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Antibiotic resistance profile of the subgingival microbiota following systemic or local tetracycline therapy

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Abstract

Background: Tetracyclines have been extensively used as adjunctives to conventional periodontal therapy. Emergence of resistant strains, however, has been reported. This study evaluated longitudinally the tetracycline resistance patterns of the subgingival microbiota of periodontitis subjects treated with systemic or local tetracycline therapy+scaling and root planing (SRP).

Methods: Thirty chronic periodontitis patients were randomly assigned to three groups: SRP+500 mg of systemic tetracycline twice/day for 14 days; SRP alone and SRP+tetracycline fibers (Actsite®) at four selected sites for 10 days. Subgingival plaque samples were obtained from four sites with probing pocket depths (PPD) $\geqslant$ 6 mm in each patient at baseline, 1 week, 3, 6 and 12 months post-therapy. Samples were dispersed and diluted in pre-reduced anaerobically sterilized Ringer's solution, plated on Trypticase Soy Agar (TSA)+5% blood with or without 4 μg/ml of tetracycline and incubated anaerobically for 10 days. The percentage of resistant microorganisms were determined and the isolates identified by DNA probes and the checkerboard method. Significance of differences among and within groups over time was sought using the Kruskal–Wallis and Friedman tests, respectively.

Results: The percentage of resistant microorganisms increased significantly at 1 week in the tetracycline groups, but dropped to baseline levels over time. The SRP+Actsite® group presented the lowest proportions of resistant species at 6 and 12 months. No significant changes were observed in the SRP group. The predominant tetracycline-resistant species included *Streptococcus* spp., *Veillonela parvula*, *Peptostreptococcus micros, Prevotella intermedia, Gemella morbillorum* and *Actinobacillus actinomycetemcomitans (Aa)*. A high percentage of sites with resistant *Aa*, *Porphyromonas gingivalis* and *Tanerella forsythensis* was observed in all groups at baseline. However, *T. forsythensis* was not detected in any group and *P. gingivalis* was not present in the SRP+Actsite® group at 1 year post-therapy. *Aa* was still frequently detected in all groups after therapy. However, the greatest reduction was observed in the SRP+Actsite® group.

Conclusion: Local or systemically administered tetracycline results in transitory selection of subgingival species intrinsically resistant to this drug. Although the percentage of sites harboring periodontal pathogens resistant to tetracycline were quite elevated in this population, both therapies were effective in reducing their prevalence over time.

Key words: antimicrobial resistance; local drug delivery; periodontal therapy; periodontitis; randomized controlled trial; subgingival microbiota; tetracycline

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Over the years, evidence supporting the microbial etiology of periodontal diseases has been documented (Haffajee & Socransky 1994). Studies have revealed that most forms of periodontal diseases are treated predictably by conventional periodontal therapy and periodontal health can be maintained for a long period of time with proper maintenance care programs (Greenstein 2000). Nevertheless, recolonization of the subgingival area with pathogens frequently occurs following treatment, which may result in recurrence of disease (Sbordone et al. 1990). Particularly in the cases of conventional treatment failure and more aggressive diseases, antimicrobial agents have been used as adjuncts to mechanical therapy (van Winkelhoff et al. 1996, Walter & Karpinia 2002). The effective use of these agents requires an adequate drug concentration at the site of infection for sufficient period of time to allow for the agent to eliminate the periodontal pathogens (van Winkelhoff et al. 1996). In addition, periodontal infections may require different antibiotic regimens for a favorable clinical treatment outcome (Haffajee et al. 1996). Systemically administered antibiotics have been successfully used in combination with scaling and root planing (van Winkelhoff et al. 1996, Walter & Karpinia 2002). Alternatively, several local delivery devices have been developed for the topical application of different antimicrobial agents into the periodontal pocket (Killoy 2002). These systems present advantages over the systemic administration, such as a dramatically increased and sustained drug concentration in the crevicular fluid, as well as a reduction of systemic undesirable side effects (Rams & Slots 1996). Systemic and locally administered tetracyclines, as well as its derivatives doxycycline and minocycline, are the most commonly used antimicrobial agents in the treatment of periodontal infections (Goodson 1994, Rams & Slots 1996, van Winkelhoff et al. 1996). Several studies have demonstrated a suppression of the pathogenic subgingival microbiota during and after tetracycline administration associated with clinical improvement (Christersson & Zambon 1993, Haffajee et al. 1995, Michalowicz et al. 1995, Walker et al. 2000). However, others have shown that tetracycline failed to completely eliminate periodontal pathogens from periodontal pockets (Asikainen et al. 1990, Maiden et al. 1991, Mombelli et al. 1996, Feres et al. 1999a, Wong et al. 1999).

