibiotic resistance in each concentration of every antibiotic tested.

Minimum inhibitory concentrations (MICs) for reference strains

The MICs of amoxicillin and doxycycline for 40 reference strains of subgingival microorganisms were determined. The lyophilized bacterial stocks presented in Table 2 were rehydrated in *Mycoplasma* broth base (BBL). All strains were grown on *Mycoplasma* agar base supplemented with 5% defibrinated sheep blood, 5 μg/ml hemin, 0.3 μg/ml menadione and 10 μg/ml *N*-acetyl muramic acid at 35°C under anaerobic conditions. The growth from 7 day-cultures was harvested and placed in individual tubes containing 1 ml of *Mycoplasma* broth base supplemented with 5 μg/ml hemin, 0.3 μg/ml menadione and 10 μg/ml *N*-acetyl muramic acid. The optical density in each tube was adjusted to 1 at 600 nm in a spectrophotometer. Using a 96-pin stainless-steel replicator (Nalge Nunc, Rochester, NY, USA), each reference strain was transferred in duplicate to agar plates without antibiotic and others containing 0.5, 1, 2, 4, 8, 16, 32, 64 and 128 μg/ml of either amoxicillin or doxycycline. Plates were incubated at 35°C for 7 days under anaerobic conditions. MICs of each antibiotic for every reference strain were determined by visual examination of the bacterial growth.

Data analysis

Descriptive statistics of the subject population including age, number of missing teeth, gender and percentage of current-smokers were calculated and are expressed as mean values ± standard error of the mean (SEM) and range.

The proportion of resistant CFUs in subgingival plaque samples for each concentration of the antibiotics tested was calculated based on the total number of CFUs present in the non-antibiotic-containing plates, which was considered as 100% growth in each sample. Proportions were averaged within samples of each subject and then across the subject population. Data are expressed as mean values ± SEM. Differences in the proportion of CFUs resistant to amoxicillin and doxycycline in each concentration tested were determined using the Wilcoxon test.

Table 1. Characteristics of the subject population ($n = 20$)

	Mean ± SEM	Range
Age (years)	39.1 ± 2.1	28–64
Number of missing teeth	2.8 ± 0.5	0–7
Gender (% females)	55	
% current smokers	30	
Mean pocket depth (mm)	3.3 ± 0.3	1.9–5.9
Mean attachment level (mm)	3.6 ± 0.3	1.9–6.0
% sites with		
Plaque accumulation	29.8 ± 6.5	2.6–9.7
Gingival erythema	3.6 ± 1.8	1.9–40
Bleeding on probing	19.1 ± 3.8	0.6–45.3
Suppuration	3.8 ± 1.2	0–24

SEM, standard error of the mean.

Results

Antibiotic resistance in subgingival plaque samples

The mean proportion (± SEM) of CFUs resistant to different concentrations (0.5, 1, 2, 4, 8 and 16 μg/ml) of doxycycline and amoxicillin in subgingival plaque samples from 20 subjects is summarized in Fig. 1. On average,

Table 2. Reference strains used for the determination of MICs

Species	Strain*	Species	Strain*
Actinobacillus actinomycetemcomitans stp. a	43717	*Gemella morbillorum*	27824
A. actinomycetemcomitans stp. b	43718	*Leptotrichia buccalis*	14201
Actinomyces israelii	12102	*Neisseria mucosa*	19696
A. naeslundii stp. 1	12104	*Peptostreptococcus micros*	33270
A. odontolyticus	17929	*Porphyromonas endodontalis*	35406
A. viscosus	43146	*P. gingivalis*	33277
Campylobacter gracilis	33236	*Prevotella intermedia*	25611
C. rectus	33238	*P. melaninogenica*	25845
C. showae	51146	*P. nigrescens*	33563
Capnocytophaga gingivalis	33624	*Propionibacterium acnes*	6919
C. ochracea	27872	*Selenomonas noxia*	43541
C. sputigena	33612	*Streptococcus anginosus*	33397
Corynebacterium matruchotii	14266	*S. constellatus*	27823
Eikenella corrodens	23834	*S. gordonii*	10558
Eubacterium saburreum	33271	*S. intermedius*	27335
E. sulci	35585	*S. mitis*	49456
Fusobacterium nucleatum ss *nucleatum*	25586	*S. oralis*	35037
F. nucleatum ss *polymorphum*	10953	*S. sanguinis*	10556
F. nucleatum ss *vincentii*	49256	*Tannerella forsythia*	43037
F. periodonticum	33693	*Veillonella parvula*	10790

*Reference strains from the American Type Culture Collection, Rockville, MD, USA.
MICs, minimum inhibitory concentrations.

