Comparison of viable plate count, turbidity measurement and real-time PCR for quantification of Porphyromonas gingivalis

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Abbreviated running headline:

***Quantification of* Porphyromonas**

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Significance and impact of the study

Various clinical and research settings require fast and reliable quantification of bacterial suspensions. The viable plate count method (VPC) is generally seen as "the gold standard" for bacterial enumeration. However, VPC-based quantification of anaerobes such as *Porphyromonas gingivalis* is time-consuming due to their stringent growth requirements and shows poor repeatability. Comparison of VPC, turbidity measurement and TaqMan-based qPCR demonstrated that qPCR possesses important advantages regarding speed, accuracy and repeatability.

Abstract

The viable plate count (VPC) is considered as the reference method for bacterial enumeration in periodontal microbiology but shows some important limitations for anaerobic bacteria. Since anaerobes such as *Porphyromonas gingivalis* are difficult to culture, VPC becomes time-consuming and less sensitive. Hence, efficient normalization of experimental data to bacterial cell count requires alternative rapid and reliable quantification methods. This study compared the performance of VPC with that of turbidity measurement and real-time PCR (qPCR) in an experimental context using highly concentrated bacterial suspensions. Our TaqMan-based qPCR assay for *P. gingivalis* 16S rRNA proved to be sensitive and specific. Turbidity measurements offer a fast method to assess *P. gingivalis* growth, but suffer from high variability and a limited dynamic range. VPC was very time-consuming and less repeatable than qPCR. Our study concludes that qPCR provides the most rapid and precise approach for *P. gingivalis* quantification. Although our data were gathered in a specific research context, we believe that our conclusions on the inferior performance of VPC and turbidity measurements in comparison to qPCR can be extended to other research and clinical settings and even to other difficult-to-culture microorganisms.

Keywords

Enumeration, PCR (polymerase chain reaction), rapid methods, detection, optimization.

Introduction

The oral microflora consists of numerous anaerobic bacteria that may play a role in periodontal diseases. Under laboratory conditions, these anaerobes are difficult to culture because of their fastidious nature and slow growth (Lau *et al.* 2004; Urban *et al.* 2010). The stringent culture requirements hamper viable plate counting (VPC), which is "the gold standard" technique for bacterial quantification (Jervoe-Storm *et al.* 2005; Urban *et al.* 2010; Davey 2011). VPC allows the simultaneous detection of multiple bacteria and antimicrobial resistance profiling, but tends to be time-consuming and less sensitive for anaerobic bacteria (Siqueira and Rocas 2003; Boutaga *et al.* 2005; Urban *et al.* 2010). In addition, anaerobic incubation requires specialized laboratory techniques, which augment experimental costs significantly (Urban *et al.* 2010; Sutton 2011). Since many clinical and research settings demand bacterial quantification in a fast and reliable way, alternative counting techniques for anaerobes should be carefully assessed. Fundamental research often requires the exact determination of bacterial counts, even for highly concentrated samples. In the frame of ongoing research, the enzyme activities of the periodontal pathogen *Porphyromonas gingivalis* needed to be normalized to bacterial cell count (Clais *et al.* 2014). As for many other anaerobes, *P. gingivalis* grew very slowly on agar and was associated with variable viable plate counts. This encouraged us to explore other methods to count bacterial cells using *P. gingivalis* as a model microorganism for difficult-to-culture anaerobes. Turbidity measurement is a commonly used approach that allows fast and inexpensive assessment of microbial growth, while real-time quantitative PCR (qPCR) enables bacterial enumeration independent of growth characteristics. As such, qPCR can quantify unculturable, slow-growing and fastidious strains (Boutaga *et al.* 2003; Siqueira and Rocas 2003; Maeda *et al.* 2003). To identify the most appropriate technique to count anaerobes, this study systematically

compared VPC, turbidity and qPCR for the quantification of *P. gingivalis*. A Taqman-based qPCR that targets *P. gingivalis* 16S rRNA was developed and analytically validated. Given the experimental setting, the focus was on the rapid enumeration of concentrated suspensions of a difficult to culture bacterium.

Results and discussion

This study evaluated the applicability of three frequently used microbiological counting methods to quantify the anaerobe *P. gingivalis*. VPC was used as a gold standard method to enumerate a *P. gingivalis* starting suspension of 10^{10} colony forming units (CFU) ml^{-1} from which a tenfold serial dilution series was made containing 10^9 to 10^6 CFU ml^{-1} (Table 1). Each dilution was quantified using VPC, turbidity and qPCR. To assess the accuracy of these methods, the experimental counts were compared with the expected values using regression analysis.

