

REVIEW ARTICLE

Shifts in *Campylobacter* species abundance may reflect general microbial community shifts in periodontitis progression

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Background: Oral *Campylobacter* species have been found to be associated with periodontitis progression. While the etiological significance of *Campylobacter rectus* is quite established, the association of *C. gracilis*, *C. concisus*, and *C. curvus* with health or disease remains contradictory.

Objectives: This study hypothesizes that the proportion of species within the *Campylobacter* genus rather than the absolute abundance of a single species is a suitable indicator for periodontitis progression.

Design: Subgingival plaque from 90 periodontitis patients and gingival sulcus fluid of 32 healthy individuals were subjected to a newly developed nested PCR approach, in which all *Campylobacter* spp. were amplified simultaneously. The resulting mixture of 16S-rRNA-gene-amplicons were separated by single-stranded conformation polymorphism (SSCP) gel electrophoresis, followed by sequencing and identification of excised bands and relative quantification of band intensities. In all samples, the abundance of selected periodontitis marker species was determined based on DNA hybridization on a microarray.

Results: The highly prevalent *Campylobacter* community was composed of varying proportions of *C. rectus*, *C. gracilis*, *C. concisus*, and *C. curvus*. Cluster analysis based on SSCP-banding pattern resulted in distinct groups which in turn coincided with significant differences in abundance of established periodontitis marker species (*Tannerella forsythia*, *Porphyromonas gingivalis*, and *Fusobacterium nucleatum*) and progression.

Conclusions: The shift in the *Campylobacter* community composition seems to display the general microbial community shift during clinical progression in a simplified manner. The focus on members of the *Campylobacter* in this study suggests that this genus can be an indicator of ecological changes in the subgingival oral microflora.

Keywords: oral *Campylobacter*; periodontitis progression; microbial ecology; SSCP; *ParoCheck* genechip

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Periodontitis is an inflammatory disease of the periodontium, caused by different bacterial consortia, the so-called ‘microbial complexes’ (1). Socransky et al. visualized the progression of periodontitis as a pyramid with ‘colored’ components representing each a distinct microbial complex (2). While the complexes at the base of the pyramid (e.g. green and yellow complexes) comprise species that are thought to thrive under healthy conditions or at a very early state of periodontitis, the species of the orange complex become more dominant in an advanced stage and are thought to bridge base complexes and the red complex. The latter is

forming the pyramidal roof at a late or final stage in progression.

Evidence exists that different oral *Campylobacter* species are associated with different stages of periodontitis progression. For instance, *Campylobacter rectus* and *C. gracilis* could be detected at elevated levels in diseased subgingival sites compared with healthy sites (3–6). Throughout the literature, *C. rectus* (former name *Wolinella recta*) is consistently described as being more prevalent and abundant in initial and established periodontitis. *C. gracilis*, although one of the most dominant *Campylobacter* species associated with periodontitis (4),

was found according to other studies with higher prevalence and abundance in rather shallow subgingival sites (6, 7). The third most prevalent oral *Campylobacter*, *C. concisus*, seems to play an even more contradictory role in health and disease. While *C. concisus* is a recognized intestinal pathogen associated with Crohn's disease or gastroenteritis (8), at oral sites this species seems to be present predominantly in healthy subjects (6, 7).

According to Socransky's pyramid model, *C. concisus* is a member of the 'green complex', while *C. gracilis* and *C. rectus* are members of the 'orange complex'. Based on correspondence analysis, *C. gracilis* showed more proximity to bacteria of the 'green complex' than to those of the 'red complex'. In contrast, *C. rectus* showed strong association with members of the 'red complex' and further progression (1).

During periodontitis progression, the actual bacterial composition is thought to change gradually forming distinct microbial complexes. This may be accompanied by a continuous change of *Campylobacter* species within but also between each microbial complex (Fig. 1). Therefore, we hypothesize that the proportion of species within the genus *Campylobacter*, rather than the absolute abundance of a single species, is a suitable and dynamic parameter for periodontitis progression.

