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Long-term treatment with sub-antimicrobial dose doxycycline has no antibacterial effect on intestinal flora

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Abstract

Aim: The purpose of this study was to determine if a 9-month regimen of sub-antimicrobial doxycycline (20 mg, bid) had an effect on either the intestinal or the vaginal microflora.

Material and Methods: A total of 69 periodontally diseased subjects were randomized to receive drug or placebo control for a 9-month period. Stool specimens and vaginal swabs were collected at baseline and after 3 and 9 months of therapy. Samples were examined for total anaerobic counts, opportunistic pathogens, and doxycycline-resistant ($\geqslant 4$ μg/ml) bacteria. All isolates that survived sub-culture were identified and their susceptibilities determined to six antibiotics. Analyses were performed to determine if treatment differences were present.

Results: The only statistically significant differences ($p < 0.05$) between the two treatment groups occurred in the doxycycline-resistant counts at the baseline sample period for the faecal samples. This imbalance was before treatment initiation and the administration of the study drug. No between-treatment differences were detected at either the 3- or 9-month sample period either in the predominant bacterial taxa present or in their antibiotic susceptibilities.

Conclusions: There was no evidence that sub-antimicrobial doxycycline treatment exerted an effect on the composition or doxycycline resistance level of either the faecal or the vaginal microflora.

Key words: antibiotics; doxycycline; human flora; microbiology; sub-antimicrobial dosage

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Substantial evidence indicates that the adjunctive use of sub-antimicrobial dose doxycycline (SDD) consisting of 20 mg doxycycline hyclate (Periostat™, CollaGenex Pharmaceuticals Inc., Newtown, PA, USA), bid, provides a significant benefit to scaling and root planning (SRP) in the treatment of periodontitis because of the anticollagenase and anti-inflammatory activities of doxycycline. However, serious concern has been expressed that even sub-antimicrobial levels of doxycycline may exert a detrimental antimicrobial effect on the normal flora. Such an effect could result in the disruption or suppression of the normal flora and lead to its colonization or overgrowth by opportunistic pathogens as well as the development of non-susceptible microorganisms. Previous studies have rigidly documented the safety of SDD on the sub-gingival flora and have clearly shown that SDD exerts no detectable effect on the normal oral bacteria or on the antibiotic susceptibilities of these bacteria (Thomas et al. 1998, Thomas & Walker 2000, Walker et al. 2000). However, there have been some questions regarding the effect of long-term SDD therapy on the intestinal flora.

The normal microflora of the large intestine and the vagina consists predominantly of anaerobic bacteria. Various species belonging to the genera *Bacteroides*, *Prevotella, Clostridium*, and *Eubacterium* tend to be the predominant bacteria isolated from the large intestine and stool samples (Gall 1970, Moore & Holdeman 1974, Holdeman et al. 1976, Franks et al. 1998, Mai & Morris 2004). The normal vaginal flora contains *Bacteroides* and *Prevotella* species as well as various *Lactobacillus*, *Peptostreptococcus*, and *Peptococcus* species (Masfari et al. 1986, Dominque et al. 1991,

Table 1. Target microorganisms, media, incubation conditions, and confirmatory tests for the recovery and enumeration of bacteria from human stool samples

Target microorganisms	Medium	Incubation conditions	Confirmatory tests
Total anaerobic counts	Trypticase soy blood agar (TSBA)	Anaerobic, 37°C, 5–7 days	None
Total doxycycline-resistant counts (4 μg/ml)	TSBA-doxycycline	Anaerobic, 37°C, 5–7 days	None
Enterics	McConkey's agar	Aerobic, 37°C, 2–3 days	Colonial and cellular morphologies
Staphylococcus aureus	Mannitol salt agar	Aerobic, 37°C, 2–3 days	Yellow pigmented colony
Candida	Mycosel agar	Aerobic, 37°C, 2–3 days	Colonial and cellular morphology

Larsen 1993, Keane et al. 1997, Zhou et al. 2004). The use of broad-spectrum antibiotics may lead to the suppression or disruption of the normal flora of the intestines and/or the vagina and result in colonization or overgrowth by opportunistic pathogens. In the intestines, such pathogens may include *Candida* and other yeasts, *Clostridium difficile*, enteric bacteria, and/or *Staphylococcus aureus* (Barza et al. 1987, Giuliano et al. 1987, Stark et al. 1995). In the vagina, vaginosis because of *Candida albicans* and/or *Gardnerella vaginalis* is a frequent side effect of systemic antibiotic therapy (Di Rosa & Masttrantonio 1993, Rosenstein et al. 1996).