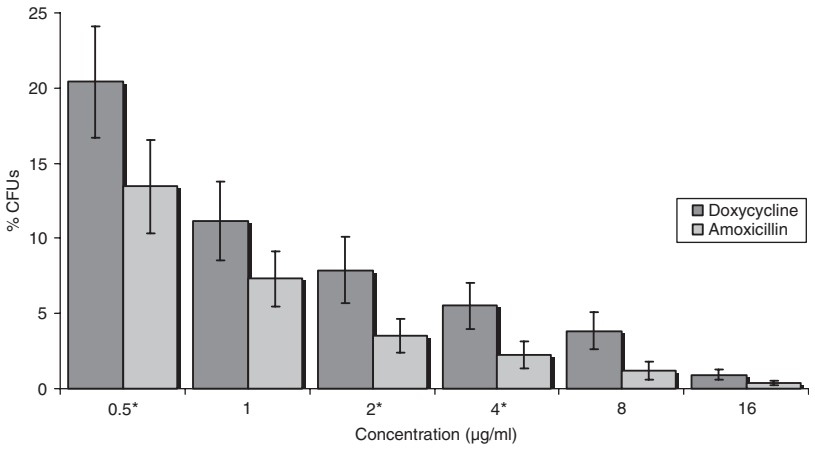

Fig. 1. Bar chart of the mean proportion (± standard error of the mean) of colony-forming units resistant to six concentrations of doxycycline and amoxicillin in subgingival plaque samples from Mexican subjects ($n = 20$). *$p < 0.05$; Wilcoxon signed ranks test.

$0.4 \pm 0.1\%$ to $13.4 \pm 3.1\%$ and $0.9 \pm 0.3\%$ to $20.4 \pm 3.7\%$ of the total cultivable subgingival microbiota was resistant to the concentrations tested of amoxicillin and doxycycline, respectively. At a break-point concentration of 8 μg/ml, $1.2 \pm 0.6\%$ and $3.8 \pm 1.2\%$ of CFUs were resistant to amoxicillin and doxycycline, respectively. A lower proportion of CFUs was resistant to amoxicillin than to doxycycline in all of the concentrations tested. The difference in the proportion of CFUs resistant to these antibiotics, however, was only statistically significant for the 0.5, 2 and 4 μg/ml concentrations ($p < 0.05$).

MICs for reference strains

Table 3 summarizes the MICs of amoxicillin and doxycycline for 40 reference strains. None of the reference strains tested exhibited resistance to both antibiotics. Only *Fusobacterium nucleatum* ss *vincentii* and *F. periodonticum* were resistant to amoxicillin and *Streptococcus constellatus* to doxycycline. No recognized – or putative-periodontal pathogens, such as *Campylobacter rectus*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Actinobacillus actinomycetemcomitans* serotypes a and b, *Peptostreptococcus micros*, *Selenomonas noxia*, *Eikenella corrodens* and *Tannerella forsythia*, were resistant to either antibiotic tested.

Discussion

The present study evaluated the proportion of the total cultivable subgingival microbiota resistant to amoxicillin and doxycycline in 20 Mexican subjects, and determined the MICs for 40 type strains in order to obtain a presumptive reference in terms of the identity of resistant species. To our knowledge, this is the first report in which antimicrobial resistance in subgingival plaque samples has been examined in the Mexican population. A low proportion of the subgingival microbiota was resistant to both amoxicillin and doxycycline. However, amoxicillin appeared to inhibit bacterial growth more efficiently than doxycycline in all of the concentrations tested. In accordance with our findings, a number of studies have reported that amoxicillin is capable of inhibiting ~95% of the microbial species recovered from subgingival plaque samples (Walker et al. 1983, Pacini et al. 1997, van Winkelhoff et al. 2000, Feres et al. 2002). Considering a break-point concentration of 8 μg/ml, only 1.2% of the total cultivable subgingival microbiota from Mexican subjects was resistant to amoxicillin, whereas 3.8% was resistant to doxycycline. Furthermore, of the 40 reference strains tested, only *F. nucleatum* ss *vincentii* and *F. periodonticum* were resistant to amoxicillin, and *S. constellatus* to doxycycline.

