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Innovative application of nested PCR for detection of *Porphyromonas gingivalis* in human highly calcified atherothrombotic plaques

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ABSTRACT

Atherothrombosis, leading to stroke and myocardial infarction, is responsible for most of the deaths in the world. An increased risk of atherothrombotic vascular events has been reported in patients with periodontitis. Periodontitis is a chronic multifactorial inflammatory disease, which involves a dysbiotic microbiota, and leads to a progressive destruction of the tooth-supporting apparatus. Transcient periodontal pathogen blood translocation, mainly bacteremia, has been associated with the severity of gingival inflammation. The identification of periodontal bacteria within atherothrombotic plaques is challenging and unpredictable. This review aims to summarize existing molecular technics for identifying periodontal microbiota in human atherothrombotic samples. A secondary objective is to describe a protocol for the identification of *Porphyromonas gingivalis* from highly calcified, atherothrombotic human samples that is based on our experience in translational cardiovascular research. Compared to direct real-time PCR, our protocol based on nested PCR has increased the detection of *Porphyromonas gingivalis* by 22.2% with good specificity.

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Introduction

Atherothrombotic cardiovascular diseases have been linked to periodontitis primarily via associations with periodontal bacteria [1]. Several hypotheses have been proposed to explain this link: (1) the direct role of bacteria or their byproducts (endotoxins, proteases, DNA), damaging the vascular wall and driving atherothrombotic plaque development [2]; and (2) the role of the innate immune response to oral dysbiosis by establishing a prothrombotic state and intraplaque hemorraghes [3,4]. To decipher these mechanisms, many studies first investigated the presence of periodontal pathogens within human atherothrombotic plaques using techniques based on cellular and molecular biology [5]. Although the identification of periodontal microbiota in other less complex types of human samples, like subgingival swab and blood, are now well established [6], detection of periodontal bacteria within atherothrombotic plaques remains challenging, because of the characteristics of the pathological tissue highly enriched with oxidation, protease activity and cellular death leading to haemorraghes and calcifications.

Techniques based on amplification of the *16S* rRNA bacterial gene by polymerase chain reaction (PCR) offer large scale applications in clinical vascular samples [7]. Given the variety of PCR protocols used for identification of periodontal bacteria in atherothrombotic plaque samples, the outcomes across human studies show a wide range of heterogeneity [5]. This review aims to summarize existing molecular protocols for identifying periodontal microbiota in human atherothrombotic samples. A secondary objective is to describe a protocol for the identification of *Porphyromonas gingivalis* from human highly calcified atherothrombotic samples, which is based on our experience in translational cardiovascular research.

Polymerase chain reaction

All of the PCR steps and reagents are points of potential variability that lead to different results across clinical studies that focus on identifying periodontal bacteria in human atherothrombotic plaques. Thus, we analyzed the literature regarding the charatecteritcs of the samples and the PCR protocols used (Tables 1–3). With regards to the

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Table 1. Identification of periodontal pathogens in atherothrombotic samples using PCR methods.

Authors (year)	Country	Vascular sample	n	Aa (%)	Pg (%)	Tf (%)	Pi (%)
Kurihara et al. (2004) [26]	Japan	Aneurysmal wall	32	0	85	22	31
Marques da Silva et al. (2005) [27]	Norway		56	7.1	0	0	/
Nakano et al. (2009) [28]	Japan		86	30.2	<20	<15	<15
Delbosc et al. (2011) [29]	France		16	/	43.8	/	/
Pyysalo et al. (2013) [30]		Finland	36	<20	<20	/	<20
Haraszthy et al. (2000) [11]	USA	Carotid	50	18	26	30	14
Cairo et al. (2004) [12]	Italy		19	0	0	0	0
Fiehn et al. (2005) [13]¶	Denmark		11	0	0	0	100
Kozarov (2005) [2]	USA		1	100	100	/	/
Aimetti et al. (2007) [14]	Italy		33	0	0	0	0
Figuero et al. (2011) [15]	Spain		42	66.6	78.6	/	/
Rangé et al. (2014) [4]	France		157	0	39	35	33
Fernandes et al. (2014) [16]	Brasil	Carotid, aneurysmal wall	14	/	0	/	7.1
Kozarov et al. (2006) [17]	USA	Carotid, aortic, femoral	9	55.5	88.8	22.2	77.7
Aquino et al. (2011) [18]	Brazil	Carotid, coronary, femoral	30	0	0	/	/
Padilla et al. (2006) [8]	Chile	Carotid, femoral, tibial, popliteal	12	1.7	0	0	0
Toyofuku et al. (2011) [9]	Japan	Carotid, iliac, femoral	53	0	51	0	15
Figuero et al. (2018) [10]‡	Sweden	Carotid, peripheral, aneurysmal wall	70	2.8	0	2.8	/
Ishihara et al. (2004) [19]	USA	Coronary	51	23.3	21.6	5.9	/
Pucar et al. (2007) [20]	Serbia		15	26.7	53.3	13.3	33.3
Gaetti-Jardim et al. (2009) [21]	Brasil		44	46.2	53.8	25.6	59
Mahendra et al. (2009) [22]	India		51	/	45.1	/	/
Rath et al. (2014) [23]	India		7	42.8	71.4	100	0
Mahalakshmi et al. (2017) [24]	India		65	0	52.3	46.2	32.3
Atarbashi-Moghadam et al. (2018) [25]	Iran		23	17.4	13	/	/
Fiehn et al. (2005) [13]¶	Denmark	Femoral	13	0	7.7	0	100
Okuda et al. (2001) [32]	Japan	Not detailed	26	0	0	0	/
Taylor-Robinson et al. (2002) [33]	UK		32	21.9	0	0	9.4

