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ORIGINAL ARTICLE

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Intraoral dissemination of treponemes after periodontal therapy

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Abstract Oral treponemes are related to chronic periodontitis, but the effect of periodontal therapy on the majority of treponemal species is unknown. The aim of this prospective study was to evaluate the dynamics in prevalence profiles of treponemes in different habitats of the oral cavity. Thirty-five patients with chronic periodontitis were randomly assigned to mechanical debridement alone (control group) or systemic amoxicillin/metronidazole plus chlorhexidine (test group). Subgingival and mucous membrane plaque samples were taken at baseline, after 10 days, and during supportive periodontal therapy at 3, 6, 9, 12, 18, and 24 months. *T. denticola*, *T. lecithinolyticum*, *T. maltophilum*, *T. socranskii*, *T. vincentii*, and treponemal phylotypes I–VII were detected using polymerase chain reaction (PCR) and dot blot analysis. For the majority of the assessed treponemes, a significant intragroup increase in prevalence in the different habitats ($P<0.05$) occurred over the study course but, compared to debridement alone, adjunctive antimicrobial therapy resulted in a nonsignificant trend toward lower prevalence in the subgingival habitat. In no case were treponemes eradicated from the oral cavity. After both therapies, possibly new infection with and/or dissemination of *Treponema* ssp. occurred, which led to treponemes recovering in different habitats and to increased intraoral prevalence. The prescribed adjunctive antimicrobial therapy may limit this increase in the subgingival region.

Keywords Antimicrobial therapy · Chronic periodontitis · Periodontal pathogen · Prevalence · Treponemes

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Introduction

Periodontitis is a chronic infectious disease characterized by inflammation related to intraoral biofilms harboring a variety of putative periodontopathogenic micro-organisms. These biofilms may be found in the subgingival area covering the root surface and the epithelial lining of the periodontal pockets as well as on oral mucous membranes. Biofilms detected in patients suffering from severe chronic periodontal disease are characterized by great microbiological diversity. One important group associated with periodontitis are the *Treponema* species. These are commonly present in periodontally affected and extracrevicular sites [17], and there is strong evidence that these bacteria are involved in the etiology of chronic periodontal disease. This assumption was based on the finding that treponemes were detected in sites with periodontitis [15, 24] and that microbiological complexes containing *T. denticola*, for example, are strongly related to increased pocket probing depths and bleeding on probing [23].

In past periodontal research, oral spirochetes have usually been identified by dark-field microscopy or culture-based methodologies. While spirochetal morphotypes were identified by dark-field microscopy in proportions representing up to 60% of the total bacterial count [13, 22], culture-based methods resulted in a recovery rate of only about 1% of the total cultivable microflora [18, 27]. These technical deficiencies complicated the longitudinal assessment of their etiological role and the effect of periodontal therapy on this group of putative periodontal pathogens.

Up to now, the effect of periodontal therapy on oral treponemes has been evaluated for single species only,

but the influence of mechanical debridement and adjunctive antimicrobial therapeutic approaches is unknown at species or group level for the majority of these organisms. However, recently developed molecular biological techniques such as 16S rDNA analysis facilitate the identification of oral treponemes at the group- or species-specific level and may also enable even uncultivable treponemes to be detected [3, 5, 19, 20].

The aim of the present study was therefore to evaluate the short- and long-term effects of mechanical debridement with and without adjunctive antimicrobial therapy on the prevalence profiles of five treponemal species (*T. vincentii*, *T. denticola*, *T. maltophilum*, *T. lecithinolyticum*, *T. socranskii*) and members of phylotypes I–VII in the subgingival habitat and on oral mucous membranes.

Patients and methods

Patients

Thirty-five patients with untreated, moderate-to-severe chronic periodontitis entered this study. The subjects were from a consecutive sample of patients with a mean age of 51.1±10.7 years (Table 1) recruited from the Department of Periodontology, University of Würzburg, Germany, and on whom clinical reports have been published previously [6, 9]. All subjects signed informed consent forms approved by the Ethics Committee of the Medical Faculty, University of Würzburg, Germany. Patients receiving systemic antibiotics or periodontal therapy during the 6 months prior to baseline investigation or receiving systemic antibiotics during the course of the study were excluded, as were those with systemic conditions affecting periodontal health.

