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Validation of a multiplex qPCR assay for the identification and quantification of *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*: *In vitro* and subgingival plaque samples

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ABSTRACT

Objective: To validate a multiplex qPCR (m-qPCR) assay for the simultaneous identification and quantification of *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* in subgingival samples.

Material and methods: ***In vitro samples***: DNA combinations of *A. actinomycetemcomitans* and *P. gingivalis* in similar or different concentrations were prepared. qPCR and m-qPCR were performed using the same primers and hydrolysis probes specific for 16SrRNA genes. Results were analyzed using intra-class (ICCs) and Lin's correlation coefficients (r) based on quantification cycle (Cq) values. ***Subgingival plaque samples***: a cross-sectional study analyzing subgingival plaque samples harvested from periodontally-healthy and chronic periodontitis patients. Samples were processed by either qPCR or m-qPCR targeting both bacteria. Sensitivity, specificity, predictive values and Liń́s correlation coefficients (r) were calculated using CFU/mL as primary outcome.

Results: ***In vitro samples***: m-qPCR yielded a good reproducibility (coefficients of variation around 1% and ICCs > 0.99) for both bacterial species. m-qPCR achieved detection limits and specificity similar to qPCR. An excellent concordance (r = 0.99) was observed between m-qPCR and qPCR for *A. actinomycetemcomitans* and *P. gingivalis* without statistical significant differences between both methods ***Subgingival plaque samples***: a high sensitivity (above 80%) and specificity (100%) was obtained with the m-qPCR for both bacteria. The m-qPCR yielded a good concordance in Cq values, showing a good level of agreement between qPCR and m-qPCR.

Conclusion: The tested m-qPCR method was successful in the simultaneous quantification of *A. actinomycetemcomitans* and *P. gingivalis,* with a high degree of sensitivity and specificity on subgingival plaque samples.

1. Introduction

Periodontitis is a chronic inflammatory condition characterised by the destruction of tooth supporting tissues. The primary etiological factor of this disease is the pathogenic bacterial species within the subgingival biofilm (APP, 1996). Although there are more than 700 different bacterial species in symbiosis with the host within the human oral cavity (Aas, Paster, Stokes, Olsen, & Dewhirst, 2005), only a small fraction of these bacteria have the potential to cause dysbiosis and induce the metabolic changes responsible of periodontal tissue destruction (Sanz et al., 2017). Amongst these bacteria with pathogenic potential, two species, *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* have been clearly associated in many clinical studies with the initiation and progression of periodontitis (Slots & Ting, 1999). Moreover, in recent years, *P. gingivalis* has been identified in experimental studies as a key pathogen in promoting the dysbiotic enviroment leading to the chronic inflammatory changes characteristic of the periodontitis lesion (Hajishengallis & Lamont, 2012). The appropriate detection and quantification of these periodontal pathogens in subgingival plaque samples, therefore, seems appropriate for the diagnosis, risk assessment and treatment planning of periodontitis patients (van Winkelhoff & Winkel, 2005).

For many years, bacterial culturing was considered the gold standard method for detecting bacterial pathogens residing in subgingival biofilms (Sanz, Lau, Herrera, Morillo, & Silva, 2004). This technique, however, has clear limitations, namely it is relatively time-consuming and expensive, it needs experienced personnel and it is difficult to identify and isolate cultivable species when present in low abundance or those species with stringent growing conditions (Morillo, Lau, Sanz, Herrera, & Silva, 2003; Sanz et al., 2004). In fact, most of the bacterial species present in the subgingival biofilms are considered as non-cultivable, since they have only been identified by molecular methods. To overcome these limitations, techniques based on molecular methods such as the polymerase chain reaction (PCR) assays have been

