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Microbiological changes associated with four different periodontal therapies for the treatment of chronic periodontitis

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Background/aims: To examine subgingival microbiological changes in chronic periodontitis subjects receiving scaling and root planing (SRP) alone or with systemically administered azithromycin, metronidazole or a sub-antimicrobial dose of doxycycline.
Methods: Ninety-two periodontitis subjects were randomly assigned to receive SRP alone or combined with azithromycin, metronidazole or sub-antimicrobial dose doxycycline. Subgingival plaque samples taken at baseline, 2 weeks, and 3, 6, and 12 months were analyzed for 40 bacterial species using checkerboard DNA–DNA hybridization. Percentage of resistant species and percentage of sites harboring species resistant to the test antibiotics were determined at each time-point.
Results: All treatments reduced counts of red complex species at 12 months, although no significant differences were detected among treatment groups for most species at all time-points. Both antibiotics significantly reduced counts of red complex species by 2 weeks. Percentage of resistant isolates increased in plaque samples in all adjunctive treatment groups, peaking at the end of administration, but returned to pretreatment levels by 12 months.
Conclusion: The significant reduction of red and orange complex species at 2 weeks in the subjects receiving SRP plus azithromycin or metronidazole may have contributed to a better clinical response in these treatment groups. Therapy did not appear to create lasting changes in the percentage of resistant isolates or sites harboring resistant species.

Key words: antibiotics; antibiotic resistance; azithromycin; chronic periodontitis; metronidazole; periodontal pathogens; periodontal therapy; scaling and root planing; sub-antimicrobial dose doxycycline

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In an earlier paper, the clinical effects of systemically administered azithromycin, metronidazole or sub-antibacterial dose doxycycline (SDD) as adjuncts to scaling and root planing (SRP) were examined (10). It was found that subjects receiving the systemically administered antibiotics, azithromycin or metronidazole, showed greater clinical improvement at 12 months post-therapy compared with subjects receiving SRP only and that the difference among groups was more marked at sites with initially deeper pockets. The dosage, duration, and mechanism of action of the three adjunctive agents are quite different and thus, the effects on the subgingival microbiota were examined. To date, there are few studies examining the changes in the subgingival microbiota in subjects receiving these adjunctive agents and no 'head to head' comparison.

Although it has been shown that bacterial species residing in biofilms are much more resistant to antibiotics than the same species in a planktonic state (1, 2, 11, 20), antibiotics have been used frequently in the treatment of periodontal infections. Of the single antibiotics that have been evaluated, systemically administered metronidazole appeared to have the most consistent beneficial effect on clinical parameters of periodontal diseases and on subgingival plaque composition (29). Metronidazole is attractive for the treatment of patients with chronic periodontitis because the narrow spectrum of this agent is thought to work specifically on the anaerobic microbiota associated with periodontal diseases. Indeed, several investigations suggested that

systemically administered metronidazole used as an adjunct to SRP in the treatment of chronic periodontitis offers a clinical benefit over SRP alone (8). Few studies have documented the microbiological changes associated with the systemic administration of metronidazole. Lindhe et al. (13) demonstrated that systemically administered metronidazole in combination with mechanical therapy was more effective in reducing spirochetes than SRP alone. Other investigators have shown that periodontal therapy that included systemically administered metronidazole led to clinical improvements and a reduction in the proportions or levels of several periodontal pathogens including *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, *Prevotella intermedia*, members of the genera *Campylobacter*, *Fusobacterium*, *Eubacterium nodatum*, *Peptostreptococcus micros* and *Streptococcus constellatus* (5, 14–16, 31). Feres et al. (4) examined the changes in metronidazole resistance over time. The percentage of isolates resistant to metronidazole increased at 2 weeks, the end of antibiotic administration, but at 6 months post-therapy the levels were similar to pretreatment values.

The effects of azithromycin on the oral microbiota have been examined in a limited number of studies. Smith et al. (23), in a randomized double-blind, placebo-controlled study of 46 chronic periodontitis subjects, demonstrated that subjects receiving azithromycin as an adjunct to SRP showed a better clinical response than subjects not receiving this agent. In addition, they found that compared with the placebo group, counts of spirochetes and 'pigmented anaerobes' in subgingival plaque samples were significantly more reduced in subjects receiving azithromycin at 22 weeks post-therapy (22).

The effect of long-term SDD on the subgingival microbiota was examined by Thomas et al. (28). Subgingival plaque samples were taken before and after therapy that consisted of SRP or not, and 20 mg twice daily (bid) doxycycline or placebo for 9 months and were evaluated using dark-field microscopy and culture. The E-test (AB Biodisk, Solna, Sweden) and minimum inhibitory concentrations (MICs) were used to determine doxycycline resistance. Morphotype distribution was somewhat similar among treatment groups at each time-point; there was no marked decrease in species recovery and no overgrowth of opportunistic species. Furthermore, the administration of SDD was not associated with the development of doxycycline-resistant species. Walker et al. (30) examined the effect of a 9-month administration of SDD on the intestinal and vaginal microbiota in 69 periodontally diseased subjects. No changes in the level of resistant species in either fecal or vaginal samples from these subjects were detected after long-term SDD administration.

The purpose of the current study was to examine microbiological changes associated with treatment groups receiving SRP alone or in combination with one of systemically administered azithromycin, metronidazole, or SDD. In addition, the effect of the different therapies on the proportion of antibiotic-resistant species post-therapy and the identity of these species was also examined.

Material and methods

Subject population

Ninety-two subjects were recruited into the study. Subjects were >20 years of age, in good general health, had at least 20 natural teeth, including at least one molar tooth in each quadrant, and at least eight sites with pocket depth >4 mm. The subjects did not have a history of unsuccessful periodontal therapy that may have indicated 'refractory' periodontitis and the history of the subjects precluded a diagnosis of aggressive periodontitis. Subjects were excluded if they had any known allergies to the test antimicrobial agents, had received periodontal therapy in the previous 3 months, were pregnant or nursing, or had systemic conditions that required antibiotic premedication or could influence the outcome of periodontal therapy. The subjects had a mean age of 46 years (range 22–77 years), 36% were female, 63% were White, 26% were African American, 7% were Hispanic and 4% were Asian. The baseline clinical characteristics of the subjects are presented in Table 1. The study was approved by the Forsyth Institute Institutional Review Board. The nature of the study was described thoroughly to all subjects and each provided signed informed consent before entry into the study.

Experimental design and treatment

This was a randomized, single-blind study. Subjects were randomized to one of four treatment groups receiving either SRP alone or combined with systemically administered azithromycin at the dosage of 500 mg once daily for 3 days, systemically administered metronidazole at the dosage of 250 mg thrice daily (tid) for 14 days, or 20 mg doxycycline (SDD, Periostat®; CollaGenex Pharmaceuticals, Newtown, PA) bid for 12 weeks. SRP was performed a quadrant at a time under local anesthesia at approximately weekly intervals. The adjunctive agents were started at the first SRP visit. Subjects were clinically monitored at baseline (before therapy) and at 3, 6, and 12 months post-therapy. In addition, all subjects received maintenance SRP at the three post-therapy monitoring visits.