One of the major causes for treatment failure is the emergence of resistant human pathogens as a result of the widespread use of antibiotics in medicine and dentistry (Walker 1996, van Winkellhoff et al. 1997). Subgingival species resistant to commonly used antibiotics have been isolated from subgingival biofilms (Walker 1996). In addition, an increase in resistance of periodontal species to tetracycline has been reported by several investigators (Fiehn & Westergaard 1990, Olsvik & Tenover 1993, Olsvik et al. 1995a, b, Walker 1996, van Winkelhoff et al. 2000, Walker et al. 2000). In particular, the long-term use of tetracycline seems to favor the development of resistant strains in periodontal pockets (Kornman & Karl 1982). Other studies evaluated the resistance of periodontal bacteria after local administration of tetracycline (Larsen 1991, Goodson & Tanner 1992, Wade et al. 1992). Various mechanisms of resistance to tetracycline have been recognized on the genetic level in oral microorganisms (Olsvik & Tenover 1993). At least 27 different tetracycline resistance genes have been described in a number of periodontal species, such as oral streptococci, *Veilonella* spp., *Actinomyces* spp., *Peptostreptococcus anaerobius* and *Fusobacterium nucleatum* strains [*tet*(M)]; species of *Prevotella*, *Capnocytophaga* [*tet*(Q)], *Porphyromonas* [*tet*(Q); *tet*(W)] and *Eubacterium* [*tet*(K)] (Roberts & Lansciardi 1990, Speer et al. 1992, Olvisk et al. 1995a, b, 1996, Lacroix & Walker 1995, 1996, Roberts 2002). These data support the fact that the presence of tetracycline-resistant determinants in conjugal elements in several bacterial strains in the oral cavity and further spread of these elements with emergence of multresistant species is evident. Thus, antimicrobial agents should be reserved for use in refractory and/or agressive forms of periodontal disease. In addition, susceptibility testing of the subgingival microbiota should be indicated prior to antibiotic administration (Olsvik & Tenover 1993). Based on that, the present investigation evaluated the changes in the tetracycline resistance profile of the subgingival microbiota in subjects treated with local or systemic tetracycline combined with scaling and root planing (SRP) up to 1 year post-therapy. Tetracycline-resistant species were identified by DNA probes and the checkerboard DNA–DNA hybridization method.

Material and Methods

Subject population

Thirty chronic periodontitis patients (mean age 46 ± 11 years) from the Dental School at the Federal University of Rio de Janeiro (UFRJ) were selected. All subjects had at least 20 teeth and four sites with probing pocket depths (PPD) $\geqslant 6$ mm at baseline. Exclusion criteria included pregnancy, nursing, allergy to tetracycline, systemic conditions that could affect the progression or treatment of periodontal diseases, any type of periodontal intervention and use of antibiotics 6 months prior to the beginning of the investigation. In order to participate in the study, all patients were informed about the nature of the study and a signed consent form was obtained from each individual. The study protocol was approved by the Review Committee for Human Subjects of the University Hospital Clementino Fraga Filho (UFRJ).

Clinical monitoring

Full-mouth clinical measurements, including PPD and clinical attachment level (CAL) measured with a conventional North Carolina periodontal probe (Hu-Friedy, Chicago, IL, USA), as well as presence or absence of visible plaque and bleeding on probing (BOP) were performed at six sites per tooth at all teeth in all patients, excluding third molars, at baseline, 3, 6 and 12 months post-therapy. The clinical monitoring and treatment were performed by two calibrated periodontists. One operator performed all clinical measurements in a group of 15 randomly selected patients blind to the treatment delivered by the other, and vice versa.

Therapeutic procedures

The selected subjects were randomly assigned to three groups, including 10 subjects in each group. After initial clinical and microbiological assessments, group 1 received full-mouth SRP plus systemically administered tetracycline (Tetraciclina, INFABRA, Rio de Janeiro, Brazil), 500 mg twice/day for 14 days, starting on the first day of mechanical therapy (SRP+Syst tet). Group 2 received full-mouth SRP only

and group 3 received full mouth SRP followed by placement of tetracycline fibers (Actsite®, ALZA Corporation, Palo Alto, CA, USA) at four randomly selected non-adjacent sites with PPD ⩾ 6 mm for 10 days (SRP+Tet fib). Patients in this group were instructed to rinse twice daily with 0.12% chlorexidine solution (Periogard®, Colgate, São Paulo, Brazil) for 2 weeks. SRP was performed under local anesthesia in two to four weekly sessions of approximately 1 h. All patients received maintenance therapy, including supragingival prophylaxis and reinforcement in home care procedures during the post-therapy period. No subgingival interventions other than clinical measurements were performed at the maintenance visits.