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developed. Among them, the quantitative real-time PCR (qPCR) provides a precise and sensitive method for an accurate quantification of individual periodontal pathogenic bacterial species with high sensitivity and specificity using fluorescence signals (Boutaga, Savelkoul, Winkel, & van Winkelhoff, 2007). These assays allow the detection of pathogens with slow or difficult growth in culture (Boutaga, van Winkelhoff, Vandenbroucke-Grauls, & Savelkoul, 2003; Yoshida et al., 2003). Previous studies from our research group have shown a high sensitivity and specificity of qPCR technology in detecting and quantifying target periodontal microorganisms (Morillo et al., 2003, 2004) and when compared to standard culturing techniques they demonstrated a positive predictive value (0.92) in detecting *P. gingivalis* in periodontitis patients (Lau et al., 2004).

Recently, a different modality of qPCR, the multiplex qPCR (m-qPCR) has being successfully used in microbiological diagnosis (Puenpa, Suwannakarn, Chansaenroj, Vongpunsawad, & Poovorawan, 2017) and specifically in periodontology (Figuero et al., 2014; Miranda et al., 2017; Montero et al., 2017; Nagaoka et al., 2017; Zhou et al., 2015, 2017). The m-qPCR assay takes the analysis one step further, allowing simultaneous amplification of different target sequences from the same sample, what implies a clear reduction in time and cost (Bolivar, Rojas, & García, 2013). These m-qPCR assays, however, have not been validated in the identification and quantification of periodontal pathogenic bacteria. Only Milne et al. (2016) developed a multiplex assay aimed to subgingival plaque samples, but using primers targeting single copy genes (Rgpa and IlkA), instead of 16SrRNA sequences (Milne et al., 2016). Most real-time q-PCR assays for the quantification of *A. actinomycetemcomitans* and *P. gingivalis* use 16SrRNA sequences, a small-subunit of DNA present in all bacterial species that contains highly conserved species-specific sequences (Al-Hebshi, Shuga-Aldin, Al-Sharabi, & Ghandour, 2014; Heid, Stevens, Livak, & Williams, 1996; Marin et al., 2017; Sánchez et al., 2017). The study by Milne et al. (2016) only reported results from m-qPCR, without presenting the comparison with the gold-standard technique, qPCR. It is, therefore, the objective of this study to validate the m-qPCR for the simultaneous detection of *A. actinomycetemcomitans* and *P. gingivalis*.

2. Material and methods

2.1. In vitro samples

The primer-hydrolysis probe set were designed to target 16SrRNA gene from *A. actinomycetemcomitans* and for *P. gingivalis* strains. To test the sensitivity (detection limit) of this primer/hydrolysis probe combination, we used a series of 10-fold dilutions of known DNA concentrations of the reference strains. To test the specificity (cross-reactions) qPCR assays were performed with DNA from the reference strains of non-target species: *Tannerella forsythia* (strain ATCC 43037), *Fusobacterium nucleatum* (strain DMSZ 20482), *Campylobacter rectus* (strain NCTC 11489), *Streptococcus oralis* (strain CECT 907T) and *A. actinomycetemcomitans* (strain DSMZ 8324) (in assays with primers/hydrolysis probe of *P. gingivalis)* or *P. gingivalis* (strain ATCC 33277) (in assays with primers/hydrolysis probe of *A. actinomycetemcomitans)*. DNA of each non-target species was included in qPCR reactions individually or pooled as a DNA mixture of all non-target bacteria. Primer and probe specificity was also checked in silico by BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

To compare the quantification data (Cq) between qPCR and m-qPCR, pure culture samples of *A. actinomycetemcomitans* and *P. gingivalis* the following experiments were used:

a Serial dilutions from 10^8 to 10^1 colony forming units (CFU)/mL of purified genomic DNA of *P. gingivalis* (n = 8).
b Serial dilutions from 10^8 to 10^1 CFU/mL of purified genomic DNA of pure *A. actinomycetemcomitans* (n = 8).
c A combination of DNA from serial dilutions of *A. actinomycetemcomitans* and *P. gingivalis* in similar concentrations (from 10^8 to 10^1 CFU/mL) (n = 8).
d A combination of DNA from serial dilutions of *A. actinomycetemcomitans* and *P. gingivalis* in different concentrations:
   - *P. gingivalis* (10^8 CFU/mL) and *A. actinomycetemcomitans* (serial dilutions from 10^5 to 10^1 CFU/mL) (n = 5).
   - *A. actinomycetemcomitans* (10^8 CFU/mL) and *P. gingivalis* (serial dilutions from 10^5 to 10^1 CFU/mL) (n = 5).