Treatment

At baseline, the patients were randomly assigned to mechanical debridement and oral hygiene instruction, either alone (control group n=17) or in combination with an 8-day adjunctive antimicrobial regime consisting of systemic administration of 250 mg of metronidazole t.i.d., 375 mg of amoxicillin t.i.d. [26], and supragingival irrigation with 0.06% chlorhexidine digluconate once daily (test group n=18). The adjunctive antimicrobial therapy was started immediately after completion of scaling. Supra- and subgingival debridement was carried out with Gracey curettes under local anesthesia by senior dental students under supervision of a qualified resident specializing in the field of periodontology until all root surfaces felt smooth. If a dental student was unable to reach this goal, the treatment was completed by the qualified periodontal resident.

Ten days after completion of supra- and subgingival scaling, the test patients were asked to return the remaining tablets of their prescribed medications. Compliance with the antibiotic therapy was estimated by counting the numbers of tablets remaining. All patients received supportive periodontal therapy consisting of full-mouth supragingival debridement at 3, 6, 9, 12, 18, and 24 months. At baseline and the 12- and 18-month appointments, attachment level measurements were performed, and all sites exhibiting a probing attachment loss of 2 mm or more from baseline underwent repeated subgingival scaling.

Microbiological sampling

Swab samples were taken from the subgingival plaque and oral mucous membranes of all patients before mechanical debridement at baseline, after 10 days, and during systemic periodontal therapy at 3, 6, 9, 12, 18, and 24 months. At all study appointments, subgingival plaque samples were taken with sterile curettes from the same four sites (deepest site per quadrant at study onset, with a pocket probing depth of $\geq$6 mm). The samples were pooled and placed in 1 ml of reduced transport fluid [25]. Plaque samples from oral mucous membranes (dorsum of the tongue, both tonsils, and both buccal mucosae) were collected with sterile cotton swabs and suspended each in 1 ml of reduced transport fluid. Before processing, all samples were stored in liquid nitrogen.

Microbiological analysis

The prevalence of different oral treponemes at any given time was assessed using 16S rDNA PCR amplification followed by dot blot hybridization with species- or group-specific oligonucleotide probes as described earlier [19]. Five *Treponema* species (*T. vincentii*, *T. denticola*, *T. maltophilum*, *T. lecithinolyticum*, *T. socranskii*) and seven phylogenetic groups of oral treponemes were detected with species-specific (TVIN, TDEN, TMAL, TLEC, TSOC) and group-specific (TRE I–VII) probes, respectively. All probes except TLEC (5'-CAC TCT CAG AAA GGA GCA AGC TCC-3') have been published earlier [19]. Their sequences were deposited in ProbeBase [16], an online resource for rRNA-targeted oligonucleotide probes allowing fast specificity checks (http://www.microbial-ecology.de/probebase/index.html).

For PCR amplification and dot blot analysis, the samples were processed as previously described. Briefly, bacterial DNA was extracted and amplified in vitro by PCR [19]. Successful amplification was verified by agarose gel electrophoresis. Dot blot hybridization of PCR-amplified plaque material was used to detect minute quantities of treponemes and determine their presence in individual patients. A total of 34 amplicons from either recombinant clones retrieved from the original 16S rRNA gene library [3], known cultivable treponemes, or other putative periodontal pathogens were included as controls in all dot blot hybridizations.

All probes were labeled nonisotopically with digoxigenin (DIG)-ddUTP (Boehringer, Mannheim, Germany) and detected by chemiluminescence according to the manufacturer's recommendations. All hybridizations were done at 54°C. Stringency washes were performed at temperatures ranging from 56°C to 64°C with a washing buffer containing 5× sodium saline citrate (SSC) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.2% sodium dodecyl sulfate (SDS) or 0.1× SSC-0.1% SDS, depending on the respective probe. Digoxigenin-labeled probes were detected with anti-DIG-alkaline phosphatase conjugates after adding the appropriate sub-

Table 1 Demographic patient data and clinical parameters at baseline (means±SD). *MD AB* Patients receiving mechanical debridement plus adjunctive antimicrobial therapy, *MD* patients receiving mechanical debridement alone