Material and methods

Subjects

Subgingival plaque samples of 90 consecutive periodontitis patients (aged 40–69, mean 52.7) were collected at the Clinic of Operative and Preventive Dentistry and Periodontology, Rheinisch-Westfälische Technische

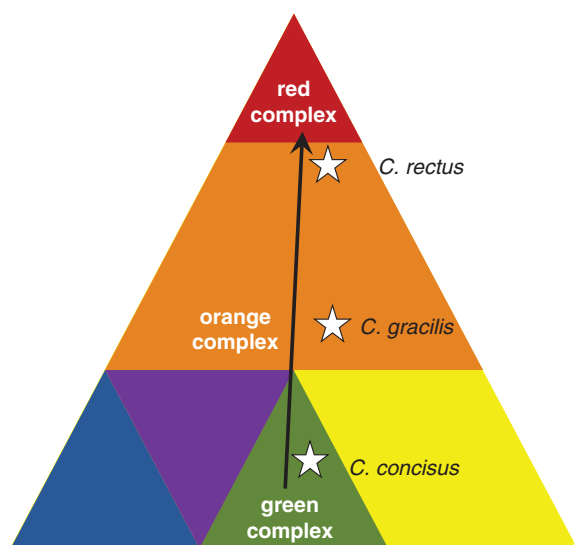


Fig. 1. Pyramid of periodontitis complexes and the approximate positions (and the dynamic change) of typical *Campylobacter* species visualizing the hypothesis of this study. Modified from Ref. 2.

Hochschule (RWTH) Aachen, Germany, for microbial analysis. In order to display the whole range of periodontitis-associated diseases, all patients that were diagnosed with 'periodontitis' were included. As a control, 32 samples of subgingival crevicular fluid were collected from dental students of the RWTH Aachen University Hospital (aged 21–33, mean 23.7) with a healthy periodontal status. Subjects who had taken antibiotics within the last 3 months were excluded. All participating subjects were informed about the study and signed an informed consent that had been approved in accordance with the guidelines of the Ethics Committee of the RWTH University Hospital, Aachen. From the periodontitis group, subgingival plaque was collected from 1 to 4 inflamed and deep periodontal pockets (>5 mm) using 1–4 sterile paper points ISO 45 (Alfred Becht GmbH, Offenburg, Germany). From the healthy control group, sulcus fluid was sampled from region 24 and 36 with the same method. Bacterial cells were recovered from paper points by vortexing with glass beads (diameter 1–2 mm) and subsequent centrifugation. All bacteria suspensions were stored frozen at -20°C until further analysis.

DNA extraction and chip hybridization

For each sample, DNA was extracted and purified with the Qiampl DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with few changes as described previously (9). DNA was stored at -20°C until further use.

For abundance determination of *Tannerella forsythia*, *Porphyromonas gingivalis*, and *Fusobacterium nucleatum* (and seven other species not discussed here in depth) the ParoCheck[®] detection system (Greiner Bio One, Frickenhausen, Germany) based on universal 16S-rRNA-gene amplification and subsequent chip-hybridization was used. The amplification and hybridization were performed according to the instructions of the manufacturer which has also been described in detail previously by our group (9). Based on the resulting fluorescence signal, the signal-to-noise ratio (SNR) was calculated and used as an indication of species abundance.

Seminested PCR amplification

Purified DNA was subjected to a preamplification-PCR based on the universal forward primer 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') (10), and based on the reverse primer EPS914R specific to *Epsilonproteobacteria* (5'-GGT CCC CGT CTA TTC CTT-3') (11), resulting in an amplicon length of 907 bp. PCR was carried out using 1 μl DNA template, 4 μl 10X PCR buffer, 1.6 μl DMSO, 0.8 μl of each primer (20 pmol/ μl), 0.5 μl dNTPs (40 mM), and 0.5 μl Taq-polymerase in a final volume of 40 μl . After initial denaturation of 2 min at 95°C and subsequent 25 cycles (30 s at 95°C , 30 s at 60°C , 70 s at 72°C) a final elongation followed for 10 min at 72°C . One microliter of this PCR mixture was then subjected to a second PCR