The purpose of the microbial portion of this study was to monitor the effects of a 9-month regimen of 20 mg doxycycline, bid, relative to a placebo control, on the intestinal and vaginal microflora to determine whether the use of SDD resulted in (a) shifts in the normal flora, (b) increases in the proportion of the flora resistance to doxycycline (4 μg/ml), and/or (c) increases in the quantity of doxycycline required to inhibit the bacteria, e.g. increases in the minimum inhibitory concentrations (MICs).

Methods

Subjects

Up to 35 adult subjects diagnosed with periodontitis who met the inclusion criteria were entered into the study following informed consent at each of two centres, The University of Pittsburgh (Pitt) and The Ohio State University (OSU) (69 subjects in total). Subjects were aged 30–75 years with evidence of moderate–severe chronic periodontitis manifested by attachment loss and probing depths 5–9 mm in two sites in each of two quadrants for a total of four sites per subject. Exclusion criteria were pregnancy or lactation; serious, chronic medical conditions (e.g. diabetes mellitus, kidney or liver disease); acute systemic infection; previous dental prophylaxis or periodontal treatment within 90 days of the baseline visit; requirement for antibiotic prophylaxis before dental procedures; use of non-tetracycline antibiotics within 6 weeks of baseline; use of tetracycline antibiotics within 3 months of baseline; hypersensitivity to tetracyclines; the need for long-term (>2 weeks) daily antacid therapy with antacids containing aluminium, calcium, or magnesium. Patients requiring chronic (2 weeks or more) antibiotic therapy or who participated in a periodontal clinical trial within 12 months of baseline were also excluded. Following screening to confirm eligibility, the subjects received sub-gingival SRP and then were randomly assigned to either the SDD or the placebo group. Subjects took either SDD or placebo bid for the 9-month duration of the study, and returned to the study centres for assessments at months 3, 6, and 9. Samples for microbiological analyses were collected at baseline, month 3, and month 9 at each of the two clinical centres and then shipped to the University of Florida where all microbial analyses were performed.

Faecal samples

Sample collection kits, containing a commode collection kit, latex gloves, a sterile pre-weighted sample tube, ziplock bag, a sterile 1 ml sample spatula, and written instructions for the collection and handling of the stool sample, were provided for each subject at the two sites. The subject was instructed to collect the stool sample either on the evening before or the morning of their scheduled clinical appointment. The sample was to be delivered to the clinic, refrigerated until shipped, and sent on ice by an overnight courier to arrive at the UF-PDRC microbiology laboratories the next day. The clinic was provided with foam-insulated shipping containers, freezer packs, and fax forms. The clinics were asked to notify the microbiology labs by fax when a sample was shipped.

Once received, the sample was unpacked and logged in as to subject number, sample period, and date collected. The sample was weighed, approximately 1 g was placed into 50 ml of anaerobically sterilized, pre-reduced (PRAS) Ringers solution, sonicated briefly to dispense the sample, and then serial 10-fold dilutions were made in PRAS Ringers solution. The target microorganisms, the media, incubation conditions, and confirmatory tests are given in Table 1.

Colony counts

Following the prescribed incubation period, the plates were examined for colony-forming units (CFUs). Anaerobic counts were determined from the plate dilution that gave rise to 30–300 CFUs. For all other media, colony counts were taken from plates with 30–300 CFUs if available. If less than 30 colonies were present on the least diluted plate, the colony number present was counted providing that more than a single colony was detected. A single colony on a plate was considered a ''0'' count.

Doxycycline-resistant colonies

The value of 4 μg of doxycycline was arbitrarily selected as a cutoff value based on the level of doxycycline achieved in the gingival fluid following normal dosage (Pascale et al. 1986). The number of colonies resistant to 4 μg of doxycycline per ml was determined as described above. The proportion was determined relative to the total anaerobic count.

