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Validation of a quantitative real-time PCR assay and comparison with fluorescence microscopy and selective agar plate counting for species-specific quantification of an *in vitro* subgingival biofilm model

T. W. Ammann[1], N. Bostanci[2], G. N. Belibasakis[1], T. Thurnheer[1]
[1]Oral Microbiology and Immunology, Institute of Oral Biology, Center of Dental Medicine, University of Zürich, Zürich, Switzerland and [2]Oral Translational Research, Institute of Oral Biology, Center of Dental Medicine, University of Zürich, Zürich, Switzerland

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Background and Objective: Subgingival biofilms are the prime etiological factor of periodontal disease. Owing to their complex polymicrobial nature, quantification of individual bacterial species within the biofilm for research and diagnostic purposes can be methodologically challenging. The aims of this study were to establish a quantitative real-time PCR (qPCR) assay to quantify the bacteria used in our 10-species *in vitro* 'subgingival' biofilm model and to compare the quantitative outcome with fluorescence microscopy and colony-forming unit (CFU) counts on selective agar plates.

Material and Methods: The 10 species included in the *in vitro* biofilm were *Streptococcus oralis*, *Streptococcus anginosus*, *Veillonella dispar*, *Fusobacterium nucleatum*, *Treponema denticola*, *Tannerella forsythia*, *Actinomyces oris*, *Campylobacter rectus*, *Porphyromonas gingivalis* and *Prevotella intermedia*. The numbers of each species were quantified at two time points using qPCR, microscopy counting following fluorescence *in-situ* hybridization (FISH) or immunofluorescence staining, and counting of CFUs after growth on selective agar plates.

Results: All 10 species were successfully quantified using qPCR and FISH or immunofluorescence, and the eight species culturable on selective agar plates were also quantified by counting the numbers of CFUs after growth on selective agar. In early biofilm cultures, all methods showed a significant correlation, although the absolute numbers differed between methods. In late biofilm cultures, measurements obtained using qPCR and FISH or immunofluorescence, but not by CFU counts, maintained significant correlation. CFU counts yielded lower values than did measurements made using the other two methods.

Thomas W. Ammann, Oral Microbiology and Immunology, Institute of Oral Biology, Center of Dental Medicine, University of Zürich, Plattenstrasse 11, 8032 Zürich, Switzerland
Tel: +41 44 634 32 56
Fax: +41 44 634 43 10
e-mail: thomas.ammann@zzm.uzh.ch

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Conclusion: Quantitative PCR and epifluorescence microscopy can be easily combined with each other to determine species-specific bacterial numbers within biofilms. However, conventional bacterial cultures cannot be as efficiently combined using these molecular detection methods. This may be crucial in designing and selecting appropriate clinical diagnostic methods for subgingival biofilm samples.

One of the main requirements in microbiology is to quantify microorganisms quickly and reliably. This has direct implications in the field of periodontology, encompassing both basic microbiological research and clinical microbial diagnostics. Although culture techniques have been used for more than a century, these are being replaced to an ever-increasing extent with molecular techniques, the use of which has expanded rapidly since they were first introduced in the 1990s. Molecular methods offer the possibility not only to quantify organisms that are currently unculturable using the known classic methods, but also to enable the rapid screening of very complex samples. However, despite the technological advancements, all methods currently available still have their specific limitations. Comparison or combination of data generated using different methods can lead to the misinterpretation of results, and therefore knowledge of how different methods do correlate with each other is crucial.

Counting of colony-forming units (CFUs) on selective or nonselective agar culture plates only quantifies viable organisms. Moreover, as only visible colonies are counted, the microorganisms must be able to grow until they become visible. However, not only single cells, but also large aggregates or chains of cocci, result in a visible colony. Species-specific quantification is possible using selective agar plates. As selectivity is achieved by repressing the growth of undesired species, the bacteria intended for quantification are also affected, which leads to generally lower counts. Fluorescence *in-situ* hybridization (FISH) was developed in the 1990s (1) and has had a major impact in all fields of microbiology. The species-specific fluorescence labeling enables the quantification of nonculturable organisms; however, both viable and nonviable (i.e. dead) cells are stained. This problem can be circumvented by using FISH in combination with live/dead staining (2). An advantage shared by quantitative real-time PCR (qPCR) and FISH is the quantification of nonculturable organisms. However, as dead bacteria are also quantified, qPCR has the same limitation as quantification by FISH. The use of compounds such as propidium monoazide and ethidium monoazide can reduce the amplification of DNA from dead organisms. However, this effect is limited because the quantification in heat-killed samples shows values just 2–4 logs lower compared with those of living samples (3).

While *in vivo* samples often have a complexity that renders species-specific quantification redundant, *in vitro* samples are of a different nature. Studying well-defined microbial communities demands precise monitoring at a species-specific level. To achieve accurate results, the quantification techniques have to be selected carefully and with respect to their advantages and disadvantages. The results obtained using different methods are largely influenced not only by the viability of the microorganisms but also by their growth state (4).

The aims of this study were to develop a qPCR assay that could be used for species-specific quantification of the bacteria in an *in vitro* 10-species subgingival biofilm model, and to compare the results with those obtained using microscopy and CFU counting.