using the forward primer c-gsp8 (5'-GCG TGG AGG ATG ACA CTT TTC-3') specific to the genus *Campylobacter*. The reverse primer EPS914R was kept identical as in the first-round PCR except that it was phosphorylated at the 5' end. PCR was carried out using 4 µl 10X PCR buffer, 0.8 µl of each primer (20 pmol/µl), 1.6 µl dNTPs (40 mM), and 0.5 µl Taq-polymerase in a final volume of 40 µl. Starting with an initial denaturation of 2 min at 95°C, 32 cycles (30 s at 95°C, 30 s at 60°C, 40 s at 72°C) were followed by a final elongation for 10 min at 72°C. The length of the amplicon was 509 bp.

SSCP gel electrophoresis

Single-stranded conformation polymorphism (SSCP) is based on single-stranded DNA, which adopts a secondary/tertiary structure, determined by sequence-dependent intramolecular interactions. The migration through the gel matrix is affected by the secondary structure; it is hampered by spread out molecules and fast with compact molecules. The result of an SSCP electrophoresis is a pattern of bands in a lane, with each band ideally corresponding to one species/phylogroup in the original community.

SSCP gel electrophoresis was performed with slight modifications according to the protocol from Schwieger and Tebbe (12). Briefly, in order to obtain single-stranded DNA, the phosphorylated reverse strand of the amplicons from the semi-nested PCR was digested by lambda exonuclease (New England Biolabs, Schwalbach, Germany) and further purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. Vacuum-dried single-stranded DNA was then resuspended in denaturing SSCP-loading buffer (47.5% formamide, 5 mM sodium hydroxide, 0.12% bromophenol blue, and 0.12% xylene cyanol), heated to 95°C for 5 min, and immediately chilled on ice. MDE-Gels for electrophoresis (6 ml 2X MDE®-Gel-Solution; Lonza, Cologne, Germany), 2 ml 10X TBE buffer, 12 ml bidistilled water, 8 µl TEMED (tetramethylethylenediamine), and 80 µl APS (ammonium persulfate, 10%) were cast between quadratic glass plates (20 cm) with 0.4 mm spacer. Running conditions were 400 V for 18 h at 20°C in 1X TBE buffer. After electrophoresis, the gels were silver stained with the protocol proposed by Bassam et al. (13).

Sequence analysis and identification of bands

To confirm the specificity of bands, three or more bands per running distance were excised and eluted in 30 µl gel-extraction buffer (10 mM Tris, 5 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X 100, pH 9.0) at 95°C for 20 min. One microliter of this eluate was used as template for the *Campylobacter*-specific PCR described above. The resulting amplicons were purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany) and subsequently sequenced with the BigDye Terminator

Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany) followed by purification using DyeEx 2.0 Spin Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The sequences were analyzed on the ABI PRISM™ 310 Genetic Analyser (Applied Biosystems, Darmstadt, Germany) and post edited with Bioedit (V. 7.1.3) (14). Sequences were then classified with the 'sequence match' function of the Ribosomal Database Project website (RDP) (15) and the 'Nucleotide Blast' tool of the NCBI (National Center for Biotechnology Information) website. Because *C. rectus* and *C. showae* cannot clearly be distinguished based on a 509 bp long amplicon, and because of the overall rareness of *C. showae*, corresponding sequences were consistently assigned to *C. rectus*.

Data analysis

Silver-stained SSCP gels were scanned with incident light conditions at 300 dpi. Resulting scans were edited with ImageJ software in four steps (16): 1) the image was inverted and rotated by 90° anticlockwise. 2) For noise reduction, the 'median-filter' option with one pixel radius was used. 3) For the background subtraction, we used the 'sliding paraboloid' option with 50 pixel radius. 4) Finally, we extracted the XY-data of the electropherogram with the 'plot profile' option. A self-programmed excel sheet was used to model Gauss' distributions into the peaks of the electropherogram, and from the distributions' parameters, the areas under the peaks could be calculated. The area of a species-specific peak divided by the total area of the electropherogram equals the relative proportion of this species inside the *Campylobacter* genus. The Pearson distance was calculated based on this relative proportion of species for each sample and subsequently used to create an UPGMA (Unweighted Pair Group Method with Arithmetic mean) tree with MEGA5 (17). Tests for significance were calculated with SPSS using the non-parametric Wilcoxon–Mann–Whitney Test.