Regression analysis indicated a good linear correlation between the VPC results and the expected values (R^2 = 0.9645, *p*-value<0.0001) (Figure 1). Nevertheless, this experiment clearly indicated repeatability problems for dense bacterial suspensions. The coefficient of variation (CV%) exceeded 15% for suspensions with more than 10^8 CFU ml^{-1} (suspensions A, B, C and D), indicating low precision (Table 1). When such dense suspensions are subjected to VPC, they undergo more dilution steps before plating than low concentration suspensions. Since every dilution step induces variation, the higher dilution factor might have contributed to the higher CV% of the concentrated bacterial suspensions. However, we know from previous experiments that variability may also occur for less concentrated suspensions. Furthermore, anaerobic culture techniques are characterized by a low level of sensitivity (Boutaga *et al.* 2003). The overall variability can be linked to the difficult *in vitro* culture of anaerobes. VPC reflects how many bacteria grow under the existing laboratory conditions, but does not allow the detection of latent microorganisms that cease to replicate on routine nutrient media (Davey 2011). The presence of these viable but nonculturable bacteria may be important as they often maintain their virulent properties and enzymatic activities (Davey 2011; Fakruddin *et al.* 2013). Other

drawbacks of VPC include the potential underestimation of the amount of bacteria when a single colony originates from large aggregates or the occurrence of difficult to interpret results when multiple plates show colonies in the countable range (Sutton 2011; Ammann *et al.* 2013). In our laboratory set-up, formation of visible colonies of *P. gingivalis* took about 7 to 14 days, while many experimental procedures, such as inoculum preparation, require fast enumeration. Moreover, keeping the anaerobic bacteria viable during practical handling and incubation is more expensive and less straightforward than for aerobe microorganisms.

Measuring the turbidity of a suspension is much easier and faster than VPC. However, our experiments proved that this approach has low accuracy since the optical densities poorly correlated with the expected bacterial counts (R^2 = 0.7920) (Figure 1). Several factors indeed contribute to variable counts and thus low precision. Firstly, both live and dead bacteria contribute to turbidity, endorsing the need for the exponential growth phase at the time of analysis. However, the unpredictable anaerobic growth makes it difficult to standardize sampling. Since cell morphology and bacterial surface area are also linked to the growth phase, these variables also contribute to less repeatable measurements (Baird *et al.* 2000; Lindqvist 2006). In addition, several bacteria produce extracellular products or bacterial pigments in a variable way, which influences light scattering (Baird *et al.* 2000; Lindqvist 2006). The lack of accuracy and precision of turbidity measurements in combination with the high detection limit (10^7 CFU ml^{-1}) and poor dynamic range discourage the application of this bacterial quantification method, although it is still widely used for rapid approximation of bacterial concentrations (Baird *et al.* 2000).

As a fast and growth-independent quantification method, we developed a TaqMan-based qPCR directed against a species-specific region of the 16S rRNA gene. Primers and probe were carefully designed as their configuration is pivotal for a high-quality qPCR (Table 2). The TaqMan format was chosen to maximize target specificity compared to SYBR Green-based real-time PCR as SYBR Green dyes intercalate in any double stranded DNA. To evaluate the specificity of primers and probes, qPCR was applied to a broad array of *P. gingivalis* and non-*P. gingivalis*