Microbiological assessments

At each monitoring visit clinical measurements were taken as described in a companion paper (10). Subgingival plaque samples were taken from the mesial aspect

Table 1. Mean (±SD) clinical and demographic features of subjects with data for the four monitoring visits in the four treatment groups at baseline

	Treatment group				Kruskal–Wallis *P*-value
	Azithromycin	Metronidazole	Periostat	SRP	
n	25	24	20	23	
Age	47 ± 14	44 ± 11	47 ± 11	43 ± 15	0.51086
No. missing teeth	2.40 ± 2.50	2.58 ± 2.12	2.35 ± 2.62	1.83 ± 2.44	0.50788
No. males	15	18	10	16	0.33237
No. current smokers	3	3	1	2	0.66845
% of sites with					
Plaque accumulation	62.49 ± 23.49	65.98 ± 20.90	66.52 ± 25.61	64.26 ± 22.27	0.86874
Gingival redness	64.32 ± 29.14	73.90 ± 21.38	66.09 ± 24.57	63.95 ± 27.04	0.62136
Bleeding on probing	34.29 ± 26.04	39.37 ± 28.02	41.11 ± 24.78	32.62 ± 26.20	0.61425
Suppuration	0.94 ± 1.45	0.85 ± 2.05	2.52 ± 4.16	0.21 ± 0.68	0.00246
Mean pocket depth (mm)	3.11 ± 0.64	3.00 ± 0.45	3.33 ± 0.92	2.92 ± 0.37	0.52465
Mean attachment level (mm)	3.42 ± 0.88	3.21 ± 0.78	3.47 ± 0.93	3.03 ± 0.56	0.34108

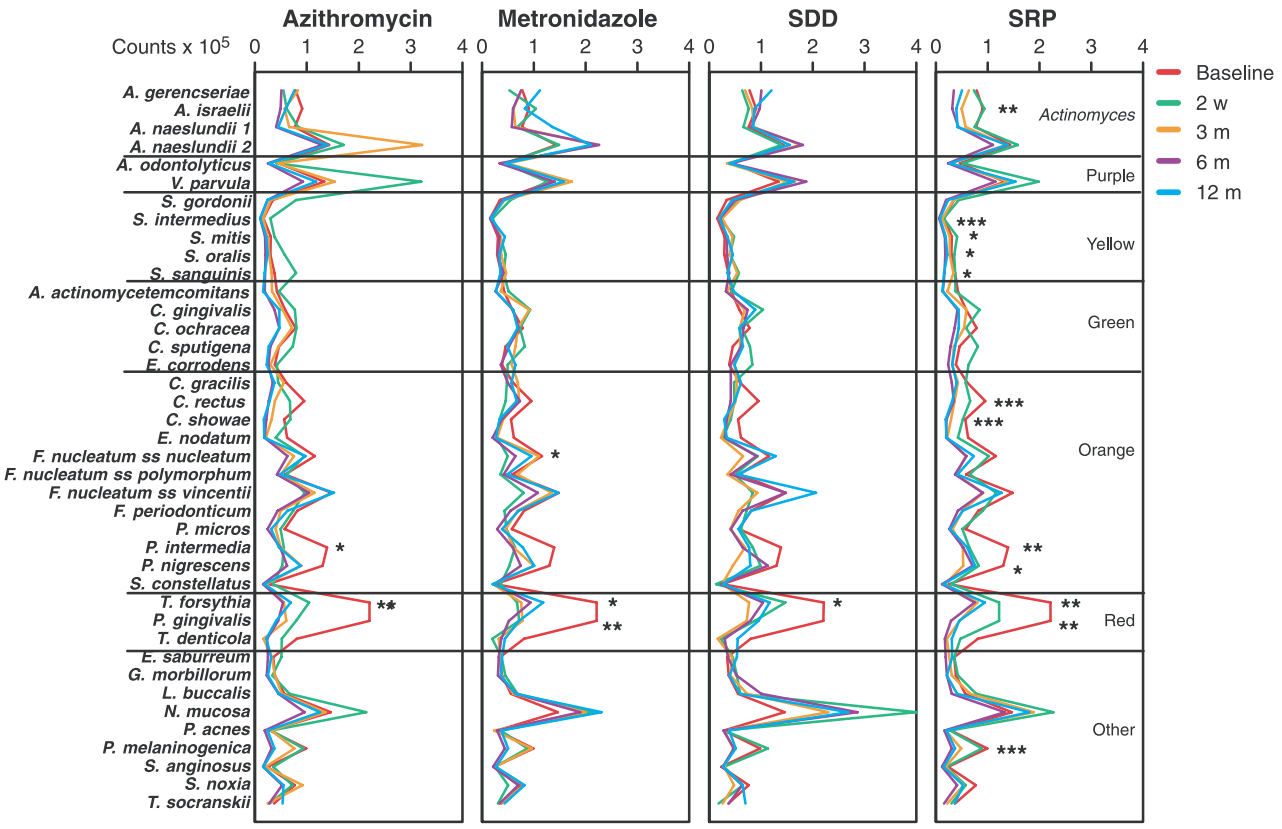

Fig. 1. Mean counts ($\times 10^5$) of 40 test species at baseline, 2 weeks, and 3, 6, and 12 months in subjects in each of the four treatment groups. The species were ordered according to the complexes described by Socransky et al. (24). Counts for individual species from up to 28 sites in each subject were averaged within a subject and then across subjects in the four treatment groups for each time-point separately. Significance of differences over time was sought using the Friedman test $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and adjusted for 40 comparisons (25).

of each tooth (excluding the third molars) for a maximum of 28 teeth in each subject at baseline, 2 weeks, and 3, 6, and 12 months. After removal of supragingival plaque, subgingival biofilm samples were taken using individual sterile Gracey curettes from the mesial surface of each tooth and placed into separate Eppendorf tubes containing 0.15 ml Tris EDTA buffer (10 mM Tris–HCl, 1 mM ethylenediaminetetraacetic acid, pH 7.6). Immediately after, 0.10 ml of 0.5 M NaOH was added to each sample. Each sample was evaluated for its content of 40 bacterial species using checkerboard DNA–DNA hybridization (26, 27). In brief, the samples were lysed and the DNA was placed in lanes on a nylon membrane using a Minislot device (Immunetics, Cambridge, MA). After fixation of the DNA to the membrane, the membrane was placed in a Miniblotter 45 (Immunetics), with the lanes of DNA at 90° to the lanes of the device. Digoxigenin-labeled whole genomic DNA probes to 40 subgingival species were hybridized in individual lanes of the Miniblotter. After hybridization, the membranes were washed at high stringency and the DNA probes were detected using antibody to digoxigenin, conjugated with alkaline phosphatase and chemifluorescence detection. Signals were detected using AttoPhos substrate (Amersham Life Sciences, Arlington Heights, IL) and were read using a Storm FluorImager (Molecular Dynamics, Sunnyvale, CA), a computer-linked instrument that reads the intensity of the fluorescence signals resulting from the probe-target hybridization. Two lanes in each run contained standards at the concentration of 10^5 and 10^6 cells of each species. The sensitivity of the assay was adjusted to permit the detection of 10^4 cells of a given species by adjusting the concentration of each DNA probe. Signals were evaluated using the Storm FluorImager and converted to absolute counts by comparison with the regression line determined from data from the standards on the same membrane. Failure to detect a signal was recorded as zero. A total of 10,913 subgingival samples were evaluated for the 92 subjects.