Microbiological assessment

In each group, four non-adjacent sites with PPD ranging from 6 to 10 mm were selected for microbiological monitoring. Individual subgingival plaque samples were taken from the 4 selected sites in each subject using sterile Gracey curettes (Hu-Friedy), at baseline, 1 week, 3, 6 and 12 months post-therapy. The samples were dispersed and serially diluted in pre-reduced anaerobically sterilized (PRAS) Ringer's solution under a flow of O_2-free gas. Following that, they were plated on two sets of plates containing 2% of Trypticase Soy Agar (BBL, Becton Dickinson, Cockeysville, MD, USA), 5% sheep blood (Fazenda Pig, Rio de Janeiro, RJ, Brazil), 1% yeast extract (BBL), 5 μg/ml hemin (Sigma Chemical Co., St Louis, MO, USA), 0.3 μg/ml menadione and 10 μg/ml *N*-acetylmuramic acid (Sigma), with or without 4 μg/ml of tetracycline HCl (Sigma), and incubated in anaerobic atmosphere for 7–10 days. Colonies on plates with and without tetracycline were counted to determine the percentage of resistant microorganisms in that sample. This direct plating method used to determine the proportion of resistant isolates has been validated by comparison with the National Committee on Clinical Laboratory Standards (NCCLS) (Feres et al. 1999b). Bacterial colonies on antibiotic-containing plates were washed off with 1 ml of TE buffer (10 mM Tris-HCL, 1 mM EDTA, pH 7.6). The suspensions were placed into individual Eppendorf tubes, sonicated for 10 s and adjusted to an optical density of 1.0 (10^9 cells). Ten microliters of the suspension (10^7 cells) were taken and placed in another tube with 140 μl of TE buffer and 100 μl of 0.5 M of NaOH. The presence of 32 subgingival species (Table 1) were determined by the checkerboard DNA–DNA hybridization method (Socransky et al. 1994). In brief, the samples were lysed and denatured DNA fixed in individual lanes on a nylon membrane (Boehringer Mannheim, Indianapolis, IN, USA) using the checkerboard slot blot devices (Minislot 30, Miniblotter 45, Immunetics, Cambridge, MA, USA). Thirty-two digoxigenin-labeled (Boehringer Mannheim) whole genomic DNA probes were hybridized at 90° to the lanes of the plaque samples in the miniblotter apparatus. Probes for *Actinobacillus actinomycetemcomitans* serotypes a and b were combined. After hybridization, the membranes were washed at high stringency and bound probes were detected using phosphatase-conjugated antibody to digoxigenin and chemiluminescence (Boehringer Mannheim). Signals were detected on X-ray films and evaluated visually by comparison with the standards at 10^5 and 10^6 bacterial cells for the test species on the same membrane. The sensitivity of this assay was adjusted to permit detection of 10^4 cells of a given species by adjusting the concentration of each DNA probe. This procedure was carried out in order to provide the same sensitivity of detection for each species. Failure to detect a signal was recorded as zero, although conceivably, counts in the 1–1000 ranges could have been present.

Statistical analysis

The statistical program SPSS, Statistical Package for the Social Sciences, version 9.0 (Chicago, IL, USA) was used for all the analyses. Statistical testing was performed on a ''subject-based'' assessment. Baseline clinical measurements were averaged for all sites in each subject and then across subjects within each group. Significance of differences in baseline clinical parameters among groups were sought using the Kruskal–Wallis test. The mean percentage of antibiotic-resistant isolates was computed by averaging these values within a subject and then in each group at each visit. Likewise, the prevalence of each species identified by DNA probes was computed for each subject and across subjects in the groups. Significance of

Table 1. Subgingival species used for whole genomic DNA probes tested against tetracycline-resistant isolates

Species	Strains*	Species	Strains*
Actinobacillus actinomycetemcomitans a	43718	*Neisseria mucosa*	19696
Actinobacillus actinomycetemcomitans b	29523	*Peptostreptococcus micros*	33270
Actinomyces naeslundii I	12104	*Porphyromonas gingivalis*	33277
Actinomyces naeslundii II (*A. viscosus*)	43146	*Prevotella intermedia*	25611
Actinomyces israelii	12102	*Prevotella nigrescens*	33563
Actinomyces odontolyticus	17929	*Prevotella melaninogenica*	25845
Actinomyces gerencseriae	23860	*Selenomonas noxia*	43541
Capnocytophaga gingivalis	33624	*Streptococcus oralis*	35037
Campylobacter rectus	33238	*Streptococcus sanguis*	10556
Campylobacter showae	51146	*Streptococcus constellatus*	27823
Eikenella corrodens	23834	*Streptococcus gordonii*	10558
Fusobacterium periodonticum	33693	*Streptococcus mitis*	49456
Fusobacterium nucleatum ss. nucleatum	25586	*Streptococcus intermedius*	27335
Fusobacterium nucleatum ss.polymorphum	10953	*Streptococcus anginosus*	33397
Fusobacterium nucleatum ss. vincentii	49256	*Tanerella forsythensis* (*B. forsythus*)	43037
Gemella morbillorum	27824	*Veillonella parvula*	10790
Leptotrichia buccalis	14201		

*ATCC, American Type Culture Collection, Rockville, MD, USA.

differences in these microbiological parameters among groups at each time point was tested using the Kruskal–Wallis test, while differences within groups over time were determined by the Friedman test. Statistical significance was determined at an $\alpha = 0.05$.