	MD+AB	MD
N subjects	18	17
Age (years)	48.9±11.0	53.2±9.9
N females	11	8
Mean *n* teeth	24.0±4.2	19.5±7.8
Mean percentage of sites with pocket probing depth $\leq$3 mm	65.2±14.2	64.2±15.6
Mean percentage of sites with pocket probing depth 4–6 mm	29.8±9.7	32.5±12.7
Mean percentage of sites with pocket probing depth $\geq$7 mm	5.0±6.0	3.3±4.7
Mean percentage of sites with bleeding on probing	44.3±17.9	63.4±17.8

strate according to the manufacturer's recommendations. X-ray films were exposed to the membranes for 2 h to 12 h. After stripping with 0.2 N NaOH-0.1% SDS (stripping buffer), identical membranes were used for multiple hybridization experiments with the probes mentioned above

Statistical analysis

The microbiological prevalence profiles of the assessed *Treponema* spp. were calculated as the percentage of patients colonized in the test or control group. Changes in the prevalence of the assessed putative pathogens were examined in the subgingival and at oral mucous membranes. Patients were considered to be subgingivally colonized if the putative pathogen were detected in the (pooled) subgingival plaque sample. The oral mucous membranes of a patient were considered to be colonized if the putative pathogen was detected on any of the assessed mucous membrane samples (dorsum of the tongue and/or both tonsils, and/or both buccal mucosae).

Intergroup differences in the antimicrobial efficacy of the two therapeutic modes performed (mechanical debridement with or without adjunctive antimicrobial therapy) on the detection frequency of the assessed treponemes were determined by two-way analysis of variance with repeated measurements for one factor. Differences in intragroup bacterial prevalence at different time points were detected by Cochran's test. Because this descriptive analysis has an explorative character, *P* values were not adjusted, despite multiple comparisons. *P* values of <0.05 were considered to be statistically significant. A putative periodontal pathogen was considered eradicated from the oral cavity (subgingival plaque and oral mucous membranes) if it were detected at baseline but not in samples taken from the same areas during further appointments.

Results

Both therapy regimens had marked influence on the prevalence in the oral cavity of most of the assessed treponemes. Changes in the prevalence profiles were most impressive in the subgingival habitat and less pronounced on oral mucous membranes. Compared with the other assessed species and groups, the intraoral prevalence of phylotypes VI and VII was low and remained unaffected by the therapeutic approaches on these levels throughout the course of the study (no significant inter- or intragroup differences, data not shown).

Prevalence dynamics in the subgingival habitat

The subgingival prevalence of all species differed significantly ($P<0.05$) within the groups at the various sample dates (Fig. 1). Initially, the subgingival detection frequency of *T. vincentii*, *T. denticola*, and *T. lecithinolyticum* in both test and control groups was low as compared to the baseline prevalence of *T. maltophilum* and *T. socranskii*. The prevalence of *T. vincentii* and *T. denticola* increased from baseline levels, dropped tran-