Determination of antibiotic resistance

Subgingival biofilm samples from the distal site of four selected teeth were taken at baseline and 12 months, and from two of the four selected teeth at 2 weeks (the third SRP visit) and 3 and 6 months in each subject. These samples were placed in separate tubes each containing 0.15 ml of Mycoplasma broth (BBL, Cockeysville, MD) supplemented with 1% glucose. One milliliter of whole saliva was collected into an empty tube at each time-point from each subject.

The plaque and saliva samples were vortexed and serially diluted 10-fold. Then, 100 µl of each dilution was plated in duplicate on antibiotic-containing and non-selective agar plates. All plates were made with 2% trypticase soy agar and 2.6% brain–heart infusion agar enriched with 5% sheep blood, 1% yeast extract, 5 µg/ml hemin, 0.3 µg/ml menadione, and 10 µg/ml *N*-acetylmuramic acid. Antibiotic-containing plates were made up with the same basal medium supplemented with one of 4 µg/ml doxycycline, 2 µg/ml metronidazole or 2 µg/ml azithromycin. These values were chosen based on data for a wide range of species presented in NCCLS publications (18, 19). Samples taken from subjects in the SRP-only group

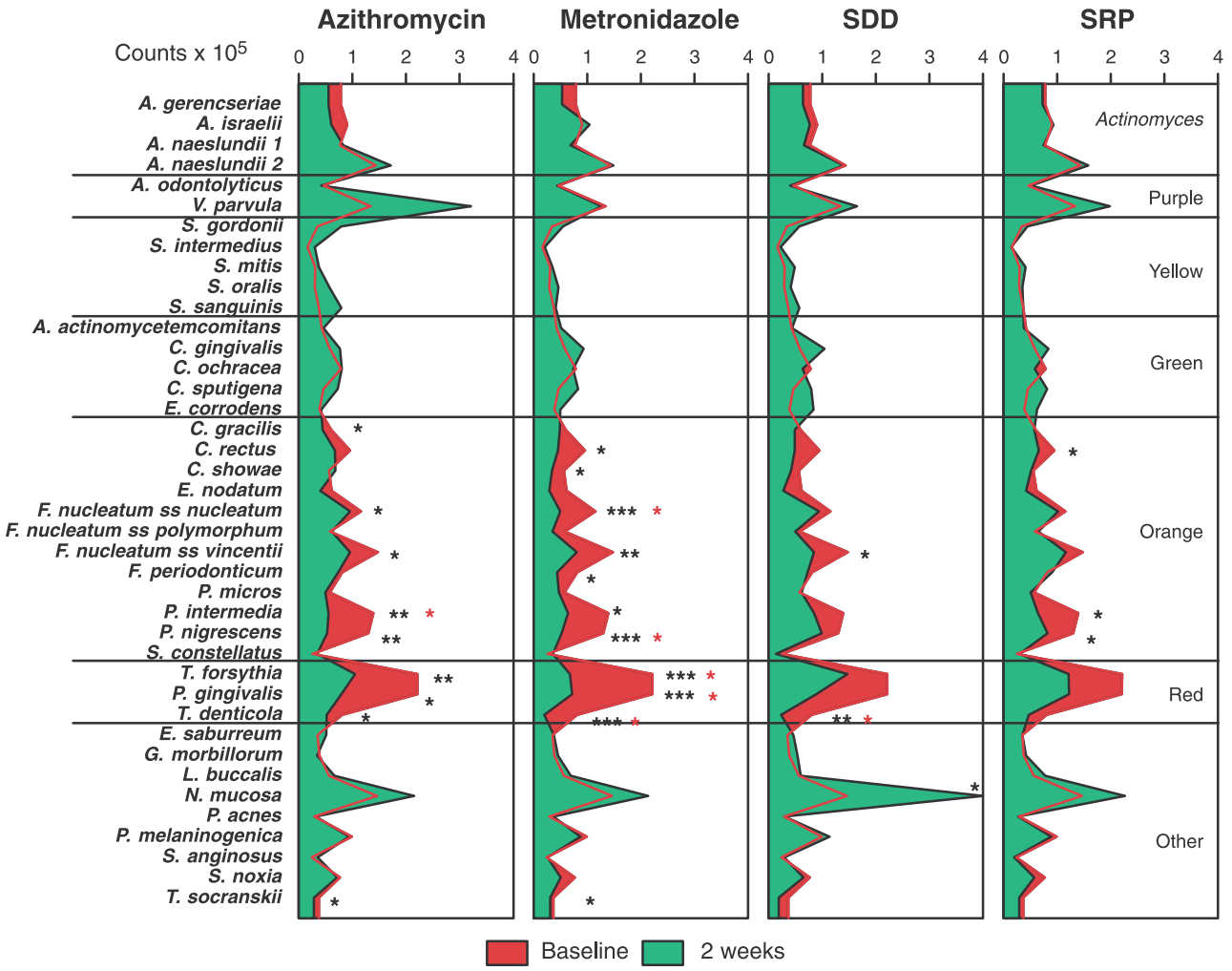

Fig. 2. Mean counts ($\times 10^5$) of 40 test species at baseline and 2 weeks in subjects in each of the four treatment groups. The species were ordered according to the complexes described by Socransky et al. (24). Counts for individual species at each sampled site were averaged within a subject, and across subjects in the four treatment groups at baseline and 2 weeks separately. Significance of differences between baseline and 2 weeks was determined using the Wilcoxon signed ranks test; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. The black asterisks represent significant differences between time-points without adjusting for 40 comparisons and the red asterisks represent significant differences between time-points after adjusting for 40 comparisons (25).

were plated on each of the antibiotic-containing plates. Samples taken from subjects receiving an adjunctive agent were plated on plates containing that agent. The inoculated plates were then placed into an anaerobic chamber and incubated in an atmosphere of 80% N_2, 10% CO_2 and 10% H_2 at 35°C. After 7 days of incubation, the plates were removed from the chamber, the colonies on the antibiotic and non-selective plates were counted, and the proportion of antibiotic-resistant species was determined.