Results

The baseline clinical features of the three therapeutic groups are shown in Table 2. The group that received SRP+systemic tetracycline presented a greater proportion of males and fewer smokers than the other two groups. Nevertheless, no significant differences among the groups for any of the clinical parameters evaluated were observed.

Fig. 1 shows the mean % of microorganisms resistant to 4 μg/ml of tetracycline in the 3 treatment groups at each time point. At baseline, no significant difference in mean % of resistant isolates was observed among groups, although subjects in the control group (SRP) presented the lowest proportion (7%). On the other hand, significant differences among groups were seen at 1 week ($p = 0.015$; Kruskal–Wallis test) and 6 months ($p = 0.023$; Kruskal–Wallis test) post-therapy. The tetracycline groups showed a significantly higher proportion of resistant microorganisms than the control group 1 week after therapy, while the tetracycline fiber group presented the lowest proportion at 6 months. At 3 and 12 months, a greater mean percent of resistant microorganisms was observed for the SRP+systemic tetracycline group in comparison to the others, however this difference was not statistically significant. The mean percentage of resistant isolates did not change significantly in plaque samples of subjects in the SRP group over time ($p = 0.582$; Friedman test). Subjects receiving systemic antibiotic showed a marked increase in the percentage of resistant isolates at 1 week, returning gradually to baseline levels one year after therapy ($p = 0.147$; Friedman test). Significant changes were observed in the tetracycline fiber group over time. The mean percentage of resistant microorganisms increased to about 43% at 1 week, dropping significantly below baseline levels, at 6 and 12 months after treatment ($p = 0.001$; Friedman test). The significant differences over time were seen between 1 week and all the other time points ($p < 0.01$; Wilcoxon signed-rank test; data not shown). The percentage of sites harboring tetracycline-resistant subgingival species was evaluated. Since the prevalence of most of the microorganisms did not differ significantly among treatment groups (40 sampled sites per group; data not shown) at each visit, the data was combined for all subjects. Table 3 presents the frequency of detection of species resistant to 4 μg/ml of tetracycline in a total of 120 sites (30 subjects × four subgingival sampled sites) at each time point. The frequency of detection of most of the resistant species diminished significantly over time. In particular, *Tanerella forsythensis*, *Capnocytophaga gingivalis*, *F. nuc. ss. vincentii*, *Neisseria mucosa*, *Prevotella nigrescens* and *Prevotella melaninogenica* were not detected in any sample one year post-therapy, while *P. micros* was not observed in any subject of the tetracycline fiber group (data not shown). Overall, *Streptococcus* spp., *Veillonella parvula*, *Peptostreptococcus micros, Prevotella intermedia, Gemella morbillorum* and *A. actinomycetemcomitans* were the most predominant resistant species before and after treatment. Although tested, *Leptotrichia buccalis* was not detected in any sample and/or visit. Of interest was the high proportion of sites presenting the pathogens *A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythensis* resistant to 4 μg/ml of tetracycline observed in all three groups at baseline (Figs 2A–C). Nevertheless, at one year post-therapy *T. forsythensis* was not detected in any group (Fig. 2B). Resistant *P. gingivalis* was present in 28% of subject sites in the SRP group, but in only 10% and

Table 2. Mean (± SEM) clinical features of subjects in the three treatment groups at baseline

	SRP+Syst tet $N = 10$	SRP $N = 10$	SRP+Tet fib $N = 10$	p
age (years)	48.3 ± 4.5	46.5 ± 2.6	45 ± 3.0	NS
percentage of smokers	10	20	20	NS
percentage of males	60	40	30	NS
attachment level (mm)	3.53 ± 0.4	3.8 ± 0.3	3.63 ± 0.2	NS
pocket depth (mm)	3.12 ± 0.2	3.4 ± 0.3	3.37 ± 0.2	NS
% sites with:				
plaque	58.0 ± 8.0	57.8 ± 9.2	53.9 ± 6.6	NS
bleeding on probing	35.1 ± 6.2	46.5 ± 6.7	31.3 ± 4.2	NS
suppuration	3.2 ± 1.9	2.0 ± 1.1	0.07 ± 0.04	NS

SRP+Syst tet, scaling and root planing+systemic tetracycline; SRP, scaling and root planing only; SRP+Tet fib, scaling and root planing+tetracycline fibers/Actisite®; p, refers to Kruskal–Wallis test; NS, no significance.