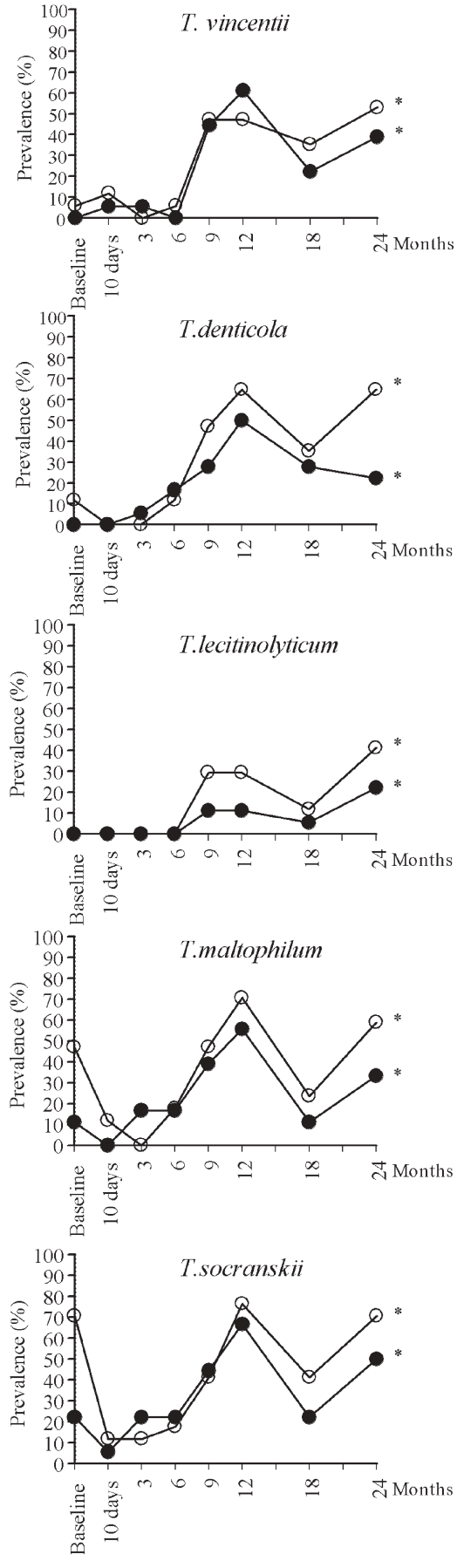
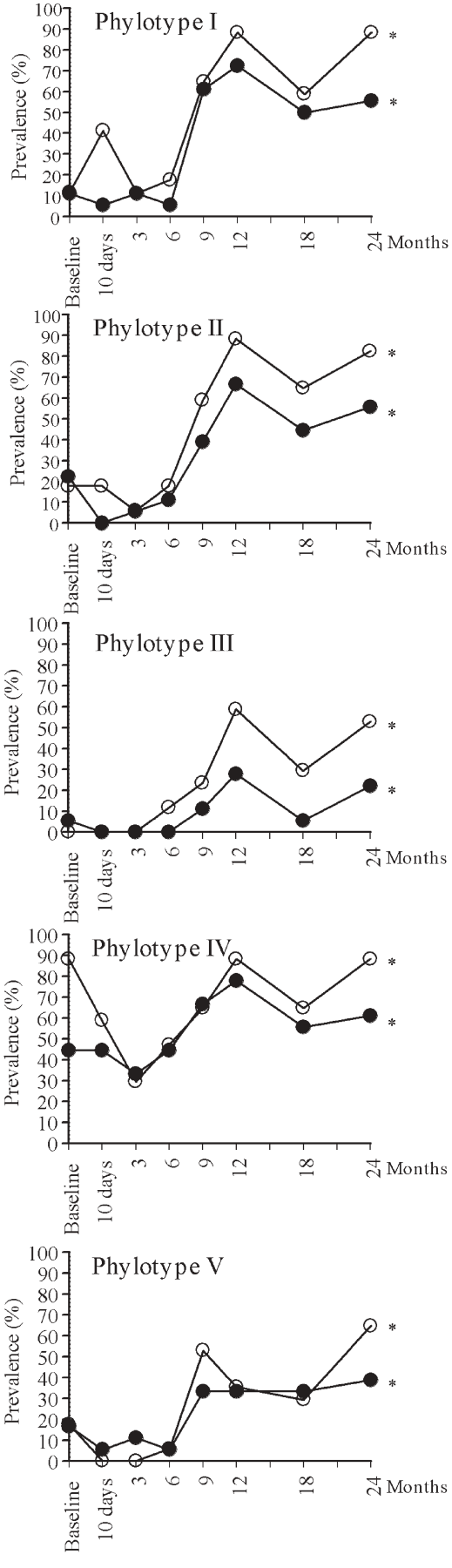


Fig. 1 Subgingival prevalence profiles of *T. vincentii* (member of phylogroup I), *T. denticola* (member of phylogroup II), *T. maltophilum* and *T. lecithinolyticum* (members of phylogroup IV), and *T. socranskii* in test (·) and control (◯) patients. *Asterisks* indicate significant intragroup differences in species prevalence over time ($P<0.05$). No significant intergroup differences were found



siently at 18 months, and increased again at 24 months toward 12-month levels. *T. lecithinolyticum* was not detected initially but, beginning at 6 months, this species was detected, and its prevalence increased up to the 12-month follow-up, and the detection frequency dynamic was similar to those of *T. vincentii* and *T. denticola*. In contrast, the prevalence of *T. socranskii* and *T. maltophilum* dropped during therapy but relapsed to baseline levels from 10 days onwards, with a transient decrease from 12 to 18 months (Fig. 1).