For each sampled site that was plated on an antibiotic plate, the dilution with the number of colonies closest to 300 was washed off with 1 ml TE buffer into a tube. The optical density was adjusted to 1.0 (approximately 10^9 cells); 0.01 ml of this suspension (about 10^7 cells) was put into a tube containing 140 µl TE buffer and 100 µl of 0.5 M NaOH was added. The samples were then analyzed for their content of 40 bacterial species using checkerboard DNA–DNA hybridization.

Data analysis

Data from a total of 92 subjects were available for analysis and included the counts of 40 bacterial species in up to 28 subgingival biofilm samples at baseline, 2 weeks, and 3, 6, and 12 months. The change in mean counts of bacterial species over time was the primary microbiological outcome evaluated. Data were averaged across sites within each subject and then averaged across subjects in each treatment group at each time-point separately. Since mean baseline values for individual species differed among treatment groups, the microbial changes among the four treatment groups were examined using ANCOVA adjusting for baseline microbial counts. The subject was the unit of analysis. Significance of differences over time in the counts of the 40 individual species was determined using the Friedman test and adjusted for 40 comparisons (25).

The proportion of resistant isolates was determined in saliva samples and in four subgingival plaque samples at baseline and 12 months and in two of these samples at 2 weeks and 3 and 6 months in subjects in the four treatment groups. Proportions of resistant isolates for the plaque samples were averaged across sites in each subject and then across subjects in the four treatment groups and at each time-point separately. The significance of differences over time in each group was sought using the Friedman test. Significance of differences between test and control samples at

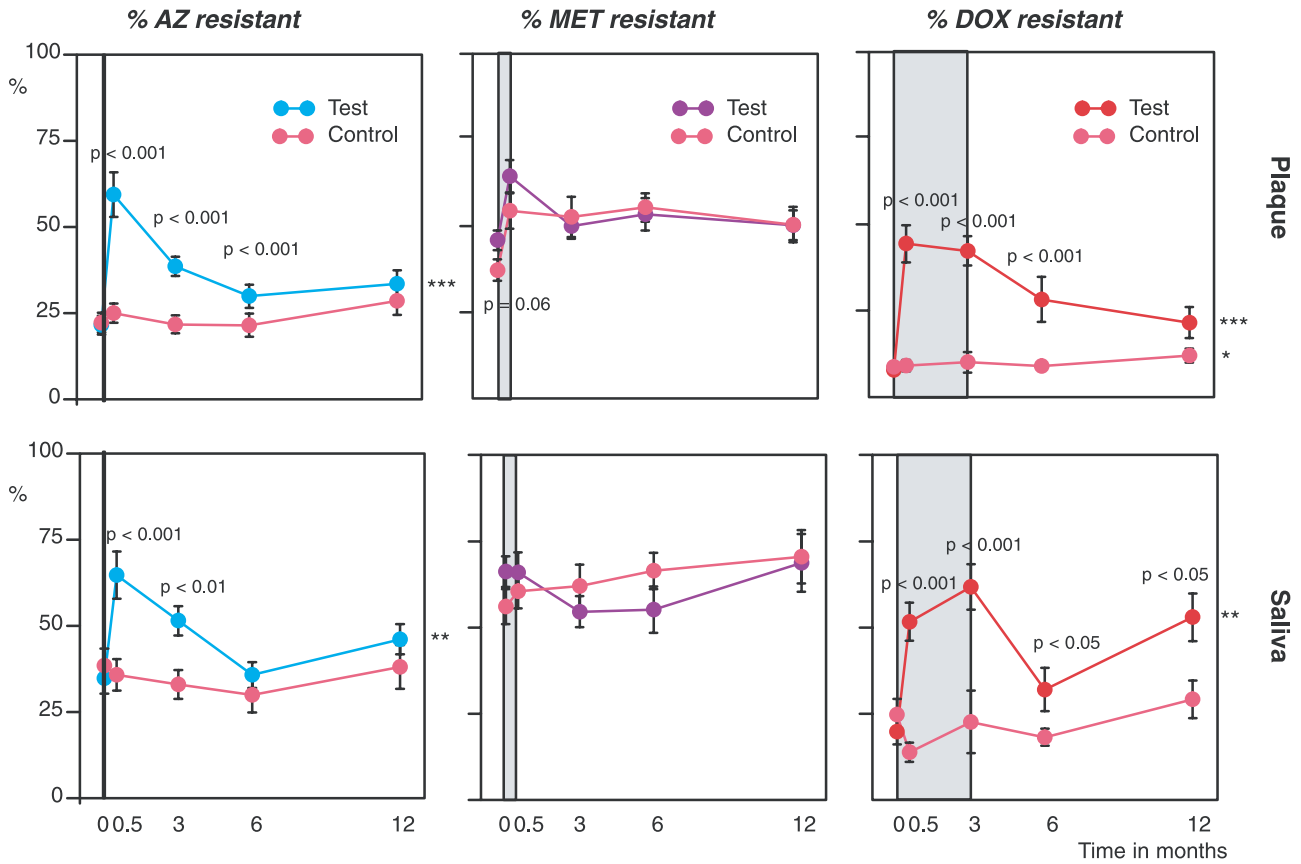

Fig. 3. Percentage of isolates resistant to 2 μg/ml azithromycin, 2 μg/ml metronidazole, or 4 μg/ml doxycycline in subgingival plaque samples (upper panels) and saliva samples (lower panels) taken at baseline, 2 weeks, and 3, 6, and 12 months from subjects in the four treatment groups. Antibiotic resistance in the SRP group was measured for all three systemic agents. Data were averaged within a subject at each time point and then across subjects in the four groups separately. The circles represent the mean and the whiskers the standard error of the mean. The shaded area represents the period of antibiotic administration in the test subjects. Significance of differences over time for each group separately was sought using the Friedman test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and between test and control subjects using the Mann–Whitney test as indicated by the *P*-values at individual time-points.

each time-point was tested using the Mann–Whitney test for each adjunctive agent separately. The mean percentage of sites colonized by resistant species in subgingival plaque samples was determined by averaging the site data for each species across subjects in the four treatment groups at each time-point separately. Significance of differences over time was determined using the Friedman test and between test and control samples at each time-point using the Mann–Whitney test.

Results

Subject retention, patient compliance and adverse events were discussed in Haffajee et al. (10).