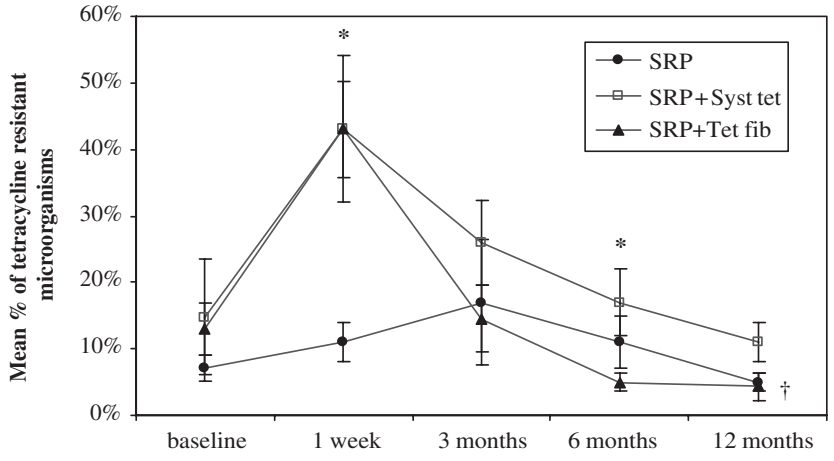

Fig. 1. Mean % (± SEM) of isolates resistant to 4 μg/ml of tetracycline in plaque samples from subjects in the SRP+Syst tet (scaling and root planing+systemic tetracycline; $N = 10$), SRP (scaling and root planing only; $N = 10$) and SRP$_+$Tet fib (scaling and root planing+tetracycline fibers/Actisite®; $N = 10$) groups at baseline (0), 1 week, 3, 6 and 12 months post-therapy. Data was averaged across subjects in each group for each visit. Significance of differences among groups at each time point was tested using the Kruskal-Wallis test ($^*p < 0.05$). Differences over time were evaluated by the Friedman test ($^\dagger p < 0.01$).

Table 3. Proportion of sites colonized by species resistant to 4 μg/ml tetracycline at each visit in a total of 120 sampled sites from subjects of all three therapeutic groups ($N = 30$)

	Baseline	1 week	3 months	6 months	12 months
*A. actinomycetemcomitans**	77 ± 7	74 ± 8	66 ± 8	45 ± 9	28 ± 9
*A. naeslundii I**	71 ± 7	58 ± 8	37 ± 8	26 ± 7	28 ± 9
A. naeslundii II (*A. viscosus*)*	50 ± 10	47 ± 11	35 ± 10	24 ± 8	16 ± 7
A. israelii	22 ± 9	30 ± 10	14 ± 6	8 ± 4	20 ± 9
*A. odontolyticus**	14 ± 6	17 ± 6	26 ± 7	12 ± 4	40 ± 8
A. gerencseriae	50 ± 9	30 ± 9	32 ± 8	21 ± 7	20 ± 7
*C. gingivalis**	33 ± 10	32 ± 9	32 ± 9	5 ± 4	0
C. rectus	29 ± 9	30 ± 9	22 ± 7	7 ± 4	10 ± 6
C. showae	26 ± 9	28 ± 9	20 ± 7	3 ± 2	6 ± 4
E. corrodens	51 ± 8	38 ± 8	31 ± 8	25 ± 7	56 ± 10
*F. periodonticum**	64 ± 9	59 ± 9	30 ± 8	7 ± 3	16 ± 6
*F. nuc. ss. nucleatum**	64 ± 8	53 ± 9	39 ± 9	26 ± 7	34 ± 8
*F. nuc. ss.polymorphum**	66 ± 8	62 ± 9	38 ± 9	19 ± 7	8 ± 5
*F. nuc. ss. vincentii**	47 ± 9	36 ± 9	23 ± 7	10 ± 5	0
G. morbillorum	84 ± 6	73 ± 7	70 ± 8	38 ± 8	54 ± 9
N. mucosa	50 ± 8	36 ± 8	20 ± 8	3 ± 3	0
*P. micros**	84 ± 6	90 ± 4	77 ± 7	50 ± 9	18 ± 7
*P. gingivalis**	75 ± 8	74 ± 8	50 ± 9	24 ± 8	12 ± 7
P. intermedia	63 ± 8	54 ± 9	66 ± 8	34 ± 7	48 ± 9
*P. nigrescens**	38 ± 8	28 ± 7	66 ± 8	17 ± 7	0
P. melaninogenica	21 ± 7	13 ± 5	12 ± 5	7 ± 4	0
*S. noxia**	70 ± 9	48 ± 9	73 ± 7	28 ± 8	20 ± 7
S. oralis	95 ± 4	90 ± 5	79 ± 7	72 ± 7	72 ± 8
S. sanguis	62 ± 12	36 ± 11	80 ± 7	62 ± 8	66 ± 8
*S. constellatus**	85 ± 6	74 ± 7	77 ± 6	55 ± 8	38 ± 10
S. gordonii	86 ± 6	78 ± 6	78 ± 6	78 ± 6	82 ± 6
*S. mitis**	87 ± 5	68 ± 8	64 ± 8	45 ± 9	16 ± 7
S. intermedius	82 ± 7	80 ± 7	84 ± 5	66 ± 7	74 ± 7
*S. anginosus**	93 ± 3	72 ± 8	71 ± 8	57 ± 9	12 ± 7
*T. forsythensis**	43 ± 8	25 ± 7	18 ± 6	14 ± 6	0
*V. parvula**	85 ± 6	84 ± 6	59 ± 8	41 ± 7	24 ± 7