Material and methods

Biofilm generation and sample preparation

The growth medium for the biofilms was composed of 60% pooled saliva, 30% modified fluid universal medium (5) and 10% heat-inactivated human serum. Biofilm was incubated for 64.5 h under anaerobic conditions at 37°C. The biofilm consortium was composed of *Streptococcus oralis* SK248 (OMZ 607), *Streptococcus anginosus* ATCC 9895 (OMZ 871), *Actinomyces oris* (OMZ 745; formerly *Actinomyces naeslundii*), *Fusobacterium nucleatum* subsp. *nucleatum* OMZ 598, *Veillonella dispar* ATCC 17748^T (OMZ 493), *Campylobacter rectus* OMZ 698, *Prevotella intermedia* ATCC 25611^T (OMZ 278), *Porphyromonas gingivalis* ATCC 33277^T (OMZ 925), *Tannerella forsythia* OMZ 1047 and *Treponema denticola* ATCC 35405^T (OMZ 661). All strains were maintained on Columbia Blood Agar plates, with the exception of *T. forsythia* and *T. denticola*, which were maintained in liquid growth medium (Table 1). To ensure high viability in the preculture phase, *C. rectus* was incubated for 64 h in liquid medium under microaerophilic conditions before the experiments were started. Two cycles of preculture were performed for all strains before they were inoculated with biofilm. In brief, bacteria were transferred into the appropriate liquid growth medium (outlined in Table 1) and incubated for 24 h (cycle 1). Then, the precultures were diluted 1 : 10 in fresh medium and incubated for another 8 h (cycle 2). The second cycle of precultures was skipped for the slow-growing strains *T. denticola, T. forsythia, C. rectus,* and *P. intermedia*. Following the preculture, all cultures were adjusted to a defined optical density (OD_{550} = 1.0 ± 0.05) in the same final volume. Before inoculation, one sintered hydroxyapatite disc was placed in each well of a 24-well plate and incubated for 4 h at room temperature in 800 µL of pooled saliva on a rotary shaker (90 rpm) in order to form

Table 1. Growth media used for cultivation of precultures and biofilms

Medium	Reference	Use
mFUM, 0.3% glucose (m/v)	(5)	Liquid precultures of *Streptococcus oralis*, *Streptococcus anginosus*, *Veillonella dispar*[a], *Fusobacterium nucleatum*, *Actinomyces oris*, *Prevotella intermedia* and *Campylobacter rectus*[b]
30% mFUM (0.3% glucose [m/v]), 60% pooled saliva, 10% human serum[c]		Growth medium for biofilms
Pg medium[d]	(13)	Liquid precultures of *Porphyromonas gingivalis*
Spirochaetes medium	(14)	Maintenance/precultures of *Treponema denticola*
Modified OMIZ-W68[e]	(15)	Maintenance/precultures of *Tannerella forsythia*

m/v, mass by volume; mFUM, modified fluid universal medium.
[a]Addition of 1% lactic acid (volume by volume).
[b]Addition of 0.1% sodium fumarate and 0.1% sodium formiate.
[c]Heat inactivated.
[d]Brain–heart infusion broth, supplemented with hemin (7.67 μM) and menadione (2.91 μM).
[e]Addition of lactose (2 g/L), caseinoglycomacropeptide (100 mg/L), *N*-acetylmuramic acid (50 mg/L) and *N*-acetylglucosamine (500 mg/L).

a pellicle. The discs were then transferred, one per well, to 24-well plates containing 1.6 mL of growth medium per well, and 200 μL of the bacterial mixture for each disc was then added. After 16.5 h of incubation, the growth medium was renewed, along with a boost of 50 μL of *T. denticola* liquid culture (OD_{550} = 1.0). The growth medium was renewed a second time, 24 h after the first renewal but without a boost with *T. denticola*.

After incubation, biofilms were exposed to keratinocyte insulin- and serum-free medium (EpiLife; Life Technologies Europe, Zug, Switzerland), under aerobic conditions for 3 and 24 h. In the present text, this medium is referred to as 'cell-culture medium'. To quantify all species, biofilms were detached from the discs by vortexing in 1 mL of 0.9% saline in a 50-mL tube. The resulting suspension was then used for quantification by plating, microscopy and qPCR.

Real-time quantitative PCR

All primers used in this study were designed using the online primer blast tool provided on the homepage of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/tools/primer-blast). All primer pairs target the 16S ribosomal RNA gene (the template sequences for the primer design are outlined in Table 2). The regions of highest divergence were detected by multiple sequence alignment using the ClustalX software (http://www.clustal.org/clustal2). All reactions were quantified individually in separate wells for each bacterial species. Primers were designed to have the same melting temperature (60°C) to enable their simultaneous use on the same plate. Primer sequences and properties are given in Table 3. All primers were ordered from Microsynth (Balgach, Switzerland).

DNA was extracted from the samples using the GenElute bacterial genomic DNA kit (Sigma-Aldrich, Buchs, Switzerland). DNA extraction for individual strains (standard curves) and biofilms was performed according to the manufacturer's guidelines following the gram-positive lysis protocol, with the following modifications. Lysis steps were expanded from 30 min to 1 h (the lysozyme/mutanolysin step) and from 10 min to 20 min (the proteinase K step). Mutanolysin (Sigma–Aldrich) was added for the extraction of streptococci and biofilm samples. DNA was eluted into the same tube in two steps, using 75 μL of elution buffer in each step.

Standard curves were generated using DNA extractions of stationary-phase cultures of all 10 species used in the biofilm model. The fast-growing *S. oralis*, *S. anginosus*, *V. dispar*, *F. nucleatum*, *A. oris* and *P. gingivalis* were cultured overnight, while the slow-growing *T. denticola*, *T. forsythia*, *C. rectus* and *P. intermedia* were cultured for 60 h. Following extraction, the DNA content was determined using a NanoDrop ND-1000 (ThermoFisher Scientific, Wohlen, Switzerland) and set to defined concentrations of 10–0.001 ng by serial dilution for the generation of standard curves. The logarithm of the corresponding quantification cycle values was used in order to obtain a linear regression. For the quantification of bacteria in biofilm samples, the concentration of the extracted DNA mixture was determined using the NanoDrop ND-1000. The qPCR was run in 7.5 μL of the qPCR was run in a total reaction volume of 15 μL, containing 7.5 μL of SYBR® Green PCR Master Mix (Life Technologies, Zug, Switzerland), 6 μL of sample (diluted to contain 1 or