Microbial changes in different treatment groups

At baseline, virtually all subjects harbored each of the test species at some level in at least one sampled site. At levels $>10^5$, fewer subjects were positive for the test species, although the red complex species were, on average, still quite prevalent in subjects in each treatment group (Table 2). There were no significant differences among treatment groups in the prevalence of the individual test species at baseline. Figure 1 presents the mean counts of the 40 test species at each visit in the four treatment groups. On average, all treatment groups showed reductions in counts of red complex species, which were maintained to 12 months. These changes were significant for *T. forsythia* in all treatment groups and for *P. gingivalis* in the metronidazole and SRP-only groups. Other species were reduced markedly at the initial post-therapy visits, particularly members of the orange complex, although an increase in counts of many species had occurred by 12 months. Indeed, several orange complex species approached or exceeded baseline values at the 12-month monitoring visit, a finding particularly noticeable in the SDD group. The data in this figure indicated that the major decrease in bacterial counts occurred between baseline and 2 weeks. Figure 2 highlights the data at these two visits for subjects who were subset into the four treatment groups. The data indicated that subjects receiving systemically administered azithromycin or metronidazole demonstrated significant reductions in red and many orange complex species at 2 weeks. There were no statistically significant differences among treatment groups for any of the test species examined at any time-point with the exception of *Actinomyces israelii*, *Streptococcus gordonii*, *Streptococcus intermedius*, and *P. gingivalis* at the 6 months post-therapy visit.

Percentage of the microbiota resistant to the test antibiotics

Figure 3 presents the percentage of the cultivable microbiota that was resistant to

Table 2. Proportion (%) of subjects positive for the 40 test species at mean levels> 10^5 at baseline

Treatment	AZ	MET	SDD	SRP
Species				
A. gerencseriae	20	25	55	26
A. israelii	24	25	45	26
A. naeslundii 1	24	17	45	30
A. naeslundii 2	68	63	70	61
A. odontolyticus	4	0	30	4
V. parvula	48	46	60	39
S. gordonii	0	0	15	9
S. intermedius	0	0	0	0
S. mitis	0	0	15	0
S. oralis	0	0	15	4
S. sanguinis	4	0	15	13
A. actinomycetemcomitans	4	4	25	9
C. gingivalis	12	8	20	4
C. ochracea	24	17	55	26
C. sputigena	12	8	30	0
E. corrodens	4	4	10	0
C. gracilis	16	8	45	22
C. rectus	28	42	50	30
C. showae	16	13	35	13
E. nodatum	16	4.17	25	26
F. nucleatum ss nucleatum	48	46	50	48
F. nucleatum ss polymorphum	4	17	22	9
F. nucleatum ss vincentii	68	58	65	65
F. periodonticum.	20	29	45	30
P. micros	12	13	25	30
P. intermedia	44	33	45	55
P. nigrescens	48	42	60	52
S. constellatus	0	0	5	4
T. forsythia	64	63	70	61
P. gingivalis	40	58	60	57
T. denticola	24	25	35	30
E. saburreum	4	8	15	9
G. morbillorum	8	8	10	0
L. buccalis	12	8	30	22
N. mucosa	72	71	55	57
P. acnes	0	4	10	0
P. melaninogenica	16	17	35	26
S. anginosus	0	0	10	4
S. noxia	32	17	40	30
T. socranskii	4	13	20	13

azithromycin, metronidazole, or doxycycline before antibiotic therapy and at 2 weeks, and 3, 6, and 12 months after initial therapy in subgingival plaque and saliva samples from subjects in the four treatment groups. Before therapy, the percentage of species resistant to azithromycin was about 22% in both the test and control samples. Forty-six and 37% were resistant to metronidazole and 8 and 9% were resistant to doxycycline in the test and control samples, respectively. In all samples from subjects receiving systemic agents, antibiotic resistance increased at 2 weeks with significant differences between the test and control samples for azithromycin and doxycycline. For both of these antibiotics, the significant difference between test and control samples remained to 6 months, but by 12 months the percentage of resistant species were reduced in the test samples and levels approached those detected before therapy. At no time-point was there a significant difference between test and control subjects in the percentage of species resistant to metronidazole. The data for the saliva samples were similar to those observed for the plaque samples except that the pretherapy proportions of resistant species were higher in the saliva samples (Fig. 3). Furthermore, there was an increase in the percentage of resistant species in the 12-month samples in subjects in the SDD group so that levels were significantly higher than those observed in control saliva samples.

Percentage of sites harboring resistant species

Figures 4–6 present the percentage of sites colonized by the 40 test species that were resistant to azithromycin, metronidazole, and doxycycline. Irrespective of treatment group, there were no significant differences over time in the percentage of sites harboring resistant species. The most commonly detected resistant species in each treatment group were the streptococci. Resistant strains of *Veillonella parvula* were detected in over 50% and 80% of sites in subjects in the SDD and azithromycin groups respectively. In general, a wider range of species was found to be resistant in the azithromycin and SDD groups.

Resistant isolates of many of the test species were found in a very small percentage of sampled sites including periodontal pathogens such as *T. forsythia*, *P. gingivalis*, *E. nodatum*, and *Campylobacter* species. The percentage of sites harboring resistant strains of *Fusobacterium* was also very low in the metronidazole group. There were a small number of significant differences between test and control samples at various time-points for each of the test antibiotics. However, when adjustments were made for multiple comparisons, these differences were no longer significant.

Discussion

The goal of the present investigation was to describe the subgingival microbial changes that took place as a result of four different treatment modalities, SRP alone or in conjunction with the antibiotics, azithromycin or metronidazole or SDD. The choice of adjunctive agents was based on their different modes of action and the different dosage regimens required, which ranged from 3 days for azithromycin to 3 months for SDD. As described in Haffajee et al. (10), there were differences among treatment groups in the clinical changes at 1 year post-therapy.

Irrespective of the treatment modality employed, there was a significant decrease in mean counts of many of the test species, particularly members of the red and orange complexes, species associated with the etiology of periodontal infections. It has been shown that a decrease in the counts of this segment of the subgingival microbiota is associated with clinical improvements (9). This was confirmed in the current investigation because a decrease in mean counts of many of the test species was significantly associated with an improvement in attachment level and a reduction in probing pocket depth (10). The most marked decrease in counts occurred between the pretreatment visit and the 2-week sampling. At this time-point, two quadrants of SRP had been completed, as well as administration of both azithromycin and metronidazole. This is in accord with data in the literature suggesting that treatment of part of the mouth has a beneficial effect on 'untreated' segments