Each DNA sample was analysed by both assays (qPCR and m-qPCR) in duplicate in different experiments in three separated days (n = 3).

2.1.1. Strains

Reference strains (*A. actinomycetemcomitans* strain DSMZ 8324, *P. gingivalis* strain ATCC 33277) were grown on blood agar plates (Oxoid No. 2 Blood Agar, Oxoid, Basingstoke, UK) supplemented with horse blood 5% (Oxoid), haemin (5.0 mg/l) (Sigma, St. Louis, MO, USA) and menadione (1 mg/l) under anaerobic conditions (10% H_2, 10% CO_2, and balanced N_2) at 37 °C for 24–72 h. BHI media (Brain Heart Infusion, Becton, Dickinson and Company, USA) were used to grow bacteria under anaerobic conditions for 24–48 h (depending on the bacterial species) until reaching the exponential phase of growth (as measured by spectrophotometry at optical density 550 nm). From each BHI medium containing *A. actinomycetemcomitans* or *P. gingivalis* at the desired concentration, 1 mL was taken and vortexed. Then, each vial was centrifuged at 13,000 rpm for 1 min, and the supernatant was discarded. The resultant pellets were frozen to −20 °C until DNA extraction.

2.1.2. DNA extraction

Bacterial DNA from each vial was extracted using a commercial kit specifically designed to extract bacterial DNA in blood samples (MoIYsis Complete5. MolzymGmbh& Co.KG. Bremen, Germany) following manufacturer's instructions. Finally the extracted DNA was eluded in 100 μL of sterile water (Roche) and frozen at −20 °C for further analysis.

2.1.3. qPCR assay

All qPCR procedures were conducted by a single experienced and blinded personnel (MJM).

2.1.4. PCR primers and hydrolysis probes

The sequence of the primers and hydrolysis probes used for *P. gingivalis* and *A. actinomycetemcomitans* targeted 16S ribosomal-RNA (rRNA) genes (Table 1) (Boutaga, van Winkelhoff, Vandenbroucke-Grauls, & Savelkoul, 2005). For qPCR, the oligonucleotide hydrolysis probes of both bacteria were labelled with the fluorescent dyes 6-

Table 1
Primers and hydrolysis probes used for the qPCR amplifications.

Bacterial species	Sequence (5′–3′)
A. actinomycetemcomitans (Boutaga et al., 2005)	Primer F: GAA CCT TAC CTA CTC TTG ACA TCC GAA Primer R: TGC AGC ACC TGT CTC AAA GC Probe: FAM-AGA ACT CAG AGA TGG GTT TGT GCC TTAGGG-TAMRA
P. gingivalis (Boutaga et al., 2003)	Primer F: GCGCTCAACGTTCAGCC Primer R: CACGAATTCCGCCTGC Probe qPCR: FAM-CACTGAACTCAAGCCCGGCAGTTTCAA-TAMRA Probe m-qPCR: HEX5′-CACTGAACTCAAGCCCGGCAGTTTCAA-BHQ1

F: Forward. R: Reverse; FAM: 6-carboxyfluorescein; TAMRA: 6-carboxytetramethylrhodamine; HEX: hexachloro-fluorescein phosphoramidita; BHQ1: Black Hole Quencher1; qPCR: quantitative polimerase chain reaction; m-qPCR: multiplex qPCR.