Table 2. Genome sizes, weight and accession numbers of the template sequences used for quantitative real-time PCR (qPCR) primer design

Organism	Genome size (kb)	Genome weight (ng)	PCR template accession number
Streptococcus anginosus	1815	1.99E-06	GU045404.1
Streptococcus oralis	1905	2.09E-06	EU156768.1
Actinomyces oris	3043	3.34E-06	GQ421308.1
Veillonella dispar	2117	2.32E-06	AY995770.1
Fusobacterium nucleatum	2175	2.32E-06	GQ301038.1
Campylobacter rectus	2513	2.75E-06	AB595133.1
Prevotella intermedia	3279	3.59E-06	L16468.1
Porphyromonas gingivalis	2355	2.58E-06	AF414809.1
Treponema denticola	2843	3.12E-06	AF139203.1
Tannerella forsythia	3406	3.73E-06	AB547708.1

All template sequences used for primer design are sequences of the 16S ribosomal RNA gene.

Table 3. Primer sequences and properties

Organism	Sequence (5′→3′)	Strand on template	T_m(°C)	Product length (bases)
Streptococcus anginosus	ACCAGGTCTTGACATCCCGATGCTA	+	59.25	76
	CCATGCACCACCTGTCACCGA	–	59.04	
Streptococcus oralis	ACCAGGTCTTGACATCCCTCTGACC	+	59.42	70
	ACCACCTGTCACCTCTGTCCCG	–	59.85	
Actinomyces oris	GCCTGTCCCTTTGTGGGTGGG	+	59.57	71
	GCGGCTGCTGGCACGTAGTT	–	60.32	
Veillonella dispar	CCCGGGCCTTGTACACACCG	+	59.70	62
	CCCACCGGCTTTGGGCACTT	–	59.83	
Fusobacterium nucleatum	CGCCCGTCACACCACGAGA	+	59.04	75
	ACACCCTCGGAACATCCCTCCTTAC	–	59.48	
Campylobacter rectus	TCACCGCCCGTCACACCATG	+	59.35	57
	CCGGTTTGGTATTTGGGCTTCGAGT	–	59.50	
Prevotella intermedia	GCGTGCAGATTGACGGCCCTAT	+	59.61	68
	GGCACACGTGCCCGCTTTACT	–	60.24	
Porphyromonas gingivalis	GCGAGAGCCTGAACCAGCCA	+	59.07	90
	ACTCGTATCGCCCGTTATTCCCGTA	–	59.44	
Treponema denticola	TAAGGGACAGCTTGCTCACCCCTA	+	58.84	55
	CACCCACGCGTTACTCACCAGTC	–	59.76	
Tannerella forsythia	CGATGATACGCGAGGAACCTTACCC	+	59.07	72
	CCGAAGGGAAGAAAGCTCTCACTCT	–	58.01	

T_m, melting temperature.

0.1 ng of DNA) and 1.5 μL of primer solution (10 μM, a mixture of forward and reverse primers). Amplification of the extracted DNA template was performed in an ABI 7000 Sequence Detection System (Applied Biosystems) by initial incubations of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min 60°C. From the obtained Cq values, the sample DNA concentration was calculated for each organism, and the abundances were calculated using the theoretical genome weight of each organism (Table 2).

Microscopy

Epifluorescence microscopy counting was performed following FISH or immunofluorescence, as described previously (6,7), using the probes and antibodies listed in Tables 4 and 5, respectively. FISH staining was performed for *S. oralis*, *S. anginosus*, *V. dispar*, *F. nucleatum* and *T. denticola*, whereas *T. forsythia*, *A. oris*, *C. rectus*, *P. gingivalis* and *P. intermedia* were stained by immunofluorescence for better visibility.

Plating on selective agars

Seven different selective agars were used to determine the CFUs for eight of the 10 species analyzed. No CFUs were determined for *T. forsythia* and *T. denticola*. Biofilm solutions were serially diluted prior to plating, and three different dilutions were plated out in order to obtain at least one plate containing 20 - 200 CFUs. *S. oralis* and *S. anginosus* were counted on Difco mitis salivarius agar (Becton, Dickinson and Company, Sparks, MD, USA), supplemented with 0.001% (weight by volume) sodium tellurite (BDH Chemicals Ltd., Poole, UK). *V. dispar*, *C. rectus* and *A. oris* were counted after culture on Difco Columbia Blood Agar Base (Becton, Dickinson and Company) supplemented with 5% whole human blood, and *P. gingivalis and P. intermedia* were counted after culture on the same medium with the addition of 0.1% phosphomycin. *F. nucleatum* was counted after culture on Fastidious Anaerobe Agar (Neogen, Lansing, MI, USA) containing erythromycin (Sigma-Aldrich), vancomycin (Teva Pharma, Aesch, Switzerland) and norfloxacin (Sigma) (5).

Results

Validation of the qPCR assay

Standard curves were generated for a range of concentrations of DNA, from 10 to 0.001 ng, in which the primers for all organisms showed high linearity

Table 4. Probes used for quantification following fluorescence *in-situ* hybridization (FISH) staining

Organism	Name	Label	FA[a]	WB[b]	Sequence (5′→3′)	Reference
Streptococcus anginosus	Sang1203 (23S rRNA)	Cy3	20	215	GGTACACCTTCACCACAC	
Streptococcus oralis	Mit447	FAM	20	215	CACYCGTTCTTCTCTTACA	(16)
Veillonella dispar	VEI217	Cy3	40	46	AATCCCCTCCTTCAGTGA	(17)
Treponema denticola	TrepG1_679	Cy3	40	46	GATTCCACCCCTACACTT	(18)

rRNA, ribosomal RNA.
[a]Formamide concentration (%) in the hybridization buffer.
[b]NaCl concentration (mM) in the washing buffer.