*Significant changes in percentage of sites with a species resistant to tetracycline over time ($p < 0.01$; Friedman test).

none of the subjects in the systemic tetracycline and fiber groups. The decrease in frequency over time for this species was significant in both tetracycline groups ($p < 0.05$; Friedman test). At 3 months after therapy, the systemic antibiotic group showed a higher proportion of sites with resistant *P. gingivalis* than the other groups ($p < 0.05$; Kruskal–Wallis test; Fig. 2A). Resistant *A. actinomycetemcomitans* was still detected in all three groups after therapy, however in lower frequency. The greatest reduction in prevalence was noted in the subjects who received the local antibiotic ($p < 0.05$; Friedman test). This group had a lower prevalence of resistant members of this species than the other groups at 6 months post-therapy ($p < 0.03$; Kruskal–Wallis test; Fig. 2C).

Discussion

The rationale for the use of systemic and/or local antibiotics in the treatment of periodontal infections is to rapidly suppress periodontal pathogens and favor the establishment of a host compatible microbiota. However, due to their overuse and misuse worldwide, antibiotic resistance has become a serious medical issue (Walker 1996). For the past two decades, the most commonly used antibiotic in periodontal treatment has been the tetracyclines (van Winkelhoff et al. 1996, Walter & Karpinia 2002). The widespread appearance of tetracycline resistance in medically important bacteria has limited their use in the treatment of medical infections (Speer et al. 1992). In the oral cavity, tetracycline resistance has been increasing in the last years (Walker 1996). This may be one of the causes of treatment failure of periodontitis when using this drug, as reported by some investigators (Asikainen et al. 1990, Mombelli et al. 1996, Colombo et al. 1998, Wong et al. 1999). The present study evaluated the changes in tetracycline resistance patterns of the subgingival microbiota from periodontitis subjects treated with local or systemic tetracycline plus SRP therapy over a period of 1 year. Given the indiscriminate use of tetracyclines in Brazil, we expected to see higher proportions of tetracycline-resistant microorganisms in this sample population prior to antibiotic administration. However, our data showed that the mean baseline percentage of isolates resistant to 4 μg/ml of tetracycline varied from 8% to 15% in all three groups. These results were in accord with Lacroix & Walker (1995) who observed a mean of 12%. Conversely, other studies have demonstrated lower proportions (1–7%) of tetracycline-resistant isolates in periodontally healthy individuals and periodontal patients not treated with antibiotics (Walker et al. 1983, Fiehn & Westergaard 1990, Goodson & Tanner 1992, Feres et al. 1999b, 2002). Regarding the changes in resistance profile after treatment, the current investigation observed a marked short-term increase in the proportions of tetracycline-resistant isolates after local and systemic antibiotic administration. These levels returned to baseline values, though, at one year post-therapy. A similar transient increase in resistance after treatment with systemic or local tetracycline has been documented by other authors (Fiehn & Westergaard 1990, Larsen 1991, Goodson & Tanner 1992, Wade et al. 1992, Feres et al. 1999b, 2002, Walker et al. 2000). Interestingly, the group that received local tetracycline therapy (Actsite®) presented the most significant reduction in the mean percentage of tetracycline-resistant isolates among the groups, whereas the systemic tetracycline therapy showed the greatest proportion of those at one year after treatment. Studies indicate that exposure to sub-inhibitory concentrations of tetracycline, especially for longer periods of time, poses a greater risk of development and persistence of resistant bacteria (Kornman & Karl 1982). It is conceivable that lower levels of this drug in other habitats within the oral cavity, such as tongue, cheek, tonsils, supragingival plaque, as well as saliva (Sakellari et al. 2000), after systemic tetracycline administration may select for more resistant microorganisms in those sites, favoring, therefore, recolonization by these species (Sbordone et al. 1990). As suggested by Feres et al. (1999b), it seems likely that the increased proportion of resistant isolates on the soft tissues and saliva might