¶No sample was positive with direct specific PCR; ‡with nested PCR, Aa: 18.% and Pg: 57.8%

sample sites, 13 studies used the carotid [2,4,8–18], 8 the coronary [18–25] and 7 the anevrysmal wall [10,16,26–30]. Other studies used various vascular samples. In 4 studies [9,20,26,31], they were immediately frozen after sampling then transported in liquid medium before being processed, or in the case of 18 studies, they were further stored until processing [2,4,8,11–14,16–19,21–25,27,28] and 6 studies did not address the processing methods [10,15,29,30,32,33]. Samples were immediately frozen after their transport in 2 studies [11,23], or kept at −20°C in 10 studies [12,14–16,18,20,21,24,28,33] or at −70°C or lower in 10 studies [9–11,23,26,27,29–31,33]. In 3 studies, samples were cultured [8], embedded in paraffin [32] or kept in conditioned medium [4] before being frozen. Samples have been mechanically homogenized in only 8 studies [4,10,14,15,22,27,29,31]. For 7 studies [8,9,12,13,23,25,26], homogenization was addressed although details were not provided and for 12 studies [2,11,16–18,20,21,24,28,30,32,33] no details on tissue homogenization were provided.

None of the studies used the exact same PCR protocol and DNA extraction kit. Seventeen studies implemented conventional qualtitative PCR to amplify DNA [8,9,11,16,18–20,22–28,31–33], 7 used quantitative PCR [2,4,12,17,21,29,30] and 4 used nested PCR [10,13–15]. The median number of cycles was 36 with a big range (30 [8,11,13,31]–60[30]). The results of the PCR were confirmed by gel only in 9 studies [8,9,12,14,18,20,26,27,29], by DNA sequencing in 8 studies [4,13,15,19,22,24,25,32] and by melting curve analysis in 4 studies [2,17,21,30].

These elements, which will be discussed below, are all parameters of variability that can explain the different results observed. Indeed, *Actinobacillus actynomyctemcomitans, Porphyromonas gingivalis, Tanerella forsythia* and *Prevotella intermedia* have been found in 58.3%, 64.3%, 55.0% and 77.8% of the studies, but were detected in a broad range in 19.2% (0–100; median: 7.1), 31.1% (0–100; median: 20.8), 15.9% (0–100; median: 4.4), 30.4 (0–100; median: 17.5) of the samples, respectively.

The heterogeneity of the protocols did not highlight one specific parameter of variability, but rather it raised the issue of the importance of each of these variables. Consequently, although PCR is a well-known technique, a strict protocol must be followed that also takes into account the proteolytic and oxidative nature of the samples, leading to hemorrages and calcifications. The second part of this manucript describes every step of our protocol based on nested PCR, from the collection to the final identification of periodontal bacteria, which is prone to bias and can lead to variable results. We then compare it to the use of direct real-time PCR.

Microbial whole genome sequencing

Microbial whole genome sequencing (WGS), a high-throughput approach to DNA sequencing using the concept of massively parallel processing, also called next-generation sequencing (NGS), has been used to explore oral bacterial communities in atherothrombotic plaques. Interestingly, the microbial community observed in

Table 2. Vascular sample preparation for identifying periodontal pathogens.