Table 5. Antibodies used for quantification following immunofluorescence staining

Organism	Cell line/monoclonal antibody	Isotype	Reference
Actinomyces oris	396AN1	Mouse IgM	(19)
Campylobacter rectus	212WR2	Mouse IgG3	(20)
Prevotella intermedia	37BI6.1	Rat IgG2b	(21)
Porphyromonas gingivalis	61BG1.3	Mouse IgG1	(22)
Tannerella forsythia	103BF1.1	Mouse IgG2b	(23)

($R^2 > 0.99$) (Table 6). Biofilm samples were diluted to contain 1 or 0.1 ng of total DNA per reaction and were therefore always in the linear range of the standards. Furthermore, the DNA concentration of each species detected in the biofilms was always higher than the lower detection limit of 0.001 ng per reaction. The DNA-extraction efficiency was estimated by comparison of microscopy counts and theoretical counts calculated from the amount of DNA extracted from stationary-phase cultures and was found to be, on average, 75% (data not shown). All described primer pairs proved to be specific when used for amplification in our model system. No nonspecific amplification was observed if the primer pairs were tested in a DNA mixture of the remaining nine untargeted species. The melting curves of the PCR product obtained showed that only one product was found in all cases, proving that the primers neither form dimers nor show amplification of regions outside the target gene (data not shown).

Detected bacterial abundance in the biofilm model

The abundance of all 10 species of bacteria was determined using three different quantification techniques: qPCR; epifluorescence microscopy following species-specific staining by FISH or immunofluorescence; and counting of CFUs following culture on selective agar plates. The abundances were determined after biofilms were exposed to a keratinocyte insulin- and serum-free cell-culture medium under aerobic conditions, as these conditions are used in biofilm–host cell interaction studies.

After 3 h of exposure to cell medium under aerobic conditions, the abundances of all species were in the range of two orders of magnitude, regardless of the quantification method used. The abundances ranged from 1.4×10^6 to 2.4×10^8 cells per biofilm when determined using CFU counts, 3.1×10^6 to 6.9×10^8 cells per biofilm when determined using microscopy counts and 8.2×10^6 to 7.7×10^8 cells per biofilm when determined using qPCR measurements (Fig. 1). After 24 h of exposure, the three quantification methods showed different values within five orders of magnitude. The abundances ranged from 4.6×10^4 to 1.8×10^8 cells per biofilm when determined using CFU counts, 1.6×10^7 to 6.8×10^8 cells per biofilm when determined using microscopy counts and 8.2×10^6 to 1.5×10^9 cells per biofilm when determined using qPCR measurements (Fig. 2).

Table 6. Quantitative real-time PCR (qPCR) standard curve values

Organism	Cells[a]	Log DNA	Cq	*a*	*b*	R^2
Streptococcus anginosus	5.03E + 05	0	14.4127	−3.400	14.421	0.99997
	5.03E + 04	−1	17.8475			
	5.03E + 03	−2	21.1929			
	5.03E + 02	−3	24.6323			
Streptococcus oralis	4.79E + 05	0	14.0411	−3.441	14.187	0.99488
	4.79E + 04	−1	17.6249			
	4.79E + 03	−2	21.5132			
	4.79E + 02	−3	24.2161			
Actinomyces oris	3.00E + 05	0	15.8122	−3.352	15.624	0.99855
	3.00E + 04	−1	19.4155			
	3.00E + 03	−2	22.3746			
	3.00E + 02	−3	25.9611			
Veillonella dispar	4.31E + 05	0	14.3359	−3.484	14.367	0.99991
	4.31E + 04	−1	17.9122			
	4.31E + 03	−2	21.3054			
	4.31E + 02	−3	24.8178			
Fusobacterium nucleatum	4.20E + 05	0	13.7294	−3.462	13.757	0.99941
	4.20E + 04	−1	17.1824			
	4.20E + 03	−2	20.8388			
	4.20E + 02	−3	24.0508			
Campylobacter rectus	3.63E + 05	0	16.2292	−3.320	16.347	0.99915
	3.63E + 04	−1	19.8333			
	3.63E + 03	−2	23.0101			
	3.63E + 02	−3	26.2384			
Prevotella intermedia	2.78E + 05	0	14.4055	−3.275	13.924	0.99785
	2.78E + 04	−1	18.0145			
	2.78E + 03	−2	21.2223			
	2.78E + 02	−3	24.1599			
Porphyromonas gingivalis	3.87E + 05	0	14.8341	−3.357	14.856	0.99989
	3.87E + 04	−1	18.2132			
	3.87E + 03	−2	21.6336			
	3.87E + 02	−3	24.8831			
Treponema denticola	3.21E + 05	0	19.5112	−3.096	19.792	0.99453
	3.21E + 04	−1	23.2655			
	3.21E + 03	−2	26.0730			
	3.21E + 02	−3	28.8948			
Tannerella forsythia	2.68E + 05	0	16.4484	−3.443	16.423	0.99973
	2.68E + 04	−1	19.8793			
	2.68E + 03	−2	23.2029			
	2.68E + 02	−3	26.8163			

[a]'Cells' are theoretical values based on the amount of DNA (which is given as logarithmized values) in ng, per reaction. Linear regressions of DNA/reaction vs. quantification cycle are characterized by their slope (a), *y*-axis intersection (b) and the R^2 values.