strains (Table 2). Only *P. gingivalis* strains could be amplified. A high-quality standard curve (y = (-3.61 ± 0.05) x + (51.0 ± 0.4), R^2 = 0.9970) indicated an amplification efficiency of 89.2%. Regression analysis indicated a linear correlation between qPCR data and expected values (*p*-value<0.0001, R^2 = 0.9548) (Figure 1). Three DNA extracts of three bacterial suspensions were analyzed in threefold to evaluate precision. The qPCR assay proved to be sensitive (limit of detection of 10^3 CFU ml^{-1}) and highly repeatable (CV% lower than 10% (0 - 2%)). To assess whether qPCR provides an accurate alternative method for VPC, the agreement between both methods was compared using Bland-Altman analysis. Goodness of fit in linear regression is often applied to compare two methods, but linearity not necessarily implies agreement (Bland and Altman 1986; Dewitte *et al.* 2002; Bunce 2009). The Bland-Altman plot showed that most data points were located within the limits of agreement, while the average discrepancy between both methods (the "bias") was close to zero, demonstrating significant agreement between VPC and qPCR (Figure 2). The variation of the VPC results for more concentrated samples explains the scatter around the bias line from 10^9 bacteria (Table 1). Although qPCR requires careful design and optimization efforts, its high speed and repeatability present important advantages, both for research and clinical purposes. In addition, qPCR is highly sensitive and enables detection of viable but nonculturable bacteria. An extra advantage of PCR strategies is the fact that they do not require single cell suspensions like VPC or flow cytometry. This is important since bacteria rather appear as an interacting community or biofilm than as a single entity. For example, *P. gingivalis* resides in the mouth as a part of dental plaque (Kuramitsu *et al.* 2007). The conversion of matrix-surrounded bacterial aggregates into homogenous single cell suspensions without influencing viability is difficult. In contrast to VPC that only detects viable bacteria, conventional qPCR amplifies DNA of both live and dead cells. To exclusively detect cells that are alive, live/dead qPCR involves the use of DNA intercalating substances like propidium monoazide to inhibit DNA amplification of dead cells (Loozen *et al.* 2011). Another approach for a more selective assessment of viable bacteria is reverse transcriptase PCR of RNA transcripts (Aellen *et al.* 2006; Martinez *et al.* 2009). After cell death, RNA degrades faster than DNA and therefore

permits a more selective detection of the viable fraction (Martinez *et al.* 2009; Reimann *et al.* 2010).

In conclusion, the slow and complex culture of *P. gingivalis* on agar and the high variability of VPC question its application for anaerobes. Turbidity measurements are easy and fast, but can only quantify highly concentrated bacterial suspensions. Our study proposes the use of TaqMan-based qPCR assay for fast and accurate enumeration of *P. gingivalis.*

Materials and methods

Bacterial strains and culture conditions

Suspensions of *P. gingivalis* were grown in Wilkins-Chalgren anaerobe broth (Oxoid, UK) supplemented with starch (2 g l^{-1}), maltose (2 g l^{-1}) (Merck, Germany), pancreatic digest of casein (30 g l^{-1}) (BD, USA) and L-cysteine (500 mg l^{-1}) (Sigma-Aldrich, Germany) under an anaerobic atmosphere (80% N_2, 10% H_2 and 10% CO_2) at 37°C for two days. Clinical isolates were obtained from the oral cavity of patients at the University Hospital of Leuven (Belgium). The ethical committee of the University Hospital approved the study (Code B32220071073).

Experimental set-up

In three independent experiments, *P. gingivalis* starting suspensions were quantified using VPC as a gold standard technique. From each starting suspension, tenfold serial dilutions were made in phosphate-buffered saline pH 7.4 (Gibco) and their corresponding expected values were calculated. Each dilution was quantified using VPC, qPCR and turbidity measurements. Using regression analysis, their experimental quantities were compared with the expected values and the agreement between the different methods was assessed.

Viable plate count

VPC was carried out by plating 50 µl of 10-fold serial dilutions on Wilkins-Chalgren agar plates (Oxoid, UK), supplemented with 5% sheep blood (Oxoid, UK) and L-cysteine (500 mg l^{-1}) (Sigma-Aldrich, Germany). Plates were incubated under anaerobic conditions for 7 to 14 days. Dilutions containing 10 to 100 colonies were counted and the concentration was calculated as CFU ml^{-1}. Each sample was analysed in duplicate.

DNA extraction and amplification

DNA was extracted from the bacterial suspensions using a QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Primers and probe were designed to target a species-specific region of the 16S rRNA gene (Table 2). Using MegAlign (DNASTAR, Lasergene 11), a conserved sequence within the 16S rRNA gene was defined for *P. gingivalis* strains. Table 2 shows the sequence homology and the locations of primers and probe (TIB MOLBIOL, Germany). Primers and probe specificity was evaluated *in silico* using BLAST analysis and *in vitro* against an array of pathogens. Both *P. gingivalis* reference strains and clinical isolates were used, but also non-*P. gingivalis* strains, which are likely to be found in the oral cavity, were assessed (Table 2). PCR reactions were performed in a total reaction volume of 20 µl, containing 10 µl of 2x TaqMan universal PCR master mix (Applied Biosystems, USA), 1µl of each primer and probe, 2 µl of PCR water and 5 µl of purified DNA using a StepOnePlus™ RT-PCR system (Applied Biosystems, USA). Primers and probe were added in a final concentration of 0.25 µmol l^{-1} each. Three technical replicates were analysed for each sample. Thermal cycling consisted of an initial amplification cycle of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec (denaturation) and 60°C for 1 min (annealing and elongation). The standard curve was constructed using a ten-fold dilution series of DNA extracted from a *P. gingivalis* starting suspension (see experimental set-up). To construct the final standard curve, three replicate standard curves were averaged to cope with inter-run variation. Visual determination of the standard curve allowed assessment of the limit of detection of the qPCR assay.