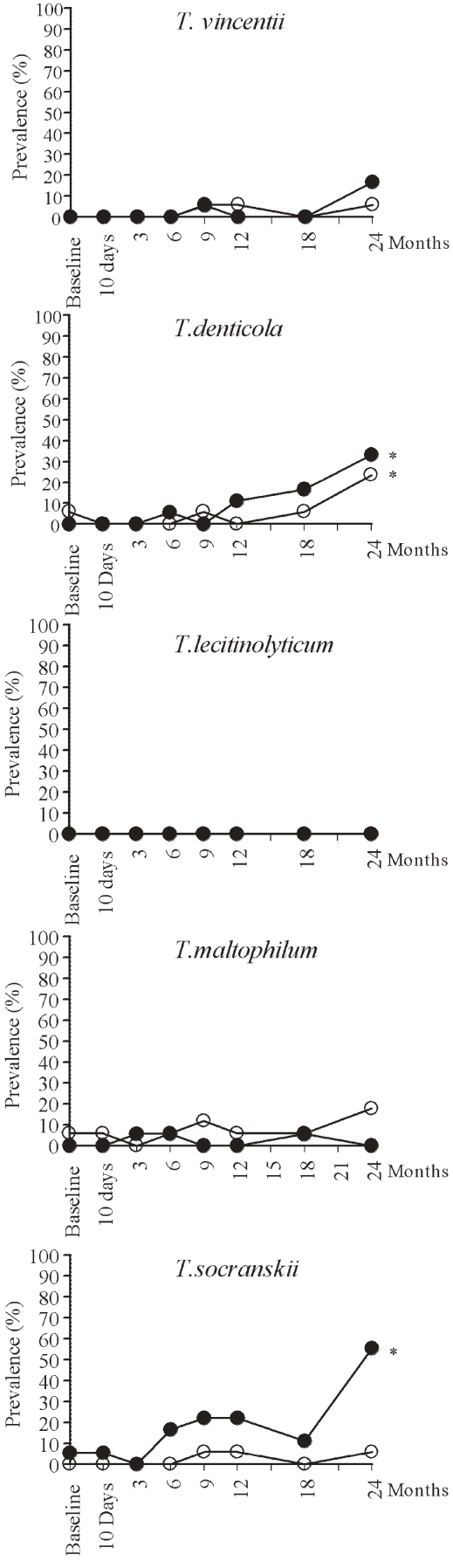
The subgingival prevalence profiles of treponemal phylotypes I, II, III, and V differed significantly ($P<0.05$) within a patient group over time. All of these phylotypes showed overall increases in subgingival detection frequency over the course of the study (Fig. 2). In contrast, phylotype IV showed a high initial prevalence at subgingival sites which, like *T. maltophilum*, decreased within the first 3 months after therapy, increased at least to baseline levels at 12 months, dropped transiently at 18 months, and returned to approximately 12-month levels at 24 months after baseline. The detection frequency of phylotype IV differed significantly ($P<0.05$) over the study course within the test and control groups.

On oral mucous membranes (Fig. 3), a continuous, significant increase in the detection frequency of *T. denticola* was recorded in both test and control groups throughout the study period. The detection frequency of *T. vincentii* and *T. maltophilum* was affected neither by mechanical debridement alone nor with adjunctive antimicrobial therapy. *T. lecithinolyticum* was the only species never detected on oral mucous membranes throughout the course of the study. The prevalence of *T. socranskii* overall increased significantly in test patients only but remained unchanged in the control group. However, on oral mucous membranes, the prevalence of all species as well as changes in their prevalence were less pronounced overall than in the subgingival habitat. The detection frequency of phylotypes I and V increased significantly ($P<0.05$) over time in the test and control groups, whereas the prevalences of phylotypes II and III remained unchanged. Similar to the prevalence dynamics at subgingival sites, phylotype IV showed a high initial prevalence on oral mucous membranes, and the detection frequency within groups was significantly ($P<0.05$) different throughout the study period (Fig. 4).