A representative of each of the three most numerous doxycycline-resistant colony types, when present, was subcultured and identified. Sub-cultures were identified to genus and species, or bacterial taxa if a species name had not been assigned, based on the quantitation and qualitation of the bacterial

cellular fatty acids by capillary gas-liquid chromotography (GLC) as described by Moore et al. (1994), using a capillary GLC (Model 5890: Hewlett Packard, Avondale, PA, USA) equipped with an HP autosampler and connected to a Dell computer with MIDI software (Microbial ID, Newark, DE, USA) for instrument control and analysis. Computer-assisted identifications were based on the MIDI Anaerobic Bacteria Library (Moore5, Microbial ID, USA).

The proportion that each colony type contributed to the total doxycycline-resistant flora recovered was calculated. Following the identification process, antibiotic susceptibilities were determined, for each isolate that survived the identification procedure, to six antibiotics (tetracycline, doxycycline, minocycline, erythromycin, clindamycin, and amoxicillin) by agar dilution methodology.

Vaginal samples

A collection system for maintaining anaerobiosis (B-D Vacutainer Anaerobic Specimen Collection Systems: B-D and Co., Boston, MA, USA) was provided for the collection of vaginal swabs. Each system consisted of a sterile swab, a plunger, and a glass tube with an anaerobic atmosphere maintained by the presence of a palladium catalyst in a nitrogen–hydrogen gas atmosphere. Instructions were given for the use of the system in the collection of anaerobic bacteria specimens.

Upon receipt in the microbiology labs, the swab was removed under anaerobic conditions and placed in 10 ml of PRAS Ringers solution for further processing. The sample was sonicated and dispensed into the Ringers solution. A series of 10-fold dilutions were performed under anaerobic conditions and the sample was plated on selective and non-selective media.

The colonies obtained were enumerated as described above for total counts on each medium. Total counts were determined from the doxycycline-containing plates, representative isolates were sub-cultured, identified, and susceptibilities determined to the six antibiotics as previously described.

Statistical methods

Because of the numbers of subjects available and the fact that identical protocols were followed, the microbial data derived from both sites were combined together and analysed as a single data set. Inferential analyses were based on wet-weight-adjusted log(10)-transformed counts. Data cleaning algorithms were programmed to detect data discrepancies or inconsistencies prior to unblinding and analysis. Based on these algorithms, a few cases were found where the estimated doxycycline-resistant colony counts exceeded the estimated total colony counts. These cases were judged to reflect unreliable data and were dropped from the analysis.

The primary analyses sought to determine whether there were statistically significant differences between treatment groups at baseline, month 3, or month 9 with regard to doxycycline-resistant colony counts. Separate analyses were conducted for each of these visits using a 2-sample Median test. This non-parametric test was chosen because distributions of the data did not meet the requirements for parametric analyses because of outliers, and it is a conservative test that provides unbiased probability estimates under these circumstances.

Because the primary analyses revealed a statistically significant between-group difference at baseline (prior to administration of the study drug), which subsequently diminished and was no longer significant at post-baseline visits, a secondary analysis was sought that would examine changes from baseline directly. This longitudinal analysis was designed to be consistent with the primary analysis strategy. For each patient, the change from baseline to month 3 in the log wet-weight-adjusted count was calculated by subtracting the log of the baseline count from the log of the month 3 count. (Note that this is equivalent to analysing ratios of the counts, and is related to analysing percent change from baseline.) The discussion refers to this parameter as a log change from baseline. The 2-sample Median test was again used to compare the two treatment groups' month 3 log changes from baseline. The same approach was used for the month 9 log change from baseline data. In all cases p-values of 0.05 or less were considered statistically significant.

Results

The number of faecal and vaginal samples received from subjects receiving SDD or placebo at each sample period is given in Table 2. A higher dropout rate than expected resulted in only 55 of 69 subjects entered completing the study. The variation seen in the number of vaginal samples collected was because of the small number of female subjects consenting for the collection of these samples and the failure of the consenting subjects to return at the appointed times at 3 and 9 months.

Analyses of faecal flora

Microbial counts

The non-parametric analysis of log wet-weight-adjusted doxycycline-resistant counts was applied to the combined data set to determine if statistically significant differences were present between treatments at each sample period. In addition, the longitudinal analysis based on changes from baseline in log wet-weight-adjusted counts was performed. The resulting p-values are given in Table 3.