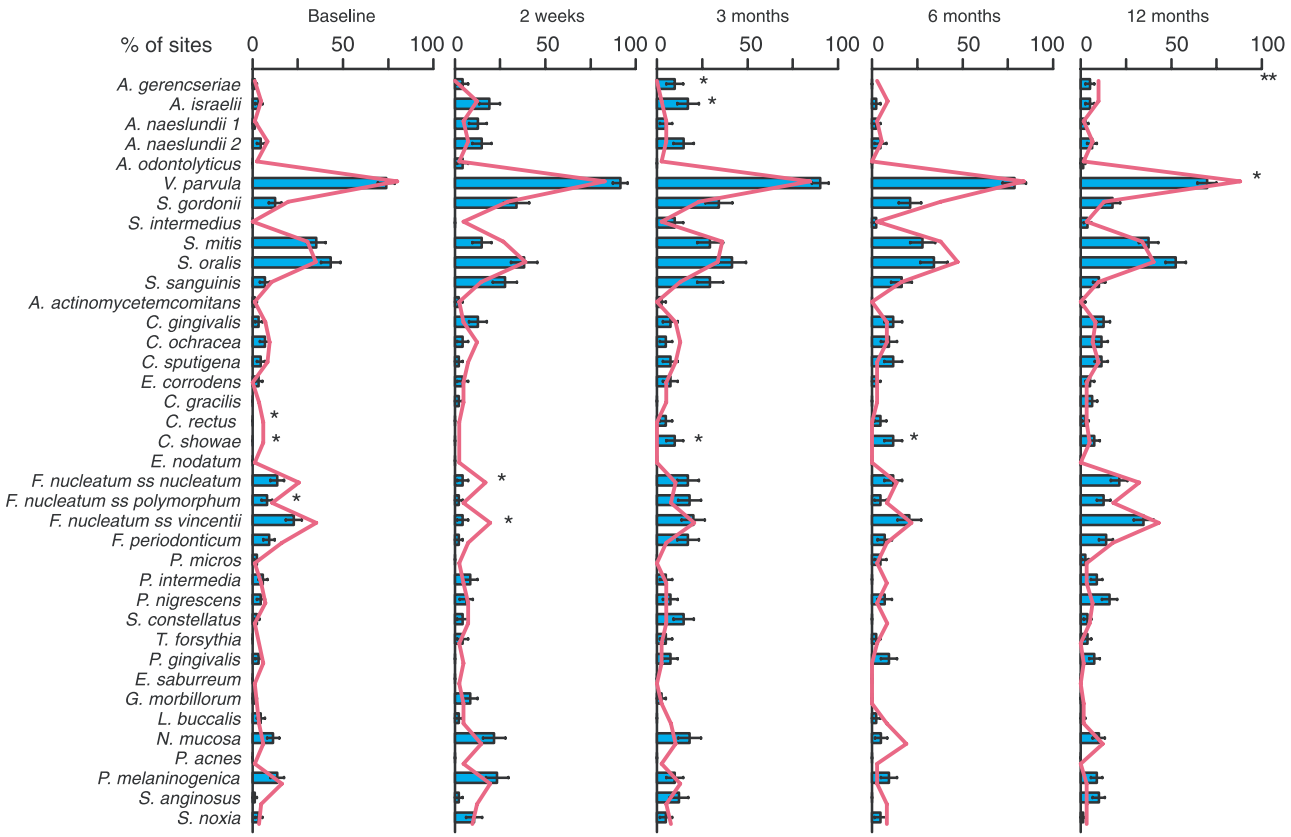

Fig. 4. Mean (±SEM) percentage of sites colonized by species resistant to 2 μg/ml azithromycin at baseline, 2 weeks, and 3, 6, and 12 months in subjects who received systemically administered azithromycin as part of their therapy. Samples were taken from two to four sites at each time-point and resistant species were averaged across subjects for each time-point separately. The pink lines represent the resistance profiles for samples tested for azithromycin resistance from subjects receiving SRP only (control group). Significance of differences over time was sought using the Friedman test. There were no statistically significant differences over time in either the test or control samples. Significance of differences between test and control samples at each time-point was tested using the Mann–Whitney test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, without adjusting for 40 comparisons. After adjustment there were no significant differences between test and control samples for any of the species at any time-point.

(12, 21). It may also suggest that systemically administered antibiotics have a marked effect on the subgingival microbiota either in the presence or absence of SRP, as suggested by Lopez et al. (17).

For many species there was a continued decrease in levels to 3 or even 6 months, with a slight rebound detected at the 12-month visit. It is interesting that at 12 months, mean pocket depth and attachment level measurements were still showing improvement overall in subjects in each of the four treatment groups, with the exception of the SDD group (10). One may speculate that the rebound in microbial counts might continue and be associated with a subsequent worsening of the clinical condition. Unfortunately, the present study was not of sufficient duration to answer this question.

This study was in accord with the findings described by Haffajee et al. (7) and Cugini et al. (3), who found that red complex species were significantly reduced by SRP. The current study extended these findings and indicated that subjects in the three treatment groups receiving adjunctive agents also showed a reduction in red complex species and that these reductions, over time, were significant for *T. forsythia* in the azithromycin group and for *T. forsythia* and *P. gingivalis* in the metronidazole group. The findings for the adjunctive groups were consistent with those in the literature. Sefton et al. (22) described reductions in spirochetes and anaerobic 'black-pigmented' species at 22 weeks after azithromycin therapy, while Feres et al. (4) described significant reductions in counts and proportions of red complex species at 12 months after initial therapy consisting of SRP and 2 weeks of systemically administered metronidazole. The antibiotic-treated subjects in the current investigation exhibited a better clinical response, suggesting that a rapid decrease in subgingival counts of periodontal pathogens may be crucial for successful periodontal therapy and long-term periodontal stability. However, there were few significant differences in the subgingival microbiota at 12 months among treatment groups.

It has been suggested that SDD does not act as an antibiotic and thus microbial changes would be limited, as suggested by the findings of Thomas et al. (28). In the current investigation there were reductions, some significant, in the mean counts of several species at 2 weeks post-therapy in the SDD group, a pattern of change similar to that observed in the other treatment groups. However, there was an increase in counts of many species in the SDD group at 6 months, the first monitoring visit after cessation of the medication. Indeed, the only statistically significant differences among groups, after adjusting for multiple comparisons, was observed at the 6-month visit for mean counts of *A. israelii*, *S. gordonii*, *S. intermedia*, and *P. gingivalis* primarily the result of the much higher levels of these species in the SDD group at this time-point. Further, counts of several orange complex species