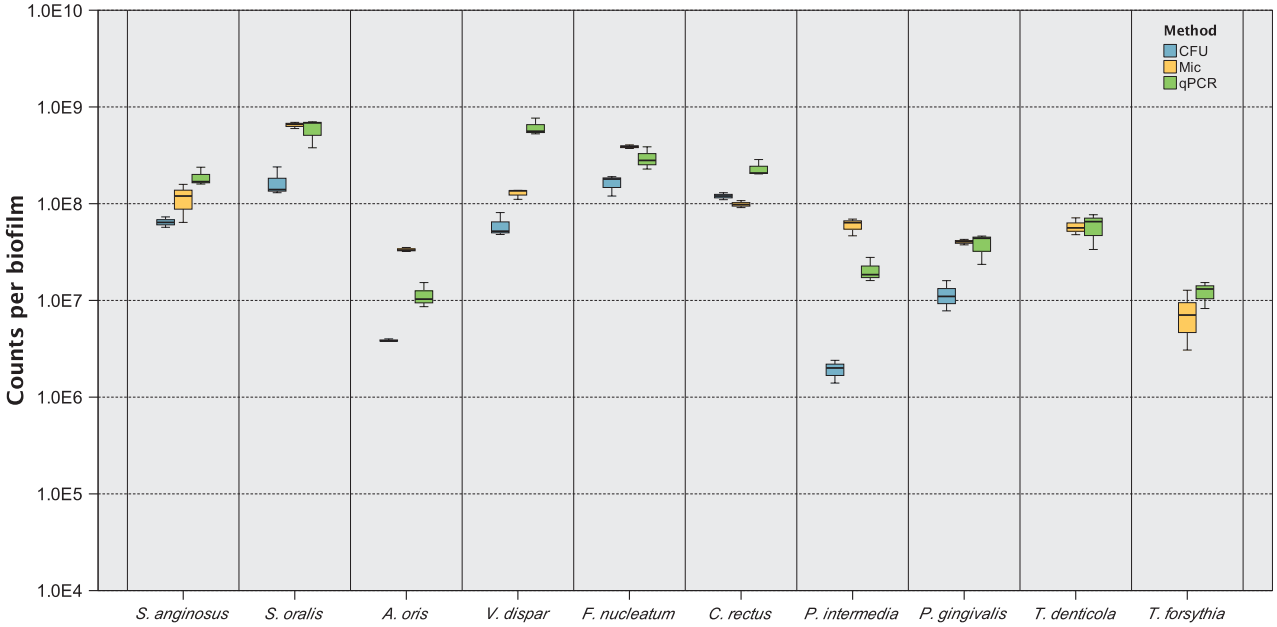

Fig. 1. Detected abundances per biofilm of the 10 species after 3 h of exposure to aerobic conditions in cell-culture medium. The data shown are representative values from one out of three independent experiments. Each box represents a triplicate of independent biofilms. Colony-forming unit (CFU) counts of *Treponema denticola* and *Tannerella forsythia* were not determined. Mic, counts determined using microscopy; qPCR, counts determined using quantitative real-time PCR. *A. oris*, *Actinomyces oris*; *C. rectus*, *Campylobacter rectus*; *F. nucleatum*, *Fusobacterium nucleatum*; *P. gingivalis, Porphyromonas gingivalis*; *P. intermedia*, *Prevotella intermedia*; *S. anginosus*, *Streptococcus anginosus*; *S. oralis*, *Streptococcus oralis*; *V. dispar*, *Veillonella dispar*.

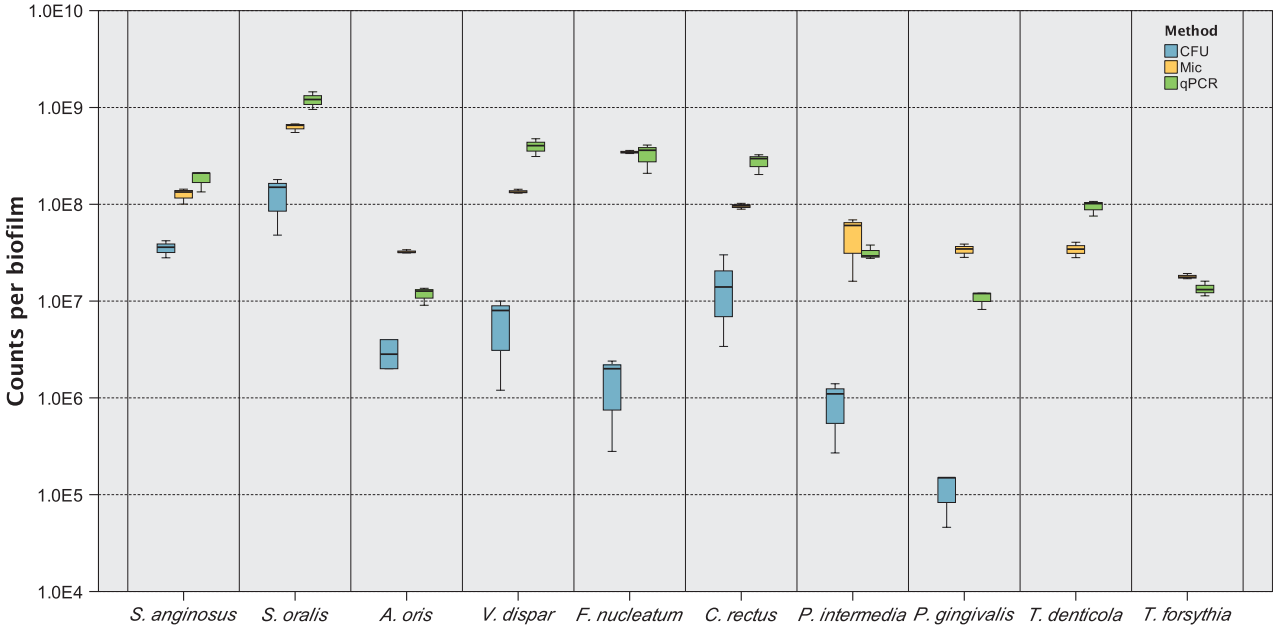

Fig. 2. Detected abundances per biofilm of the 10 species after 24 h of exposure to aerobic conditions in cell-culture medium. The data shown are representative values from one out of three independent experiments. Each box represents a triplicate of independent biofilms. Colony-forming unit (CFU) counts of *Treponema denticola* and *Tannerella forsythia* were not determined. . Mic, counts determined using microscopy; qPCR, counts determined using quantitative real-time PCR. *A. oris*, *Actinomyces oris*; *C. rectus*, *Campylobacter rectus*; *F. nucleatum*, *Fusobacterium nucleatum*; *P. gingivalis, Porphyromonas gingivalis*; *P. intermedia*, *Prevotella intermedia*; *S. anginosus*, *Streptococcus anginosus*; *S. oralis*, *Streptococcus oralis*; *V. dispar*, *Veillonella dispar*.