The only statistically significant difference ($p < 0.05$) detected between the two treatment groups occurred in the doxycycline-resistant counts present at the baseline sample period. At this point, the number of doxycycline-resistant counts recovered from the SDD treatment group was significantly higher than the placebo control. This finding reflects an imbalance between the groups prior to the administration of the study drug. However, no between-sample difference in doxycycline-resis-

Table 2. Numbers of samples received from subjects receiving SDD or placebo

Specimen	Baseline		3-month		9-month	
	SDD	Placebo	SDD	placebo	SDD	placebo
Fecal samples*	34	35	33	29	30	25
Vaginal samples†	12	10	6	8	10	7

*Of the 60 subjects entered into the study, only 55 completed the study.

†Only 22 subjects consented to vaginal sampling, of these only 14 and 17 provided samples at 3 and 9 months, respectively.

SDD, sub-antimicrobial dose doxycycline.

Table 3. Median (and inter-quartile range) log wet-weight-adjusted counts and two-sample median test analysis for differences between SDD and placebo treatments for the fecal flora

Microbial group	Baseline			3-months			9-months		
	SDD	Placebo	*p*-value	SDD	Placebo	*p*-value	SDD	Placebo	*p*-value
Total anaerobic counts	7.02 (0.92)	6.97 (1.49)	0.7173	7.16 (1.98)	7.03 (1.54)	0.9999	7.61 (1.38)	7.59 (1.15)	0.8129
Doxycycline-resistant counts	5.78 (1.07)	5.50 (1.28)	0.0279	7.19 (2.71)	5.80 (2.71)	0.0773	6.85 (0.78)	6.63 (0.70)	0.1294
Candida	1.79 (0.49)	2.10 (1.06)	0.9999	2.88 (1.12)	3.45 (0.99)	0.6256	2.43 (1.31)	2.23 (1.49)	0.9999
Total enterics	3.81 (2.24)	4.02 (1.67)	0.5963	4.02 (2.91)	3.91 (2.61)	0.8748	3.11 (2.41)	3.83 (1.50)	0.3368
Staphylococcus aureus	2.56 (0.67)	2.43 (0.88)	0.7782	3.38 (4.25)	4.42 (4.06)	0.3173	1.85 (1.70)	2.60 (0.39)	0.2542
Change from baseline: doxycycline-resistant counts	NA	NA	NA	1.71 (2.99)	0.15 (2.24)	0.0927	1.21 (1.42)	1.31 (1.42)	0.8984

SDD, sub-antimicrobial dose doxycycline; NA, not applicable.

Table 4. Percentage of the predominant microbial taxa recovered with resistance to 4 μg/ml of doxycycline (relative to total doxycycline-resistant isolates recovered)

Predominant microbial genera*	Baseline		3-month		9-month	
	SDD	placebo	SDD	placebo	SDD	placebo
Bacteroides	22.97	20.00	15.15	10.00	27.63	32.73
Bifidobacterium	6.76	12.31	19.70	18.00	10.53	3.64
Clostridium	2.70	1.54	4.55	0.00	1.32	5.45
Eubacterium	12.16	13.85	3.03	6.00	2.63	5.45
Fusobacterium	14.86	9.23	6.06	10.00	0.00	3.64
Prevotella	5.41	13.54	6.06	8.00	10.53	7.27
Unidentified Gram-negative rods†	13.51	10.77	22.73	22.00	23.68	18.18

*Identified by capillary GLC analysis of cellular fatty acids using the VPI Anaerobe database (Microbial ID Inc.).

†Gram-negative rods for which a strong identification could not be obtained.

SDD, sub-antimicrobial dose doxycycline.

tant counts was detected at either the 3- or the 9-month sample period. The baseline imbalance was no longer statistically significant at either of the post-baseline periods. This finding was confirmed in the longitudinal analysis.

Table 3 presents medians and inter-quartile range of the log wet-weight-adjusted counts for SDD and placebo treatments in the microbial groups examined from the faecal flora. Changes from baseline in doxycycline-resistant counts are also presented. The only statistically significant difference between groups was the imbalance at baseline in doxycycline-resistant counts, as noted above.

Antibiotic resistance

MICs to doxycycline, tetracycline, minocycline, erythromycin, clindamycin, and amoxicillin were determined for all isolates sub-cultured from the doxycycline-containing plates that survived the sub-culturing and identification process. These data were analysed for differences in identity of the isolates present between and within treatments, for changes in the resistance of these isolates to doxycycline, and for changes in multi-antibiotic resistance.