Results

From 90 samples belonging to the periodontitis group (designated as P-001 to P-090), we were able to amplify *Campylobacter* spp. in 72 samples (80%) with our nested PCR approach. In contrast, only 19 out of 32 samples (59%) of the healthy control group (designated as KS-01 to KS-32) were *Campylobacter* positive. The overall abundance for *Campylobacter*, if present, was found to be between 3.2×10^1 and 1.5×10^5 cells/µl, measured by q-PCR (data not shown).

Subsequent SSCP analysis resulted in reproducible banding patterns with six major bands (Fig. 2). Excision of at least three bands per running distance and gel, followed by re-amplification, sequencing, and analysis with the seqmatch tool of the RDP-Database revealed that each band-height corresponded to one distinct oral

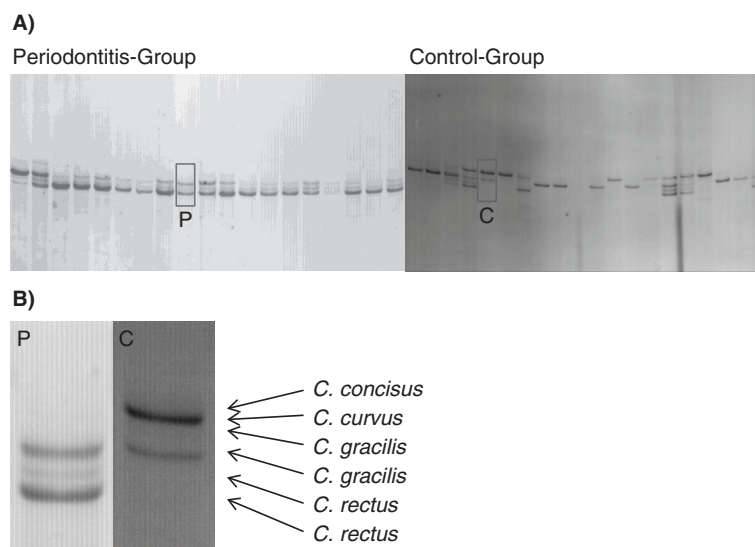


Fig. 2. (A) SSCP gels illustrating examples of periodontitis group (left) and control group (right). (B) Enlargement of Fig. 2 A. Arrows indicate species-specific running distances.

Campylobacter species. However, the two species *C. rectus* and *C. gracilis* split up into double bands, with one major and one weaker band. All analyzed sequences belonged exclusively to the typical oral *Campylobacter* species, namely *C. rectus*, *C. gracilis*, *C. concisus*, and *C. curvus*, proving the PCR specificity. No atypical *Campylobacter* species or phylotypes were detected. In the periodontitis group, of 90 cases *C. rectus* was present in 68 cases, *C. gracilis* in 60 cases, *C. concisus* in 22 cases, and *C. curvus* in 2 cases. In the healthy control group, only 10 out of 32 cases showed *C. rectus*, 17 *C. gracilis*, and 17 *C. concisus*. *C. curvus* was not found in the control group (Fig. 3).

There were distinct differences between the band intensities, reflecting species abundance, of the diseased group and the healthy control group. In the diseased group, *C. rectus* showed clearly the most intense bands, while *C. gracilis* was less abundant and *C. concisus* only rarely present. In the control group, the bands of *C.*