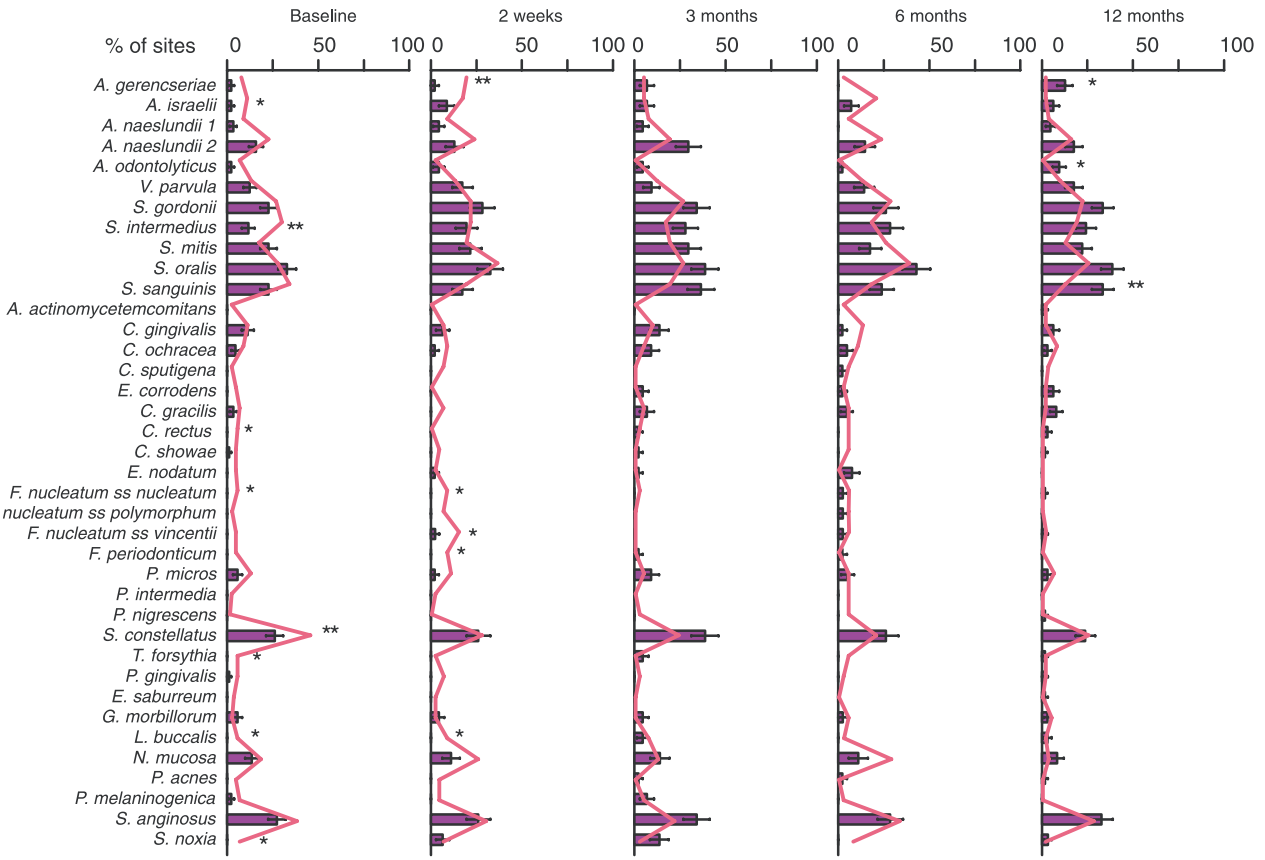

Fig. 5. Mean (±SEM) percentage of sites colonized by species resistant to 2 μg/ml metronidazole at baseline, 2 weeks, and 3, 6, and 12 months in subjects who received systemically administered metronidazole as part of their therapy. Samples were taken from two to four sites at each time-point and resistant species were averaged across subjects for each time-point separately. The pink lines represent the resistance profiles for samples tested for metronidazole resistance from subjects receiving SRP only. Significance testing was as described in Fig 4.

exceeded baseline values at 12 months in the SDD group.

Not surprisingly, the percentage of resistant isolates increased in subjects receiving adjunctive agents immediately after taking these agents, primarily because of a decrease in susceptible species. Levels had returned to baseline or close to baseline values at 12 months, with the exception of the high levels of species resistant to doxycycline observed in the saliva samples of subjects in the SDD group. The pretherapy levels, the rise in resistant species during antibiotic administration and the subsequent post-therapy decline in resistance levels were similar to the findings of Feres et al. (4, 6). The data from the present investigation indicated a similar percentage of resistant isolates pretherapy and at 12 months post-therapy but the nature of the resistant species could not be determined. The question as to whether the same strains of a given species were resistant pre- and post-therapy to the administered agents or whether new, resistant strains or strains resistant to multiple antibiotics had emerged could not be answered.

A large percentage of sites harbored strains of *V. parvula* that were resistant to azithromycin and doxycycline, both before and after antibiotic administration and in control samples taken at different time-points. This suggests an intrinsic resistance of this widely distributed species to these agents that was not observed in the samples from subjects receiving metronidazole. The species that were most likely to be resistant to all three antimicrobial agents tested were members of the genus *Streptococcus*. A large percentage of sites harbored facultative *Streptococcus* species that were intrinsically resistant to metronidazole. The proportions of sites harboring resistant *Streptococcus* species did not differ over time in the test and control groups and did not differ markedly between test and control groups at any time point. The streptococci had relatively low mean counts in the sampled sites at any time-point as shown in Fig. 1. However, the proportions of these species increased significantly at 2 weeks in the azithromycin and metronidazole groups and at 3 months in the SDD group (Fig. 3). Of clinical importance was the rather small number of sampled sites that harbored resistant species of the red and orange complexes in subjects receiving metronidazole before or during its administration. A greater percentage of sites harbored azithromycin-resistant orange and red complex species compared to metronidazole. There was a suggestion that there were more sites harboring *Neisseria mucosa*, *Selenomonas noxia*, and *S. gordonii* at 3 months in subjects receiving SDD than control subjects. However, these differences were not statistically significant after adjusting for multiple comparisons.

The study of change in species resistant to the test agents was hampered, in part, by the small number of samples that could be examined using the cultural-DNA probe assessment and the need to employ presence/absence data rather than quantitative change in proportions of resistant isolates. Examples of the greater sensitivity provided by counts or proportions are provided by the total count data of species that were resistant to the test agents over time presented in Fig. 3 and may be inferred from the shifts in proportions of species