Turbidity measurement

Bacterial dilutions were measured in a 96-well plate at 650 nm (Multiskan microplate reader, Labsystems, Finland).

Statistical analysis

Linear regression analysis was performed to correlate the experimental outcomes of VPC, turbidity measurements and qPCR with the expected values. To evaluate the agreement between VPC and qPCR, a Bland-Altman plot with limits of agreement was constructed (Bland and Altman 1986; 2007). To assess qPCR precision, three samples were subjected to DNA extraction and amplification in triplicate. Repeatability was then evaluated by analysis of the CV%, which was considered acceptable within the 10-15 per cent range as described in the European Pharmacopoeia (8th edition, 2014). All data were analysed using GraphPad Prism V.6.0 software.

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Conflict of interest

The authors declare there is no conflict of interests.

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300 **Tables**

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Table 1: Viable plate count results for the different *P. gingivalis* suspensions (A-E) after quantification on three different days.

	Experiment 1				Experiment 2				Experiment 3			
	Expected value (CFU ml^{-1})	**CFU 1 (CFU ml^{-1})**	**CFU 2 (CFU ml^{-1})**	**CV (%)**	**Expected value (CFU ml^{-1})**	**CFU 1 (CFU ml^{-1})**	**CFU 2 (CFU ml^{-1})**	**CV (%)**	**Expected value (CFU ml^{-1})**	**CFU 1 (CFU ml^{-1})**	**CFU 2 (CFU ml^{-1})**	**CV (%)**
A	3.9×10^{10}	5.8×10^{10}	1.9×10^{10}	72	3.2×10^{10}	3.0×10^{10}	3.4×10^{10}	9	1.4×10^{10}	2.6×10^{9}	2.6×10^{10}	116
B	3.9×10^{9}	6.4×10^{10}	1.0×10^{10}	103	3.2×10^{9}	6.8×10^{9}	3.0×10^{9}	55	1.4×10^{9}	2.2×10^{9}	2.0×10^{9}	7
C	3.9×10^{8}	1.7×10^{8}	2.8×10^{8}	35	3.2×10^{8}	1.2×10^{8}	1.6×10^{8}	20	1.4×10^{8}	1.6×10^{8}	1.6×10^{8}	0
D	3.9×10^{7}	1.3×10^{7}	1.6×10^{7}	15	3.2×10^{7}	1.2×10^{7}	1.1×10^{7}	6	1.4×10^{7}	1.5×10^{7}	1.5×10^{7}	0
E	3.9×10^{6}	1.2×10^{6}	1.2×10^{6}	0	3.2×10^{6}	9.0×10^{5}	1.0×10^{6}	7	1.4×10^{6}	1.2×10^{6}	1.2×10^{6}	0

302

Table 2: Overview of the qPCR assay.

Alignment data

	970	980	990	1000	1010	1020	1030	1040	1050	1060
	|	|	|	|	|	|	|	|	|	|
AB547661.1.seq*	GCGAGGAACC	TTACCCGGGA	TTGAAATGTA	GATGACGGAT	GGTGAAAACC	GTCTTCCCTT	CGGGGCTTCT	ATGTAGGTGC	TGCATGGTTG	T
AF287987.1.seq*	GCGAGGAACC	TTACCCGGGA	TTGAAATGTA	GATGACGGAT	GGTGAAAACC	GTCTTCCCTT	CGGGGCTTCT	ATGTAGGTGC	TGCATGGTTG	T
AF414809.1.seq*	GCGAGGAACC	TTACCCGGGA	TTGAAATGTA	GATGACTGAT	GGTGAAAACC	GTCTTCCCTT	CGGGGCTTCT	ATGTAGGTGC	TGCATGGTTG	T
L16492.1.seq*	GCGAGGAACC	TTACCCGGGA	TTGAAATGTA	GATGACTGAT	GGTGAAAACC	GTCTTCC-TT	CGGGGCTTCT	ATGTAGGTGC	TGCATGGTTG	T
Forward primer	----GAGGAACC	TTACCCGGGA	T							
Probe	----------	----------	---------- TA	GATGACTGAT	GGTGAAAACC	GTCTTCC				
Reverse primer	----------	----------	----------	----------	----------	----------	-------- GCTTCT	ATGTAGGTGC	TGCAT	