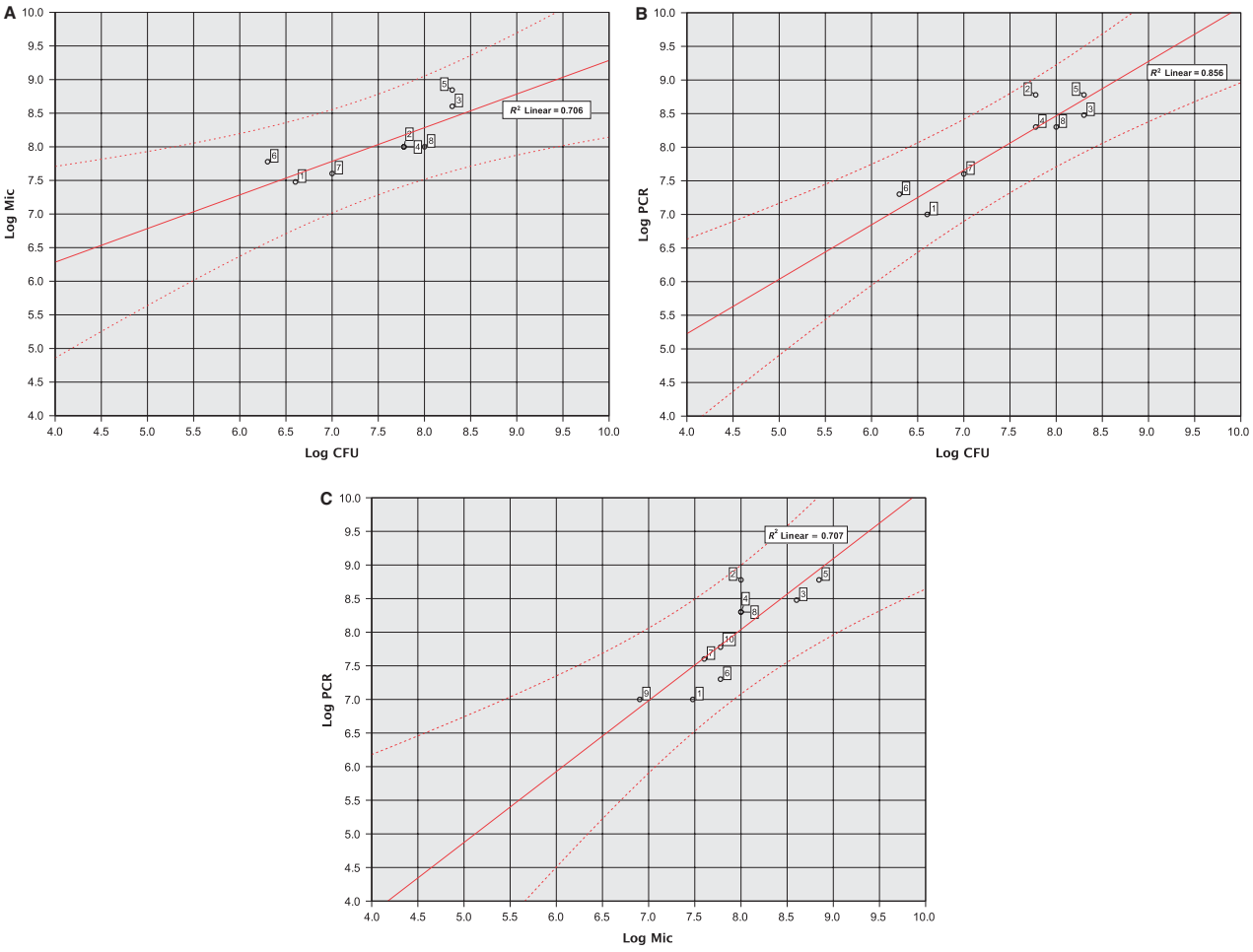

Fig. 3. Pairwise regression analysis of the quantitative data after 3 h of exposure to aerobic conditions in cell-culture medium. The values shown are logarithmized values of the measured abundances. (A) Microscopic (Mic) counts vs. colony-forming unit (CFU) counts; (B) quantitative real-time PCR (qPCR) quantification vs. CFU counts; (C) qPCR quantification vs. microscopic counts. The red solid line shows the linear regressions and the red dotted lines indicate the 95% confidence interval. Species key: (1) *Actinomyces oris*, (2) *Veillonella dispar*, (3) *Fusobacterium nucleatum*, (4) *Streptococcus anginosus*, (5) *Streptococcus oralis*, (6) *Prevotella intermedia*, (7) *Porphyromonas gingivalis*, (8) *Campylobacter rectus*, (9) *Tannerella forsythia* and (10) *Treponema denticola*.

Correlation analysis after 3 h of exposure

A Spearman correlation analysis involving all bacterial species was performed to compare the methods pairwise against each other, and a linear regression was calculated using logarithmized mean values of the abundance of each species (Fig. 3A–C). These correlations were significant for all pairs of methods: $p < 0.05$ for qPCR vs. CFU counts; and $p < 0.01$ for microscopy counts vs. CFU counts and for microscopy counts vs. qPCR (Table 7).