Amoxicillin reaches high concentrations (~ 1.5–14 μg/ml) in gingival crevicular fluid (GCF; Walker et al. 1983, Gordon & Walker 1993). The results of a number of studies have indicated that the use of systemic amoxicillin alone or in combination with other antibiotic agents as an adjunct in the treatment of periodontal infections may result in enhanced improvement of periodontal clinical parameters (Haffajee et al. 1995, Winkel et al. 2001, Rooney et al. 2002). Tetracyclines, and in particular doxycycline, are some of the most commonly used antibiotics for the treatment of periodontal diseases (Feres et al. 1999a, Ramberg et al. 2001). Their clinical use is based in part on the high concentrations reached in GCF (1–8 μg/ml; Gordon et al. 1981, Sakellari et al. 2000), and on other properties considered of value in the management of

Table 3. MICs of 40 reference strains

Species	Amoxicillin		Doxycycline	
	MIC (μg/ml)	S/R*	MIC (μg/ml)	S/R*
Actinobacillus actinomycetemcomitans a	2	S	8	S
A. actinomycetemcomitans b	4	S	4	S
A. israelii	0.5	S	0.5	S
A. naeslundii	0.5	S	0.5	S
A. odontolyticus	0.5	S	1	S
A. viscosus	0.5	S	0.5	S
Campylobacter gracilis	1	S	2	S
C. rectus	1	S	0.5	S
C. showae	0.5	S	0.5	S
Capnocytophaga gingivalis	0.5	S	0.5	S
C. ochracea	1	S	0.5	S
C. sputigena	1	S	0.5	S
Corynebacterium matruchotii	0.5	S	0.5	S
Eikenella corrodens	2	S	1	S
Eubacterium saburreum	0.5	S	0.5	S
E. sulci	0.5	S	0.5	S
Fusobacterium nucleatum ss *nucleatum*	0.5	S	0.5	S
F. nucleatum ss *polymorphum*	0.5	S	0.5	S
F. nucleatum ss *vincentii*	16	R	0.5	S
F. periodonticum	32	R	0.5	S
Gemella morbillorum	0.5	S	0.5	S
Leptotrichia buccalis	0.5	S	0.5	S
Neisseria mucosa	2	S	0.5	S
Peptostreptococcus micros	0.5	S	0.5	S
Porphyromonas gingivalis	0.5	S	0.5	S
P. endodontalis	4	S	4	S
Prevotella intermedia	0.5	S	0.5	S
P. melaninogenica	0.5	S	0.5	S
P. nigrescens	0.5	S	0.5	S
Propionibacterium acnes	0.5	S	0.5	S
Selenomonas noxia	0.5	S	0.5	S
Steptococcus anginosus	0.5	S	2	S
S. constellatus	0.5	S	32	R
S. gordonii	0.5	S	0.5	S
S. intermedius	1	S	0.5	S
S. mitis	0.5	S	0.5	S
S. oralis	0.5	S	0.5	S
S. sanguinis	1	S	0.5	S
Tannerella forsythia	8	S	0.5	S
Veillonella parvula	0.5	S	1	S

*Sensitive/resistant, based on a break-point concentration of 8 μg/ml.
MICs, minimum inhibitory concentrations.

periodontal diseases, such as their anti-inflammatory and tissue collagenase-activity-inhibiting effects (Plewig & Schopf 1975, Golub et al. 1984, Rifkin et al. 1993). Reports of the clinical effectiveness of doxycycline have shown that its use in periodontal therapy, as an adjunct to mechanical treatments, may result in significant improvement of various clinical parameters of disease measurement (Feres et al. 1999a, Ramberg et al. 2001).