Effect of therapy mode

Regarding the treatment effects in the subgingival environment, there was a nonsignificant trend toward lower species and phylotype prevalence after mechanical debridement in conjunction with antimicrobial therapy

Fig. 2 Subgingival prevalence profile of treponeme phylotypes I, II, III, IV, and V in test (·) and control (◯) group patients. *Asterisks* indicate significant intragroup differences in species prevalence over time ($P<0.05$). No significant intergroup differences were found



compared to debridement alone (Fig. 1, Fig. 2). With the exception of phylotypes I and IV, this trend was not detected on the oral mucous membranes. None of the treponemal species or groups detected at baseline were eradicated from the oral cavity over the entire study period by the therapies performed (data not shown). For clinical results and the effect of detected oral *Treponema* ssp. on the stability of attachment levels, see [6].

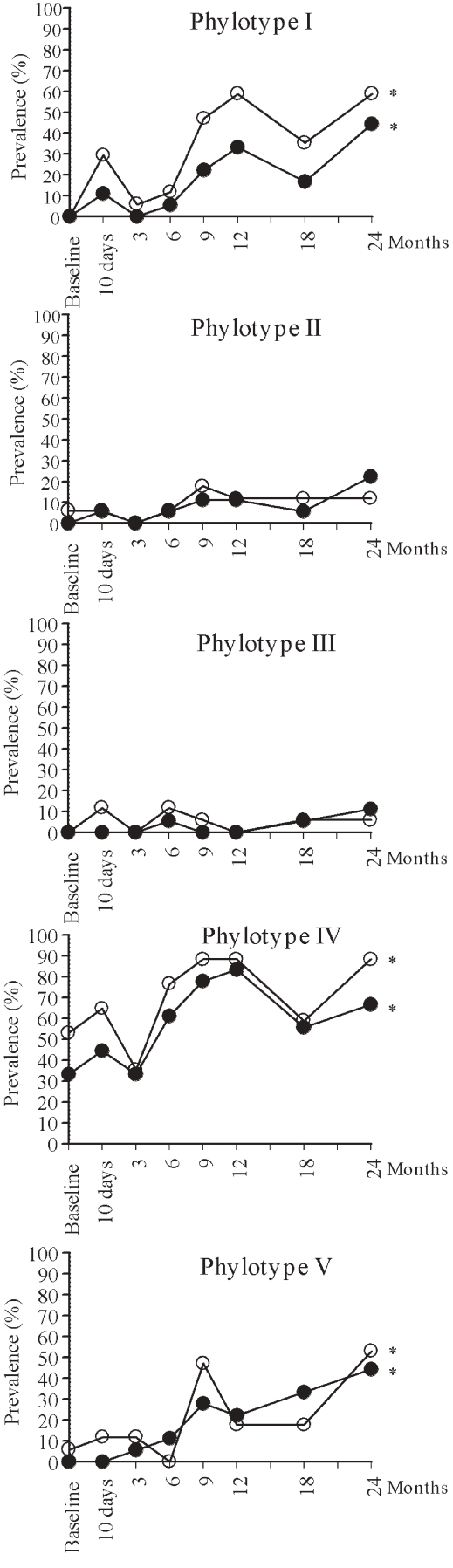
Discussion

This investigation is the first explorative approach assessing long-term changes in the detection frequency of *Treponema* spp. in different habitats in the oral cavity before and after the application of standard periodontal therapies. One finding of this study was that both therapy regimens affected the prevalence of *Treponema* spp. most notably in the subgingival habitat. Overall, two major scenarios were common throughout the study period: (1) the absence (*T. lecitinolyticum*) or low initial prevalence (e.g., in *T. vincentii*) seen from baseline to 3 months was followed by a pronounced increase beginning at approximately 6 months and (2) a high baseline prevalence, e.g., of subgingival *T. socranskii*, decreased transiently directly after therapy and was followed by a relapse to baseline levels beginning at approximately 6 months (Fig. 1).