The proportions and identity of the major bacteria taxa recovered at each sample period demonstrated no significant microbial changes either between or within treatments. These data are summarized in Table 4.

Change in antibiotic susceptibilities

The MIC_{50}, MIC_{90}, and the range for doxycycline determined for the predominant bacterial groups recovered on medium containing 4 μg of doxycycline per ml are given in Table 5. This table summarizes the doxycycline resistance level for each treatment group at each sample period. The MIC_{50} and MIC_{90} designate the doxycycline concentration required to inhibit 50 and 90% of the doxycycline-resistant isolates, respectively. There were no differences in the values obtained either within the SDD treatment group or between the SDD and placebo treatment groups. The values obtained for the doxycycline-resistant bacteria recovered from the SDD group were essentially the same as those of the placebo group.

Multi-antibiotic resistance

Correlation coefficients were calculated between the MICs obtained for doxycycline and MICs determined for each of the other five antibiotics tested against the predominant bacteria recovered on medium containing 4 μg of doxycycline per ml (Table 6). As expected because of their close molecular and pharmacodynamic similarities, the strongest correlation was repeatedly found between doxycycline and minocycline and doxycycline and tetracycline. There was no consistently strong correlation found between doxycycline and any of the three non-tetracycline antibiotics tested. There was no indication that treatment with SDD tended to promote a greater likelihood of the development of cross- or multi-antibiotic resistance with any of the five additional antibiotics tested.

Analyses of vaginal flora

It was initially projected that about 50% of the entered subjects at each site would be female and that vaginal samples would be obtained from the majority of the females entered. Unfortunately, this was not true and the number of vaginal samples received was limited (Table 2). This number was further reduced by the fact that several of the samples failed to yield any growth on preliminary culture. This may have been because of sample technique or the failure of the anaerobic sample device to maintain anaeroboisis. Regardless of the cause, the result was that only a very limited number of samples were available for analyses. This had the most impact on the doxycycline-resistant isolates that were sub-cultured from the samples. The number of isolates obtained was too few to allow any meaningful analysis in regard to the particular bacterial species present.

Table 5. Susceptibilities to doxycycline (μg/ml) of predominant bacteria recovered, on medium containing 4 μg of doxycycline per ml, from each treatment group and sample period

	SDD			Placebo		
	Baseline	3-months	9-months	Baseline	3-months	9-months
MIC_{50}* (μg/ml)	16	32	16	16	32	8
MIC_{90}† (μg/ml)	32	>32	32	32	>32	>32
Range‡ (μg/ml)	0.06 to >32	0.06 to >32	0.25 to >32	0.06 to >32	0.06 to >32	0.25 to >32

*MIC_{50}: concentration of doxycycline required to inhibit 50% of the isolates tested.
†MIC_{90}: concentration of doxycycline required to inhibit 90% of the isolates tested.
‡Range: range of the concentrations of doxycycline required to inhibit all isolates tested with the highest concentration testing being 32 μg/ml.
SDD, sub-antimicrobial dose doxycycline; MIC, minimum inhibitory concentration.

Table 6. Correlations between the MICs obtained for doxycycline with each of the other five antibiotics tested against the predominant bacteria recovered from medium containing 4 μg of doxycycline per ml at each sample period for each treatment

Sample period and treatment	Minocycline	Tetracycline	Amoxicillin	Erythromycin	Clindamycin
Baseline SDD	0.497	0.589	0.178	0.283	0.272
Baseline Placebo	0.546	0.495	0.268	0.474	0.335
3-month SDD	0.616	0.592	0.099	0.124	0.420
3-month Placebo	0.618	0.653	0.083	0.195	0.628
9-month SDD	0.683	0.364	0.266	0.415	0.114
9-month Placebo	0.500	0.393	0.069	0.510	0.435

SDD, sub-antimicrobial dose doxycycline; MIC, minimum inhibitory concentrations.

Table 7. Statistical testing by unpaired *t*-test for differences between SDD and placebo treatments in the microbial groups examined from the vaginal flora

Microbial group	*p*-values (unpaired *t*-test)		
	Baseline	3-month	9-month
Total anaerobic counts	0.2210	0.8363	0.0749
Doxycycline-resistant counts	0.6940	0.5337	0.1447
Candida	ND	ND	ND
Lactobacilli	0.3245	0.4309	0.1626
Stapylococcus aureus	0.1274	0.2643	0.2623

ND, Not detected except in one subject; SDD, sub-antimicrobial dose doxycycline.