result from a greater proportion of *Streptococcus* spp., *Veillonella* spp. and *Actinomyces* spp., species intrinsically resistant to tetracycline, in these areas. On the other hand, tetracycline fibers seemed to be more effective in reducing the proportion of resistant species probably due to the extremely high antibiotic concentration in the periodontal site. No significant changes in the percentage of tetracycline-resistant microorganisms were observed in the subgingival plaques of subjects treated with SRP only. A slight increase was noticed at 3 months, probably due to the selection and increase of beneficial species (Cugini et al. 2000), usually resistant to tetracyclines (Lacroix & Walker 1995, Olvisk et al. 1995a, b).

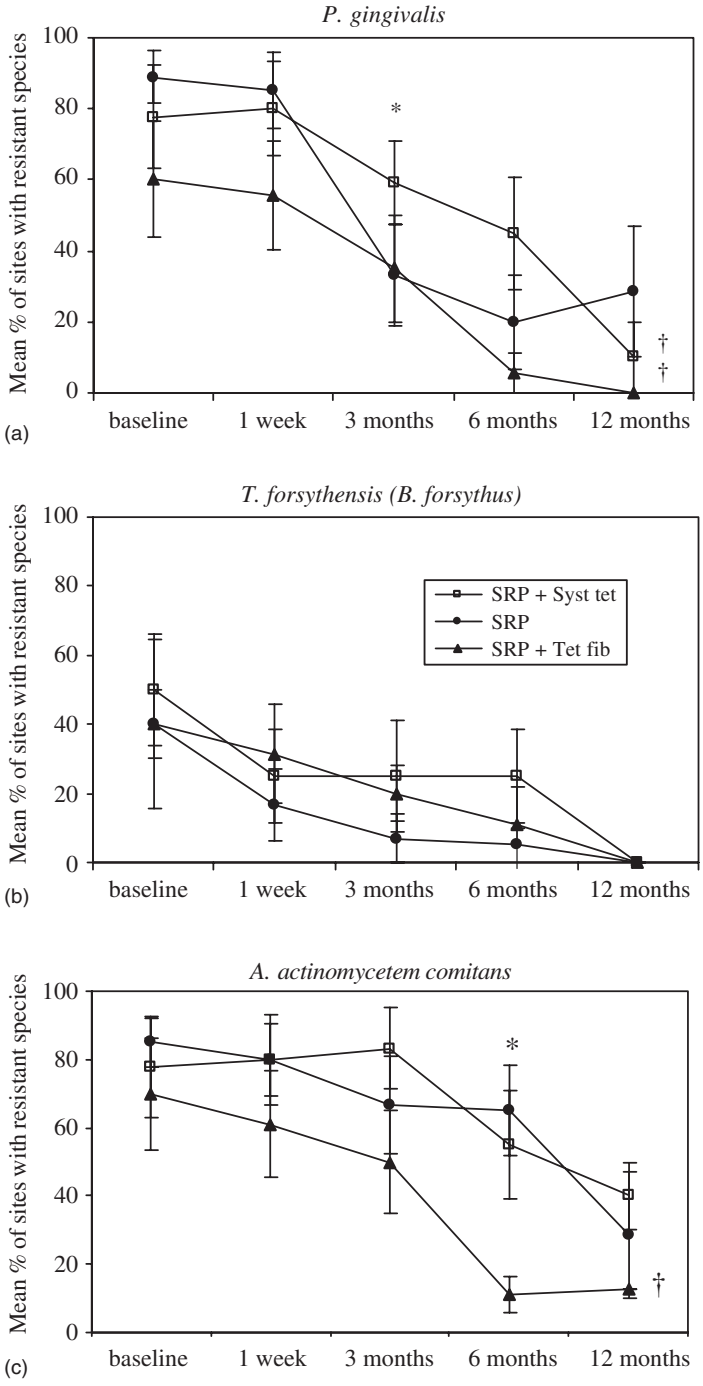

Fig. 2. Mean % (± SEM) of sites colonized by species of *P. gingivalis* (A), *T. forsythensis (B. forsythus)* (B) and *A. actinomycetemcomitans* (C) resistant to 4 μg/ml of tetracycline in subgingival plaque samples from subjects in the SRP+Syst tet (scaling and root planing+systemic tetracycline; $N = 10$), SRP (scaling and root planing only; $N = 10$) and SRP+Tet fib (scaling and root planing+tetracycline fibers/Actisite®; $N = 10$) groups at baseline (0), 1 week, 3, 6 and 12 months post-therapy. Data were averaged across subjects in each group for each visit. Significance of differences among groups at each time point was tested using the Kruskal–Wallis test ($^{*}p < 0.05$). Differences over time were evaluated by the Friedman test ($^{\dagger}p < 0.05$).