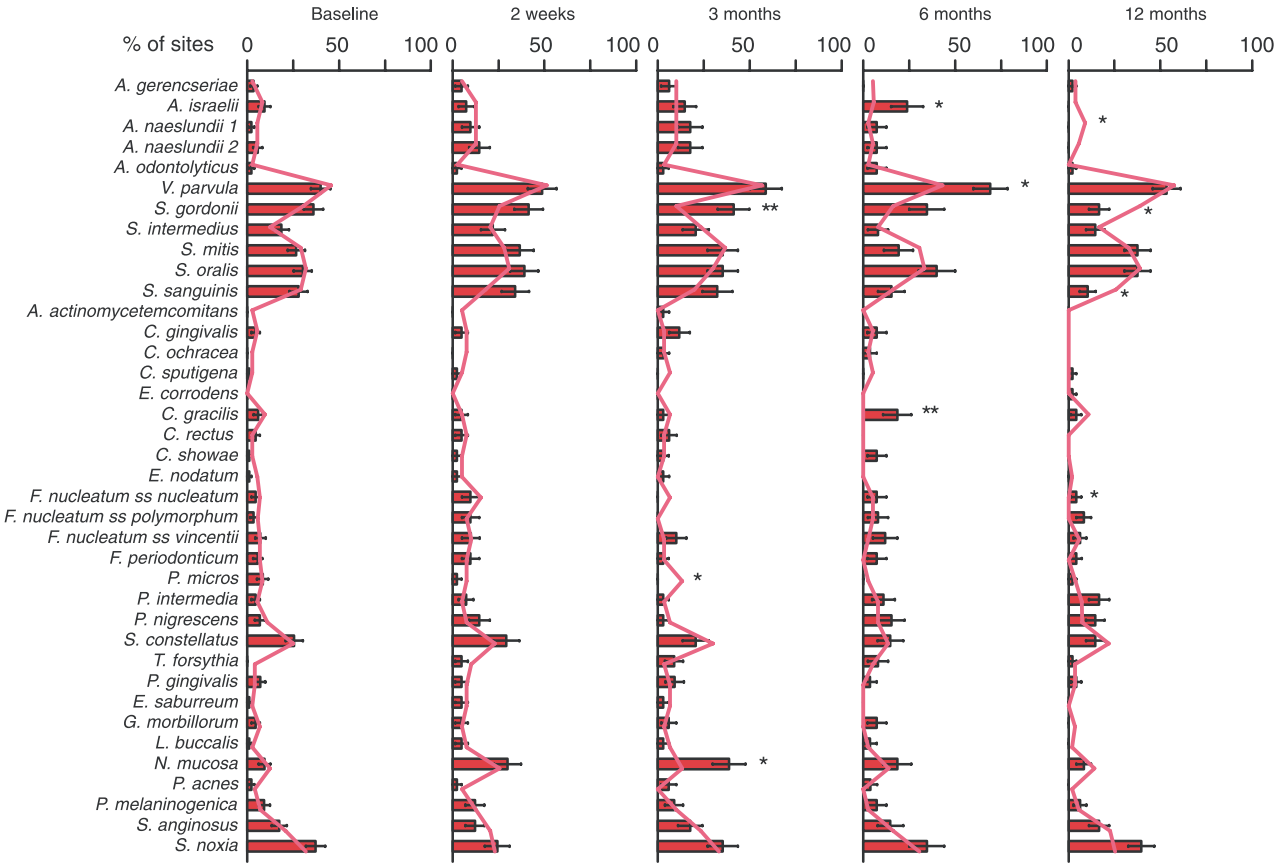

Fig. 6. Mean (±SEM) percentage of sites colonized by species resistant to 4 μg/ml doxycycline at baseline, 2 weeks, and 3, 6, and 12 months in subjects who received systemically administered SDD as part of their therapy. Samples were taken from two to four sites at each time-point and resistant species were averaged across subjects for each time-point separately. The pink lines represent the resistance profiles for samples tested for doxycycline resistance from subjects receiving SRP only. Significance testing was as described in Fig 4.

that are thought to be intrinsically susceptible or resistant, as presented in Fig. 7. This figure used the count data presented in Fig. 1 to compute the shifts in proportions of four of the test species over time in the four treatment groups. Species that were affected by mechanical debridement alone, such as *T. forsythia* and *P. gingivalis*, were decreased in mean proportions in all treatment groups. The mean proportions of a species such as *Streptococcus sanguinis*, which includes many strains that are resistant to all three adjunctive agents, rose rapidly in the subjects receiving azithromycin and metronidazole peaking at 2 weeks and at 3 months in the SDD group. Proportions of *V. parvula* increased sharply at 2 weeks in subjects receiving azithromycin, probably because most strains of this species appeared to be resistant to this agent. In contrast, the proportion of *V. parvula* declined at 2 weeks in subjects receiving metronidazole, an agent that is effective against this species. The data of Fig. 7 demonstrated the greater potential power of using proportions or counts of resistant species at

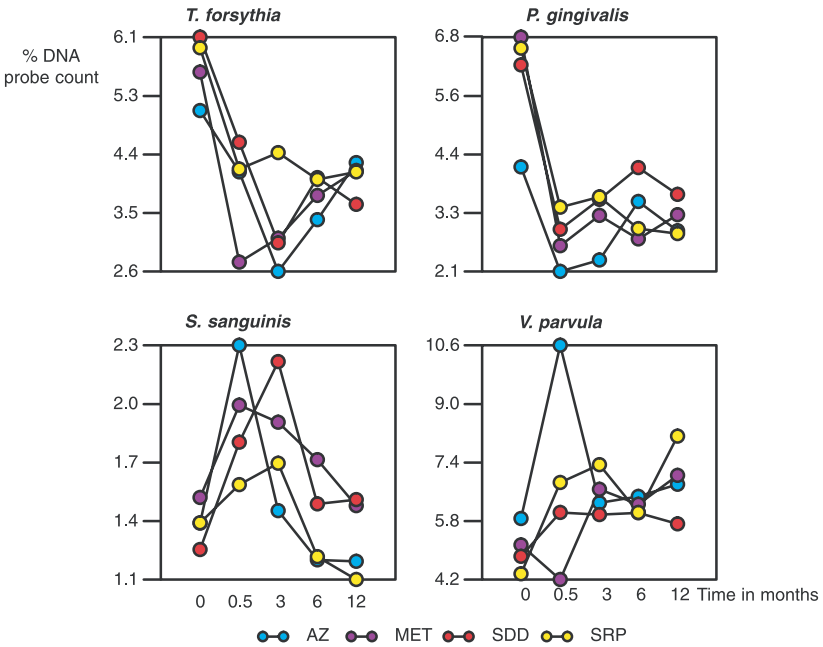

Fig. 7. Mean % DNA probe counts for *T. forsythia*, *P. gingivalis*, *S. sanguinis*, and *V. parvula* at baseline, 2 weeks, and 3, 6, and 12 months in subjects in the four treatment groups. The percentage that each species comprised of the total DNA probe count was determined at each visit for each sampled site, and was averaged across subjects in the four treatment groups and for each time-point separately.

sampled sites rather than presence/absence data. However, it must be emphasized that the data did not directly measure proportions of demonstrated resistant strains of the species, rather it provided proportions of species that are 'usually' susceptible or resistant to the tested agents.

Despite some shortcomings, this study provided a comprehensive examination of the changes that occurred in the subgingival microbiota after different therapeutic regimens. One important finding was that the better clinical outcomes observed in the antibiotic-treated subjects were accompanied by a significant and rapid decrease in counts of periodontal pathogens in these subjects by 2 weeks that may have contributed to a sustained clinical improvement to 12 months. It was also demonstrated that, within the limitations of the study, major differences in patterns of antibiotic resistance in the different treatment groups at 12 months post-therapy were not observed either in terms of percentage of isolates resistant to the test antibiotics or the percentage of sites harboring resistant species. Nonetheless, development of new antibiotic-resistant strains or strains resistant to multiple antibiotics could not be identified in the current investigation. Taken together, the clinical and microbiological findings from this study indicate that antibiotics, can provide useful adjuncts to the treatment of periodontal infections, but understanding their impact on the oral microbiota is essential to ensure responsible and optimal application.

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