The number of samples was generally too small to allow the use of the non-parametric test strategy used for the analysis of the faecal data, as this test would have very little power to detect the targeted differences in the vaginal data. However, the number of samples was sufficient to permit the use of the unpaired *t*-test to analyse the culture counts to determine if significant changes occurred between the treatments. The distribution of the data was not normal and therefore could have led to false positives (Type I errors); hence, *p*-values less than 0.050 should be interpreted cautiously. The *p*-values obtained for between-treatment differences are given in Table 7. No apparent statistically significant differences were detected between SDD and placebo treatment at any time.

Discussion

The principal objective of this investigation was to determine if SDD exerted any detectable effect on the intestinal flora that could be attributed to antimicrobial activity. Doxycycline is normally given at a daily dose of 100 mg, following a loading dose of 200 mg, which yields steady-state levels of 2.1–2.9 μg/ml in the blood and 3–5 μg/ml in the gingival crevicular fluid (Pascale et al. 1986). Studies in human volunteers have demonstrated that 20 mg doxycycline, bid, yields mean steady-state serum concentrations of 0.4 μg/ml, which translates to $\sim$ 0.04 μg/ml of free doxycycline (Collagenex Pharmaceuticals 1996). This level of free doxycycline is considerably below the MIC determined in vitro for the vast majority of the bacteria isolated from human normal florae (Sutter et al. 1983). Even so, the possibility exists that levels obtained with SDD might be inhibitory for certain bacteria that are exquisitely sensitive to the tetracyclines. Therefore, in this study, a comprehensive microbial examination of the intestinal flora was conducted in an attempt to detect differences between and within treatments that could be attributed to an antimicrobial effect.

Previous studies of the sub-gingival flora have clearly demonstrated that long-term treatment with SDD exerted no detectable antimicrobial effect on the bacteria recovered from periodontal pockets. A comprehensive study, involving 60 periodontally diseased adult subjects, conducted independently at two separate institutions over a 9-month treatment period followed by a 3-month no-treatment period failed to detect any antimicrobial effect on the sub-gingival microbial flora. There was neither (a) an indication of a shift in the normal flora, (b) overgrowth or colonization by exogenous or endogenous pathogens, nor (c) an acquisition or an increase in antibiotic resistance (Thomas et al. 1998, Thomas & Walker 2000, Walker et al. 2000).

Although SDD has been approved by the Food and Drug Administration as an adjunct to periodontal therapy in adults, it was felt that a comprehensive study of its effects on the intestinal flora was indicated as an added measure of safety. Previous reports have suggested that low doses of tetracycline may promote the transfer of tetracycline resistance in the intestinal bacteria (Speer & Salyers 1992, Stevens et al. 1993, Salyers & Shoemaker 1995, Salyers et al. 1995). Such a transfer has been postulated to occur in the *Bacteroides* group through the transfer of a tet determinant (tetQ) on the resistant bacteria's DNA to previously susceptible bacteria. If this should occur, an increase in the proportion of bacteria present with resistance to doxycycline would be detected.

There was an imbalance in the doxycycline-resistant counts between the two treatment groups prior to the administration of the drug. This imbalance was statistically significant at baseline but not at 3 or 9 months. However, a trend toward statistical significance was still noted at 3 months but not at 9 months. Analyses based on medians as well as the means reflected this trend at 3 months

but not at 9 months. Examination of the predominant taxa recovered with resistance to 4 μg/ml of doxycycline at all sample periods did not show any microbial differences. There was a slight increase in the number of *Bacteroides* that were recovered after 9 months of SDD treatment. However, this increase was less than that seen in the placebo group and is likely reflective of the variation that occurs in a complex microflora since decreases in both groups were noted at 3 months. Therefore, we concluded that the level of doxycycline present in the intestines was too low to promote or stimulate resistance. The trend detected in the number of doxycycline-resistant bacteria at 3 months is believed to be because of the initial imbalance present prior to drug administration and possibly to microbial variation because of dietary changes or to microbial sampling. As no differences between treatment groups were detected at 3 months in the predominant taxa recovered or in the MICs obtained, it was concluded that the trend observed at 3 months was not drug related.