The widespread use of antibiotics in dentistry has led to concern that frequent and repeated exposure of commensal and pathogenic bacteria to such drugs may result in increased microbial resistance (Olsvik et al. 1995, Palmer et al. 2000, Ready et al. 2002, Addy & Martin 2003). This is particularly the case in populations in which antibiotic agents are sold without prescription, potentially leading to greater misuse and overexposure of individuals to such agents (Baquero et al. 1991, Cullmann 1996, Pradier et al. 1997). While it is generally considered that individuals in Mexico, as well as in other countries in Latin America, may be overexposed to certain antimicrobial agents due to limited strategies of expenditure and usage control (Calva et al. 1993, Leyva 1999), few studies have evaluated antimicrobial resistance patterns in the Mexican population, or compared such patterns with other populations in which antibiotic usage is adequately controlled (Hernandez-Porras et al. 2004, Lopez-Merino et al. 2004, Quinones-Falconi et al. 2004, Chihu et al. 2005, Estrada-Garcia et al. 2005). Furthermore, none of these studies have evaluated microbial resistance in subgingival plaque samples.

In a comparative study of antimicrobial resistance in subgingival plaque samples from Spanish and Dutch individuals, it was reported that subjects from Spain, where there is widespread use of antibiotics, exhibited a significantly higher percentage of resistance to tetracycline and amoxicillin ($\sim$ 10% and $\sim$2%, respectively) than subjects from the Netherlands ($\sim$ 1.5% and $<$1%, respectively) where antibiotic usage is better controlled (van Winkelhoff et al. 2000). In North-American populations (from the United States of America), proportions of microbial resistance to doxycycline of 1.6–6% (Feres et al. 1999b, Walker et al. 2000) and to amoxicillin of 0.5–1% (Walker et al. 2000, Feres et al. 2002) of the total cultivable subgingival microbiota have been reported. Similarly, proportions of subgingival resistance to tetracycline and doxycycline of 4% and 7% were reported in individuals from Denmark and Brazil, respectively (Fiehn & Westergaard 1990, Rodrigues et al. 2004). Furthermore, several of the above studies reported that while the proportion of resistance increased during antibiotic intake, it returned to baseline levels 3–12 months after exposure. Our results revealed a low proportion of resistance to both amoxicillin and doxycycline in subgingival plaque samples from Mexican subjects, comparable to that reported in populations assumed to be exposed to such drugs on a limited basis. These findings suggest that, if in fact the Mexican population is overexposed to certain antibiotics, the exposure to amoxicillin and doxycycline, in particular, may not be significantly greater than in other regions of the world such as the Netherlands, Denmark and the United States.

The present study was based on MIC values of whole-plaque samples dispersed and grown on agar plates. Thus, our results, as those from other studies that have used similar methodologies, reflect antimicrobial resistance or sensitivity of rapidly growing planktonic bacterial cells. In dental plaque, bacteria are organized in a biofilm structure and exhibit a very low metabolism, which is

partly responsible for the increased antibiotic resistance observed by bacterial species that colonize such structures (Socransky & Haffajee 2002, Haffajee et al. 2003a). Therefore, while our findings may provide valuable information regarding antimicrobial resistance in subgingival plaque samples from the Mexican population, it is difficult to extrapolate them directly to a clinical setting and to ascertain the degree to which antibiotic resistance patterns by the same bacterial species may differ in vitro and in biofilms. Further studies are required to broaden the information on antimicrobial resistance by subgingival bacterial species, which may enable the establishment of more specific parameters for antibiotic usage in the treatment of periodontal diseases in the Mexican population. However, our results revealed that a significant proportion of the cultivable subgingival microbiota was sensitive in vitro to either amoxicillin or doxycycline. Thus, these antibiotics could be effective, when such agents are indicated, in the treatment of periodontal infections in Mexico.

Acknowledgements

This study was supported in part by research grants J34909-M (CONACYT) and IN205402 (PAPIIT, DGAPA, UNAM), both to Dr. Ximenez-Fyvie.

The authors wish to acknowledge the clinical support provided by Drs. Magdalena Paulín-Pérez and Guadalupe Marín-González of the Periodontology Department of the Division of Postgraduate Studies and Research of the School of Dentistry of UNAM.

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Clinical Relevance

Scientific rationale for study: Microbial resistance patterns may differ significantly in populations around the world. The study of antibiotic resistance in subgingival samples from individuals in different locations may provide a better understanding of the potential effectiveness of antibiotics in the treatment of periodontal diseases.

Principal findings: A small proportion of the total cultivable subgingival microbiota in plaque samples from Mexican subjects was resistant to amoxicillin and doxycycline.

Practical implications: Our results suggested that amoxicillin and doxycycline may be effective in inhibiting the growth of a highly significant proportion of microorganisms in subgingival plaque samples from Mexican subjects.