Quantification by epifluorescence microscopy vs. qPCR showed the highest correlation coefficient, of 0.925. In the range of 10^6–10^9 counts, the linear regression ($R^2 = 0.707$) indicates that the two methods produce very similar values. A correlation coefficient of 0.901 was found for the pairwise comparison of microscopy counts vs. CFU counts. In the range of 10^6–10^9 counts, the linear regression ($R^2 = 0.706$) indicated that microscopy counts are 13-fold higher at the lower end of the data range and that CFU counts are twofold higher at the upper end. The comparison of qPCR vs. CFU counts showed a correlation coefficient of 0.811, which was the lowest of all three pairwise methodological comparisons. The *y*-axis intersection of the linear regression ($R^2 = 0.856$) indicates that, in the range of 10^6–10^9 counts, qPCR produced values that were 8.5-fold higher at the lower end of the data range and 3.5-fold higher at the upper end of the data range.

Correlation analysis after 24 h of exposure

While after 3 h of exposure all methods showed significant correlations, this was not the case after 24 h of exposure (Table 8). The only pair still showing a significant ($p < 0.01$) correlation was that of qPCR vs. microscopy counts, with a coefficient of

Table 7. Spearman correlations of all three methods after 3 h of exposure to cell-culture medium

		qPCR	Mic	CFU
qPCR	Correlation coefficient	1.000	0.925**	0.811*
	Significant (two-sided)		0.000	0.015
	n	10	10	8
Mic	Correlation coefficient	0.925**	1.000	0.901**
	Significant (two-sided)	0.000		0.002
	n	10	10	8
CFU	Correlation coefficient	0.811*	0.901**	1.000
	Significant (two-sided)	0.015	0.002	
	n	8	8	8

CFU, colony-forming units; Mic, microscopy; qPCR, quantitative real-time PCR.
*Significant correlation with $p < 0.05$ (two-sided).
**Significant correlation with $p < 0.01$ (two-sided).

0.915. In the range of 10^6–10^9 counts, the linear regression ($R^2 = 0.806$) indicates that microscopy counts tend to be fourfold higher at the lower end of the scale, while qPCR counts are threefold higher at the upper end of the scale (Fig. 4A–C). The strict anaerobes were largely responsible for this discrepancy, as the CFU counts were reduced by more than 1 log for *P. intermedia* and *V. dispar*, and by more than 2 logs for *F. nucleatum* and *P. gingivalis*.

Discussion

In this study, a qPCR assay was developed for the species-specific quantification of all bacteria used in an established 10-species *in vitro* 'subgingival' biofilm model. We analysed the quantitative outcome of this qPCR assay by performing correlation analyses between the qPCR results and those obtained using CFU counting after growth on conventional culture plates and those obtained using microscopy counting following FISH or immunofluorescence staining.

The bacteria were quantified after exposure of the established biofilms for 3 or 24 h to aerobic conditions in cell-culture medium. The rationale for exposing the biofilms to an aerobic atmosphere and eukaryotic cell medium before quantification is that these experimental conditions are of relevance to host–biofilm interaction models, in which this biofilm is used (8,9).

The results indicate that all three methods correlate well at the early 3-h time-point, at which the bacterial viability is presumably high. However, even though significant correlations are reached when comparing the different methods with each other, they must not be freely combined without taking into consideration that the measurements obtained using qPCR and epifluorescence microscopy differ by 1 to 3 logs from those obtained using the CFU plate counts. This finding is in good agreement with the results presented in other studies, where the CFU counts were about 40-fold lower compared with qPCR while using a universal primer set in carious dentine samples (10). Another study, in which the difference between anaerobic culture and quantitative PCR was examined at a species-specific level in clinical samples, detected a difference of < 1 log in approximately 70% of the samples, while 20–30% of samples showed a 1–2 log difference and < 10% showed a > 2 log difference (11). On the one hand, this effect may be explained either by the growth-repressing effects of the selective agars or by the aggregation of the bacteria. On the other hand, the presence of dead cells in the sample could also account for this discrepancy. In previous studies we observed that, after an incubation time of 64.5 h, the viability of the bacteria was about 85% (5,9). Thus, the observed discrepancy between CFU counts and the two molecular methods at the 3-h time point would have to be accounted for by the stress induced by the aerobic conditions and the cell-culture medium. However, the impact was in a similar range for all species used in the biofilms. A significant correlation was found for the results of all three quantification methods, and no specific differences between obligate anaerobes and facultative anaerobes were observed. Thus, it seems that viability issues can be excluded at this early time point. This is also supported by the finding that the methodological gap is narrower at abundances of > 10^7 bacteria per biofilm.

Table 8. Spearman correlations of all three methods after 24 h of exposure to cell-culture medium

		qPCR	Mic	CFU
qPCR	Correlation coefficient	1.000	0.915**	0.667
	Significant (two-sided)		0.000	0.071
	n	10	10	8
Mic	Correlation coefficient	0.915**	1.000	0.571
	Significant (two-sided)	0.000		0.139
	n	10	10	8
CFU	Correlation coefficient	0.667	0.571	1.000
	Significant (two-sided)	0.071	0.139	
	n	8	8	8

CFU, colony-forming units; Mic, microscopy; qPCR, quantitative real-time PCR.
**Significant correlation with $p < 0.01$ (two-sided).