The noted variations in prevalence may suggest that depletion of the subgingival habitat may be followed by new infection (*T. lecitinolyticum*) and/or dissemination of the putative pathogens with initially low prevalence (e.g., *T. vincentii*, *T. denticola*, and phylotype III) and their recovery in habitats, resulting in increased prevalence. Due to oral treponemes having adherent properties to epithelial cells [2] and possibly their own coaggregation abilities to important biofilm-bridging organisms such as *Fusobacteria nucleatum* [11, 21], the mechanical dispersal of dental plaque containing treponemes may lead to species and phylotype diffusion, resulting in the colonization of new habitats.

Previous studies using dark-field microscopy indicated that the proportion of oral spirochetes declined after mechanical debridement [12, 14], and decreased or similar mean counts for *T. denticola* and *T. socranskii* were detected by means of DNA probes after mechanical debridement alone and in combination with adjunctive amoxicillin or metronidazole [4, 8]. While the present study represents a qualitative approach and the prevalence of the different oral *Treponema* ssp. was considered on a patient basis, it is difficult to draw direct comparisons to

Fig. 3 Oral mucous membrane prevalence profiles of *T. vincentii* (member of phylogroup I), *T. denticola* (member of phylogroup II), *T. maltophilum* and *T. lecithinolyticum* (members of phylogroup IV), and *T. socranskii* in test (·) and control (◯) group patients. *Asterisks* indicate significant intragroup differences in species prevalence over time ($P<0.05$). No significant intergroup differences were found



these studies. However, the data of the present study enable a comprehensive view of the prevalence not only of cultured species but also of yet-to-be-cultured oral treponemes and add new, previously unknown insights into the long-term changes of these putative pathogens' prevalence following periodontal therapy.

The course of the treponemes' prevalence dynamic was not expected, and it might be taken into account that storage time influenced the results. However, hybridization of the identical PCR products with a probe specific for *Fusobacteria* ssp. showed positive results in more than 90% of samples during the whole study period (data not shown). This indicates that there is no evidence for DNA degradation or decreased hybridization efficacy in the samples due to storage times, and therefore a systematic bias in the analysis of the bacterial samples is implausible.

For some of the treponemal species or groups assessed, e.g., *T. socranskii*, *T. maltophilum*, and phylogroup I, a transient and low decrease in the subgingival detection frequency was registered at 18-month follow-up. This decrease may be due to the fact that although, in the present study, principally supragingival supportive periodontal therapy was performed [9], additional subgingival debridement was performed at the 12- and 18-month follow-ups at sites showing attachment loss compared to baseline [7]. While, in a recent systematic review, supragingival and subgingival supportive therapy regimes were found to be comparable with respect to clinical results [10], it is unclear whether full-mouth, subgingivally performed, supportive periodontal therapy would have led to different results concerning the prevalence of *Treponema* spp. However, an increased detection frequency of *T. denticola* was also recorded at all periodontally affected sites in patients undergoing supra- and subgingival debridement routinely [1]. Initial periodontal therapy (approximate treatment time 2 h per quadrant) as well as the supportive treatment were performed by dental students under the supervision of a qualified periodontal resident. The overall clinical results [7, 9] were in line with what could be expected from the literature. Therefore, a systematic bias for the prevalence of *Treponema* ssp. caused by the clinicians performing the treatment can be ruled out.

The clinical effect of the overall increasing *Treponema* ssp. prevalence, i.e., their influence on stability or loss of attachment, cannot be finally estimated. A recently published paper used an explorative, multivariate approach to assess the effect of oral *Treponema* spp. prevalence profiles on the stability of periodontal attachment [6]. The recurrent detection of *Treponema* spp. at sites with initial pocket probing depths of not more than 6 mm was associated with a trend toward progression of attachment loss.

Fig. 4 Oral mucous membrane prevalence profile of treponeme phylotypes I, II, III, IV, and V in test (·) and control (◯) group patients. *Asterisks* indicate significant intragroup differences in species prevalence over time ($P<0.05$). No significant intergroup differences were found

Conclusion

After both modes of therapy, possibly a new infection with and/or dissemination of *Treponema* ssp. occurred, which led to treponemes recovering in different habitats and their increased prevalence in the oral cavity. Compared to debridement alone, the prescribed adjunctive antimicrobial therapy possibly limits this increase and may show a nonsignificant trend toward lower treponeme detection frequency in the subgingival habitat.

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