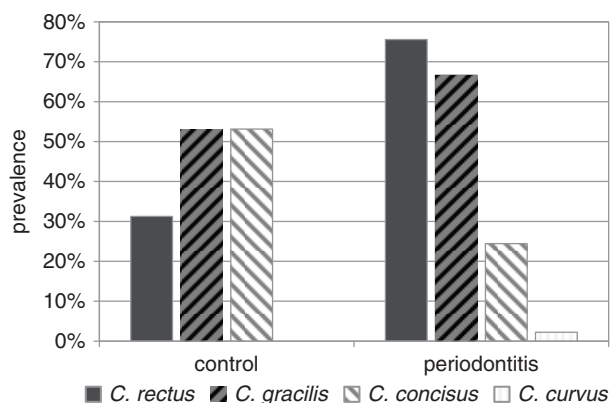


Fig. 3. Prevalence of different *Campylobacter* species in the periodontitis versus control group.

gracilis and *C. concisus* were the most intense ones, while *C. rectus* was only rarely present.

Cluster analysis based on the SSCP profiles resulted in four distinct main clusters (Fig. 4), which we refer to as 'A to D', which symbolize – according to our hypothesis – levels of an ecological shift among members of *Campylobacter* species: Cluster A is dominated by *C. rectus* with only low proportions of the other *Campylobacter* species. Most samples (71%) of the periodontitis group fall into this cluster but only two cases (KS-31 and KS-36) in the healthy controls. Cluster B is dominated by *C. gracilis*. Some members (26%) of the periodontitis group belong to this cluster as well as many members (47%) of the healthy control group. Cluster C is dominated by *C. concisus* and consists of members of the healthy control group only, except patient P-072. Cluster D exhibits elevated clusters of both *C. rectus* and *C. concisus* and is mainly composed of healthy members again with one exception (P-071).

T. forsythia showed the highest mean abundance in cluster A (28.2%, highest SNR-value was set to 100%), a low abundance in cluster B (7.6%) and cluster C (5.1%), and very low abundance in cluster D (0.1%) (Fig. 5). The same clear decrease in abundance was confirmed by data from *P. gingivalis* (A: 15.9%, B: 3.3%, C: 0%, D: 0%) and for *F. nucleatum* (A: 33.9%, B: 18.7%, C: 6.2%, D: 3.1%).

Discussion

The importance of *Campylobacter* spp. in oral communities is emphasized by the high prevalence of species we found mainly in periodontitis but also in healthy subjects. This implies that the sheer presence of *Campylobacter* cannot function as an indicator for disease. However, the prevalence of different *Campylobacter* species ('the

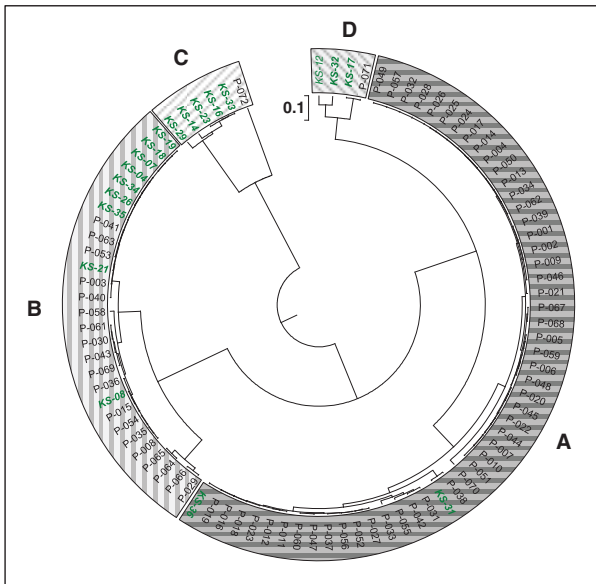


Fig. 4. Cluster analysis based on the SSCP banding pattern. Subjects of the periodontitis group (designated as P-001 to P-090) are depicted in black, control group (designated as KS-01 to KS-36) in green italic. Boxes indicate the four main clusters, referred to as ‘cluster A’ to ‘cluster D’, representing an ecological shift.

Campylobacter-mix’) differs between the healthy and the periodontitis group, with *C. rectus* as the most prevalent species in periodontitis samples and *C. gracilis* and *C. concisus* primarily present in healthy controls. Using cultivation-based methods, Macuch and Tanner showed comparable occurrences of *C. rectus* and *C. gracilis*, with a low prevalence of *Campylobacter* in the healthy control group (3). Applying molecular methods *C. rectus*, *C. gracilis*, and *C. concisus* were found in both healthy and diseased subjects in different abundances but no study so far addressed the dynamic of species mix (18–20).