After 24 h of exposure to aerobic conditions in the cell-culture medium, however, a significant discrepancy was observed between CFU counts and measurements obtained using qPCR or epifluorescence microscopy. The CFU counts of *V. dispar* and *P. intermedia* were more than 1 log lower than the numbers obtained using the other two quantification methods, whereas *P. gingivalis* and *F. nucleatum* CFU counts were reduced by more than two logs. This result may not be surprising because these organisms are strictly anaerobic and neither

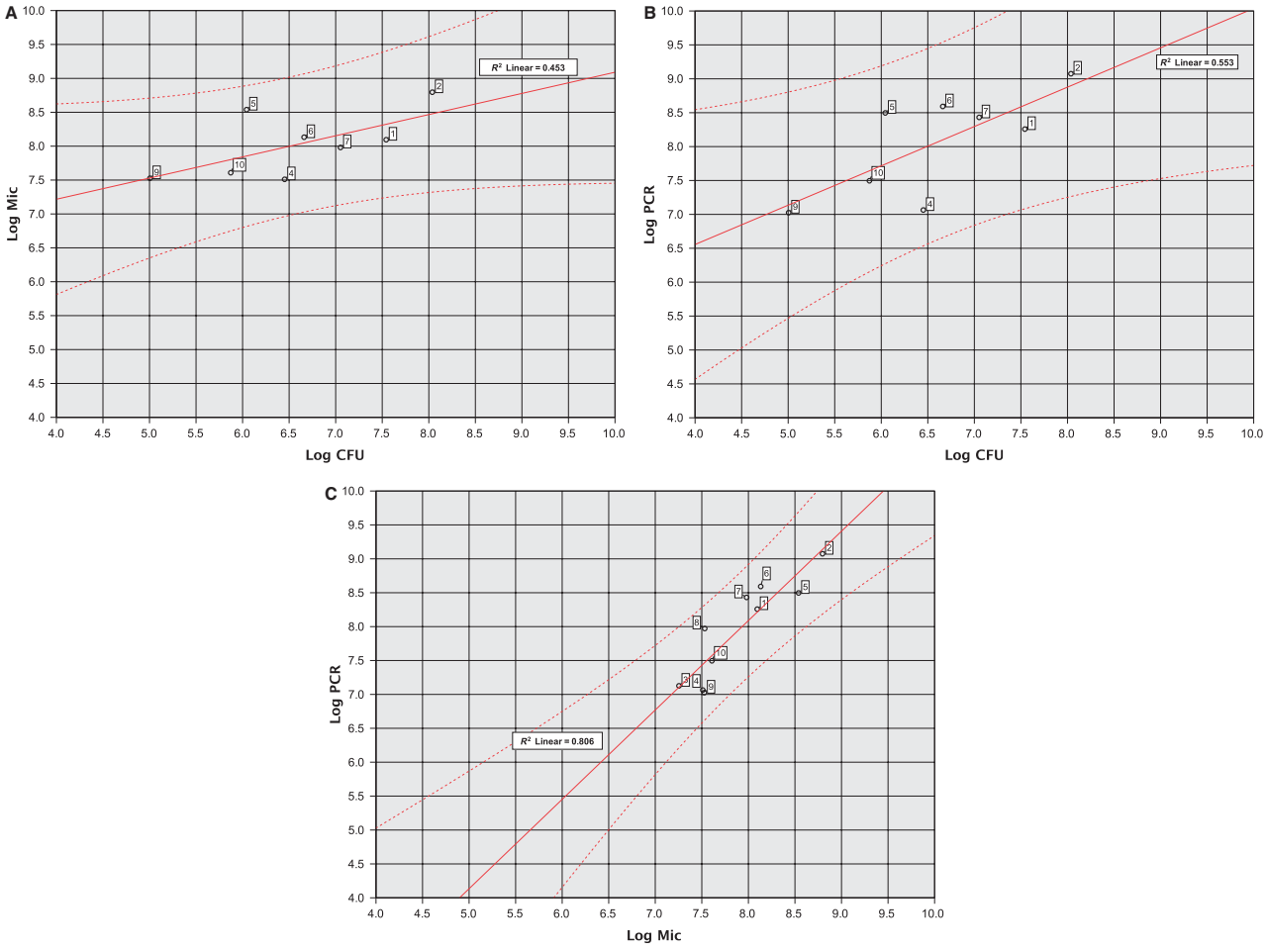

Fig. 4. Pairwise regression analysis of the quantitative data after 24 h of exposure to aerobic conditions in cell-culture medium. The values shown are logarithmized values of the measured abundances. (A) Microscopic (Mic) counts vs. colony-forming unit (CFU) counts; (B) quantitative real-time PCR (qPCR) quantification vs. CFU counts; (C) qPCR quantification vs. microscopic counts. The red solid line shows the linear regressions and the red dotted lines indicate the 95% confidence interval. Species key: (1) *Actinomyces oris*, (2) *Veillonella dispar*, (3) *Fusobacterium nucleatum*, (4) *Streptococcus anginosus*, (5) *Streptococcus oralis*, (6) *Prevotella intermedia*, (7) *Porphyromonas gingivalis*, (8) *Campylobacter rectus*, (9) *Tannerella forsythia* and (10) *Treponema denticola*.

qPCR nor epifluorescence microscopy is able to distinguish living bacteria from dead bacteria. Interestingly, these results may be in accordance with the results of a study in human subgingival dental plaque. A comparison between anaerobic culture methods and qPCR yielded a poor correlation between the methods for *P. intermedia* and *F. nucleatum*, discrepancies that were, in part, attributed to viability issues during standard culture (12).

In conclusion, CFU counts and quantification by epifluorescence microscopy or qPCR give results that correlate well as long as the viability of bacteria in the biofilms is relatively high. Furthermore, the values produced by qPCR and epifluorescence microscopy did correlate significantly, irrespective of the growth and viability states of the sample, and never differed by more than 0.5 log. This might indicate that microscopy counting following a live/dead staining of the bacteria could be used in combination with qPCR to provide an efficient tool to quantify only viable cells in a sample.

Our findings further indicate that using a combination of CFU counts and molecular methods is not ideal, as bacteria with abundances of $< 10^7$ m/L tend to yield lower CFU counts, a methodological discrepancy that may propagate further species-specific differences. These findings, using *in vitro* multispecies biofilms, indicate that for efficient comparison of the levels of different species, classic culture and molecular quantification techniques should not be applied in combination with each other. This may also have important implications in the development and selection of appropriate diagnostic methods for clinical subgingival biofilm samples.

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