The examination of the bacterial species recovered on the doxycycline-containing medium demonstrated no shift that was microbiologically significant in the SDD group relative to the placebo group. Longitudinal changes occurred, not in the major genera present, but in the proportion of these genera found in both groups over the course of the investigation. As these changes were similar in each treatment group, the differences noted over time were not thought to be related to the effect of doxycycline on the intestinal flora but most likely to changes in diet during the 9-month period.

There were no differences in the MIC range, MIC_{50} or MIC_{90} for either group at the same sample period. There was an apparent increase in the MIC_{50} and the MIC_{90} at the 3-month sample in each group. However, as susceptibility/resistance profiles are determined using increasing twofold concentrations of an antibiotic, differences of less than two dilutions are not considered microbiologically significant but fall within the error of the method.

The number of vaginal samples received was much lower than was initially expected. Added to this was the fact that some of the early samples did not survive shipping or else were not properly collected. In several instances, continuous samples were not available at all sampling periods. Although the number of samples was too low to provide a conclusive answer, the data indicate that SDD did not exert an antimicrobial effect on the vaginal flora. Clearly, there was no tendency toward vaginal candidiasis as yeast was only recovered from a single subject. The clinical data from 210 subjects in the efficacy portion of this study also support this conclusion in that there were only three incidences of vaginitis (two in the SDD group and one in the placebo group) over the 9-month course of treatment (Preshaw et al. 2004). Hence, the microbiological findings were consistent with the clinical findings.

In conclusion, there were no significant differences between treatments in either the faecal or vaginal microflora for any of the microbial parameters examined. The only statistically significant value obtained occurred in the number of doxycycline counts recovered at the baseline sample period, before the study drug was administered. This baseline imbalance was observed before the drug was administered to subjects and disappeared over time. It was therefore not related to the study drug.

Further, these data suggest that a 9-month regimen of SDD (a) did not result in a shift in the normal faecal or vaginal flora, (b) did not result in the overgrowth or colonization of either flora by opportunistic pathogens, (c) did not result in an increase in the number of doxycycline-resistant bacteria recovered, and (d) did not result in the development of multi-antibiotic resistance.

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References

Barza, M., Giuliano, M., Jacobus, N. V. & Gorbach, S. L. (1987) Effect of broad-spectrum parental antibiotics on ''colonization resistance'' of intestinal microflora of humans. *Antimicrobial Agents and Chemotherapy* **31**, 723–727.

Collagenex Pharmaceuticals I. (1996) *New Drug Application #50–744* (50–744). Food and Drug Administration.

Di Rosa, P. A. & Masttrantonio, P. (1993) Anaerobic bacteria and gynecologic infections. *Recenti Progressi in Medicina* **84**, 794–800.

Dominque, P. A., Sadhu, K., Costerton, J. W., Bartlett, K. & Chow, A. W. (1991) The human vagina: normal flora considered as an in situ tissue-associated, adherent biofilm. *Genitourinary Medicine* **67**, 226–231.

Franks, A. H., Harmsen, H. J., Raangs, G. C., Jansen, G. J., Schut, F. & Welling, G. W. (1998) Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. *Applied Environmental Microbiology* **64**, 3336–3345.

Gall, L. S. (1970) Normal fecal flora of man. *American Journal of Clinical Nutrition* **11**, 1457–1465.

Giuliano, M., Barza, M., Jacobus, N. V. & Gorbach, S. L. (1987) Effect of broad-spectrum parental antibiotics on the composition of intestinal microflora of humans. *Antimicrobial Agents and Chemotherapy* **31**, 202–206.

Holdeman, L. V., Goode, I. J. & Moore, W. E. C. (1976) Human fecal flora: variation in bacterial composition within individuals and a possible effect of emotional stress. *Applied Environmental Microbiology* **31**, 359–375.

Keane, F. E., Ison, C. A. & Taylor-Robinson, D. (1997) A longitudinal study of the vaginal flora over a menstrual cycle. *International Journal of STD & AIDS* **8**, 489–494.

Larsen, B. (1993) Vaginal flora in health and disease. *Clinical Obstetrics and Gynecology* **36**, 107–121.

Mai, V. & Morris, J. G. Jr. (2004) Colonic bacterial flora: changing understandings in the molecular age. *Journal of Nutrition* **134**, 459–464.