Primers and probe

Sequence (5' → 3')		**T_m (°C)**	**GC (%)**	**Amplicon length (bases)**
Forward	GAGGAACCTTACCCGGGAT	58.07	57.89	
Reverse	ATGCAGCACCTACATAGAAGC	58.16	47.62	82
Probe	6-FAM--TAGATGACTGATGGTGAAAACCGTCTTCC--BBQ#	64.63	44.83	

Bacterial panel for *in vitro* specificity evaluation

***P. gingivalis* strains**		**Other strains**	
3 Reference strains	ATCC 33277 W50 W83	Clinical isolates from the oral cavity	*Fusobacterium nucleatum* *Aggregatibacter actinomycetemcomitans* *Prevotella intermedia* *Streptococcus sobrinus* *Streptococcus mutans*
5 Clinical isolates	K1-K5	Reference strains	*Staphylococcus aureus* ATCC 6538 *Escherichia coli* ATCC 8739 *Helicobacter pylori* ATCC 43504

*GenBank accession numbers

#FAM = 6-Carboxyfluorescein; BBQ = BlackBerry Quencher

Figure legends

Figure 1: Regression analysis for the three methods. (A) VPC (•) versus Expected values, y = (1.1380 ± 0.0609) x – (1.2340 ± 0.5194), R^2 = 0.9645; (B) Turbidity (▲) versus Expected values, y = (1.3770 ± 0.2666) x - (4.346 ± 2.519), R^2 = 0.7920; (C) qPCR (♦) versus Expected values, y = (0.8380 ± 0.0598) x + (1.5910 ± 0.5104), R^2 = 0.9548.

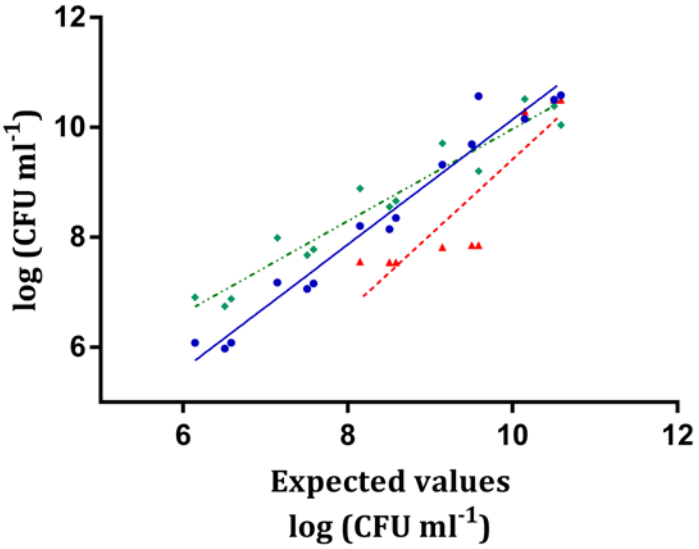
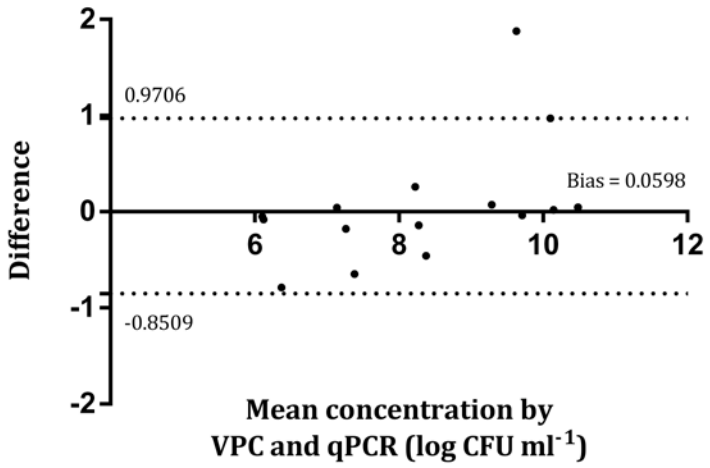


Figure 2: Bland-Altman plot for comparison of VPC and qPCR. The difference between both methods (y-axis) is represented against their average (x-axis) and the limits of agreement are represented as dotted lines.


2
1
0
-1
-2
0.9706
Bias = 0.0598
-0.8509
Difference
6
8
10
12
Mean concentration by
VPC and qPCR (log CFU ml-1)

314