In the present investigation, the tetracycline-resistant strains were identified by DNA probes to 32 oral species and the Checkerboard DNA–DNA hybridization technique (Socransky et al. 1994). The prevalence of most of the species resistant to 4 μg/ml of tetracycline did not differ significantly among groups (data not shown). In general, *Streptococcus* spp., *V. parvula*, *P. micros, P. intermedia, E. corrodens, G. morbillorum* and *A. actinomycetemcomitans* were the most predominant species before and after treatment. These results are in accordance with data reported by Abu Fanas et al. (1991), Goodson & Tanner (1992), Olsvik et al. (1995a) and Feres et al. (1999b). Moreover, studies have demonstrated an intrinsic tetracycline resistance in species of *Actinomyces*, *Streptococcus* and *Veillonella*, due to the presence of *tet* determinants (Lacroix & Walker 1995, 1996, Olsvik et al. 1995a, b). It has been suggested that this may be one of the reasons that the tetracyclines have been shown to have a beneficial effect in the treatment of periodontitis by favoring colonization of the subgingival site with a microbiota associated with periodontal health (Walker 1996). Among potential periodontopathogens, species of *Peptostreptococcus*, *Prevotella*, *Bacteroides*, *Fusobacterium*, *Selenomonas* and *Eubacterium* have also been reported to be intrinsically resistant to tetracyclines (Roberts & Lansciardi 1990, Lacroix & Walker 1995, 1996, Walker 1996). An interesting finding observed in the present study was the high prevalence of tetracycline-resistant putative pathogens including *A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythensis* prior to antibiotic therapy. Although some reports have described a relatively high in vitro susceptibility of *A. actinomycetemcomitans* for tetracycline (Walker et al. 1983), other authors have found an increase in development of resistance for this microorganism (Roe et al. 1995). Furthermore, a wide variety of *tet* genes, such as *tet*(B),

tet(M), *tet*(Q) and the newly described *tet*(W) have been detected in strains of *Actinobacillus* and *P. gingivalis* (Abu Fanas et al. 1991, Roberts 2002). The current data did not agree with other investigations which found no resistant strains of *A. actinomycetemcomitans*, *T. forsythensis* or *P. gingivalis* before and after treatment with local and systemic tetracyclines (Goodson & Tanner 1992, Listgarten et al. 1993, Feres et al. 1999b, Walker et al. 2000). It is conceivable that the much higher prevalence of these species in periodontitis lesions from individuals in our population, as recently reported by Colombo et al. (2002), and the widespread use of tetracyclines in this community would explain the greater proportions of samples with tetracycline-resistant periodontal pathogens. Clearly, geographical differences in antibiotic resistance profiles exist. For instance, van Winkelhoff et al. (2000) reported a significant higher percentage of periodontal pathogens resistant to tetracycline in Spanish patients compared to Dutch individuals. It should be pointed out, though, that in the present study a decrease in the frequency of sites presenting pathogenic species resistant to tetracycline was noticed one year after treatment in all three therapeutic groups, being more significant in subjects treated with local tetracycline. Nevertheless, this does not exclude the fact that some microorganisms of these species not resistant to the drug could still be present in the subgingival microbiota. Changes in the prevalence of these resistant species may have been a result of ecological changes in members of the subgingival biofilm required for colonization and establishment of those microorganisms. The possibility of emergence of ''new'' resistant species either by mutation or transfer of genetic elements is another fact that cannot be excluded. However, the current study could not detect novel tetracycline-resistant species, given that DNA probes were employed to identify resistant taxa. Despite that, studies using cultural techniques detected few, if any, emergent resistant species after antibiotic administration (Olsvik et al. 1995a).

In conclusion, the current findings demonstrated that local and systemic tetracycline therapy combined with SRP results in an initial transient increase in the % of resistant microorganisms, probably due to a selection of intrinsically resistant oral species. In addition, the high prevalence of resistant periodontal pathogens prior to treatment in this population suggest that this agent should be considered carefully and restricted to patients who do not respond to conventional therapy or present a more aggressive disease. Nevertheless, both therapies resulted in a decrease of the prevalence of resistant suspected periodontal pathogens over time. In particular, the prevalence of resistant species of *A. actinomycetemcomitans* was significantly reduced in the group treated with tetracycline fibers compared to the systemic tetracycline therapy.

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