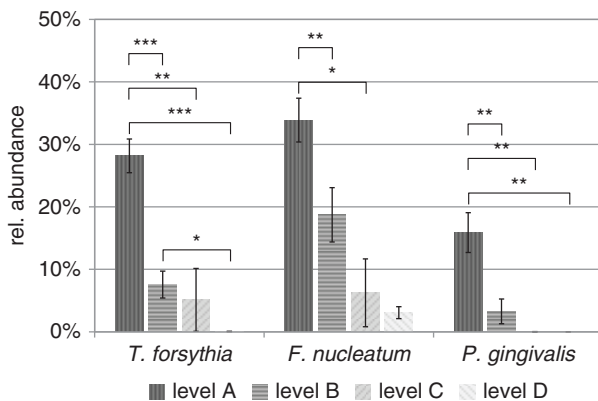


Fig. 5. Relative abundance of three prominent periodontitis marker species in proposed ecological shift clusters A–D. Highest occurring SNR value of each species was set to 100%. Asterisks indicate significant differences between ecological clusters (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Although our PCR primers were designed broad enough to cover a wide range of members out of the genus *Campylobacter*, hitherto unknown (or at least for the oral cavity unusual) *Campylobacter* species were not found here. No taxa other than *Campylobacter* were found, underlining the specificity of our approach.

The existence of four major SSCP-based clusters reflecting distinct levels of an ecological shift was supported by concurrent shifts in abundance of *T. forsythia*, *P. gingivalis*, and *F. nucleatum*. These SSCP-based clusters differed markedly from each other in distinct features, 1) clusters A to C are dominated by different *Campylobacter* species, 2) the proportion of periodontitis subjects is increasing from cluster C to A, while the proportion of healthy subjects is decreasing from cluster C to A, 3) the abundance of prominent periodontitis marker species increases significantly from cluster C to A. Cluster D, represented by only three healthy controls and one patient (P-071), is characterized by low proportions of *C. gracilis* and an elevated proportion of *C. concisus*, but shows similarities to the ecological situation in cluster C, representing health.

In total, only four samples were grouped outside their expected cluster. In the ‘disease cluster A’, the two controls KS-31 and KS-36 grouped together with 51 patients. Interestingly, KS-31 showed the highest *F. nucleatum* abundance of all control samples, which might be indicative for an ecological disturbance preceding the onset of disease. However, we have no plausible explanation for the unusual clustering of KS-36. Patient P-071 clustering in the ‘healthy cluster D’ was devoid of *P. gingivalis* and *T. forsythia* which might indicate an ‘ecological recovery’ toward a healthy condition. Finally, patient P-072 clustering in the ‘healthy cluster C’ is unique by its ecological profile as it was the only case dominated by *C. curvus*. The ecological role of *C. curvus* in periodontitis was discussed in a few publications, and it can be concluded that it is more related with (initial) periodontitis than with health, which is in concordance with our data (3, 7). However, once more individuals have been tested, samples rich with *C. curvus* probably deserve their own cluster ‘E’.

In this pilot study, we used a periodontitis group and a control group that were not age-matched, hence we consider our results as preliminary but promising which warrants further investigation. We also want to mention that cross-sectional studies – even if including a high number of consecutive periodontitis patients and controls – have their limit to estimate the actual progression. Therefore, as a next step in order to verify the results, we will compare in a future study age-matched groups in a longitudinal study. In addition, the abundance of additional recognized periodontal marker bacteria, such as *Treponema denticola*, *Aggregatibacter actinomycetemcomitans*, and *Prevotella intermedia*, will be included for assessing the overall suitability of the *Campylobacter*

ratios as a feasible and dynamic parameter for periodontitis progression.

Conflict of interest and funding

There is no conflict of interest in the present study for any of the authors.

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