Masfari, A. N., Duerden, B. I. & Kinghorn, G. R. (1986) Quantitative studies of vaginal bacteria. *Genitourinary Medicine* **62**, 256–263.

Moore, L. V. H., Bourne, D. M. & Moore, W. E. C. (1994) Comparative distribution and taxonomic value of cellular fatty acids in thirty-three genera of anaerobic gram-negative bacilli. *International Journal of Systemic Bacteriology* **44**, 338–347.

Moore, W. E. C. & Holdeman, L. V. (1974) Human fecal flora: the normal flora of 20 Japanese-Hawaiians. *Applied Microbiology* **27**, 961–979.

Pascale, D., Gordon, J., Lamster, I., Mann, P., Seiger, M. & Arndt, W. (1986) Concentrations of doxycycline in human gingival fluid. *Journal of Clinical Periodontology* **13**, 841–844.

Preshaw, P., Hefti, A., Novak, M., Michalowicz, B., Pihlstrom, B., Schoor, R., Trummel, C., Dean, J., Van Dyke, T., Walker, C. & Bradshaw, M. (2004) Subantimicrobial dose doxycycline enhances the efficacy of scaling and root planing in chronic periodontitis: a multicenter trial. *Journal of Periodontology* **75**, 1068–1076.

Rosenstein, I. J., Morgan, D. J., Sheehan, M., Lamont, R. F. & Taylor-Robinson, D. (1996) Bacterial vaginosis in pregnancy: distribution of bacterial species in different gram-stain categories of the vaginal flora. *Journal of Medical Microbiology* **45**, 120–126.

Salyers, A. A. & Shoemaker, N. B. (1995) Conjugative transposons: The force behind the spread of antibiotic resistance genes among *Bacteroides* clinical isolates. *Anaerobe* **1**, 143–150.

Salyers, A. A., Shoemaker, N. B. & Li, L.-Y. (1995) In the driver's seat: the Bacteroides conjugative transposons and the elements they mobilize. *Journal of Bacteriology* **177**, 5727–5731.

Speer, B. S. & Salyers, A. A. (1992) Bacterial resistance to tetracycline: mechanisms, transfer, and clinical significance. *Clinical Microbiology Reviews* **5**, 387–399.

Stark, C. A., Edlund, C., Sjostedt, S., Kristensen, G. & Nord, C. E. (1995) Antimicrobial resistance in human oral and intestinal anaerobic microfloras. *Antimicrobial Agents and Chemotherapy* **37**, 1665–1669.

Stevens, A. M., Shoemaker, N. B., Li, L. Y. & Salyers, A. a. (1993) Tetracycline regulation of genes on Bacteroides conjugative transposons. *Journal of Bacteriology* **175**, 6134–6141.

Sutter, V. L., Jones, M. J. & Ghoneim, A. T. M. (1983) Antimicrobial susceptibilities of bacteria associated with periodontal disease. *Antimicrobial Agents and Chemotherapy* **23**, 483–486.

Thomas, J. G., Metheny, R. J., Karakiozis, J. M., Wetzel, J. M. & Crout, R. J. (1998) Long-term sub-antimicrobial doxycycline (Periostat) as adjunctive management in adult periodontitis: effects on subgingival bacterial population dynamics. *Advances in Dental Research* **12**, 32–39.

Thomas, J. & Walker, C. (2000) Long-term use of subantimicrobial dose doxycycline does not lead to changes in antimicrobial susceptibility. *Journal of Periodontology* **71**, 1472–1483.

Walker, C., Thomas, J., Nangó, S., Lennon, J., Wetzel, J. & Powala, C. (2000) Long-term treatment with sub-antimicrobial dose doxycycline exerts no antibacterial effect on the subgingival microflora associated with adult periodontitis. *Journal of Periodontology* **71**, 1465–1471.

Zhou, X., Bent, S. J., Schneider, M. G., Davis, C. C., Islam, M. R. & Forney, L. J. (2004) Characterization of vaginal microbial communities in adult healthy women using cultivation-independent methods. *Microbiology* **150**, 2565–2573.

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Clinical Relevance

The findings in this study that no significant microbial differences could be detected between drug and placebo treatments in either the faecal or vaginal microflora lend support to the concept that long-term use of sub-antimicrobial doxycycline is safe and does not promote colonization by opportunistic pathogens or lead to an increase in resistance to doxycycline or other antibiotics.