Authors (year)	Transport medium	Storage	Sample homogenization	DNA extraction
Haraszthy et al. (2000) [11]	Sterile saline solution	Processed immediately or frozen at −70°C	Unknown	Instagen® Purification Matrix (Bio rad Laboratories, Hercules, CA)
Okuda et al. (2001) [32]	Unknown	Formalin-fixed, paraffin-embedded blocks	Unknown	Dexpat® (Takara, Otsu, Japan)
Taylor-Robinson et al. (2002) [33]	Unknown	−70°C or −20°C for up to 5 years	Unknown	Flowgen® (Novara House, UK)
Cairo et al. (2004) [12]	Sterile saline solution	−20°C	Addressed with no details	Standard protocol (with proteinase-K and cetyltri-methylammonium bromide)
Ishihara et al. (2004) [19]	Sterilized phosphate-buffered saline and mixed gently	Unknown	Tube pestle with lysis solution	Puregene® kit (Gentra Systems, Minneapolis, Minn.)
Kurihara et al. (2004) [26]	Immediately frozen	−80°C	Addressed with no details	High Pure® PCR Template Preparation Kit (Roche, Mannheim, Germany)
Fiehn et al. (2005) [13]	Reduced transport medium	Unknown	Addressed with no details	Modified SDS extraction method (Sigma-Aldrich, Vallensbaek Strand, Denmark) and DNA clean-up system (purification)
Kozarov (2005) [2]	Pre-reduced tryptic soy broth	Processed immediately	Unknown	DNeasy® Tissue kit (Qiagen Sciences, Valencia, CA)
Marques da Silva et al. (2005) [27]	Pre-reduced anaerobically sterilized Transport medium	−70°C in sterile tubes containing Todd Hewitt broth with 0.5% dimethyl sulfoxide	Sterile mortar with liquid nitrogen under a laminar flow hood	QIAamp® Mini Kit (Qiagen GmbH, Hilden, Germany)
Kozarov et al. (2006) [17]	Pre-reduced transport medium	Unknown	Unknown	QIAmp® Tissue Kit (Qiagen Inc., Valencia, CA)
Padilla et al. (2006) [8]	Saline solution	Cultivated	Addressed with no details	AquaPure® Genomic DNA Isolation kit (BioRad, Hercules, CA, USA)
Aimetti et al. (2007) [14]	Sterile saline solution	−20°C	Mechanical homogeniser (Tissue Laser, QIAGEN, Hilden, Germany)	Phenol-chloroform method and ethanol precipitation
Pucar et al. (2007) [20]	Immediately frozen	−20°C	Unknown	Standard protocol (with proteinase-K)
Elkaïm et al. (2008) [31]	Dry sterile tube immediately frozen with liquid nitrogen	−80°C	Polytron device (Kinematica, Luzern, Switzerland).	Standard protocol (with RNAse A, proteinase K, phenol–chloroform–isoamylic alcohol and EDTA)
Gaetti-Jardim et al. (2009) [21]	Sterile DNA-free saline solution	−20°C	Unknown	Charge Switch® gDNA Mini Tissue kit (Invitrogen)
Nakano et al. (2009) [28]	Sterie saline solution	−20°C	Unknown	Unknown
Mahendra et al. (2009) [22]	Sterilized phosphate buffered saline and mixed gently and then transfer to transport medium	Unknown	Tissue homogenizer (Saiki et al. 1988)	Standard protocol (with lysis solution (Tris, EDTA, Triton), temperature variation and centrifugation)
Aquino et al. (2011) [18]	Sterile microcentrifuge tubes containing trypticase soy broth and dimethyl sulfoxide	−20°C	Unknown	QIAamp® DNA mini kit (Qiagen, Valencia, Spain)
Delbosc et al. (2011) [29]	Unknown	−80°C	Cryopulverized using a freezer mill (Spex Certiprep Ltd)	QIAamp® DNA blood Midi kit (Qiagen) with modifications
Figuero et al. (2011) [15]	Sterile conditions	−20°C	Mechanical homogenizer	G-Nome® DNA kit (MP Biomedicals)

(*Continued*)

Table 2. (Continued).

Authors (year)	Transport medium	Storage	Sample homogenization	DNA extraction
Toyofuku et al. (2011) [9]	Immediately frozen under sterile conditions	−80°C	Addressed with no details	High Pure® PCR Template Preparation Kit (Roche, Mannheim, Germany)
Pyysalo et al. (2013) [30]	Unknown	−70°C	Unknown	Unknown
Fernandes et al. (2014) [16]	Sterile vial containing phosphate-buffered saline	−20°C	Unknown	Standard protocol (with cetyltri-methylammonium bromide)
Figuero et al. (2014) [10]	Sterile conditions	−80°C	Mechanical homogenizer	G-Nome® DNA kit (MP Biomedicals) and purification
Rangé et al. (2014) [4]	Cold Roswell Park Memorial Institute medium (RPMI) (4°C) containing antibiotics plus an antimycotic	Incubated (24 h at 37°C) in a standardized volume (6 mL/g of sample wet weight) of RPMI culture medium supplemented with antibiotics and an antimycotic and TPI at −80°C	Cryopulverized using a freezer mill (Spex Certiprep Ltd)	QIAamp® DNA blood Midi kit (Qiagen) with modifications
Rath et al. (2014) [23]	Saline solution in sterile vial	Frozen in a bath of liquid nitrogen at – 80°C	Addressed with no details	Qiagen® Kit method
Mahalakshmi et al. (2017) [24]	Phosphate buffered saline	−20°C	Unknown	Boiling – lysis method
Atarbashi-Moghadam et al. (2018) [25]	Soaked in saline with sulfate buffer and then placed in Stuart transport medium	Unknown	Addressed with no details	Unknown

Table 3. PCR conditions and identification methods for periodontal pathogens in atherothrombotic samples.

Authors (year)	PCR protocol	Cycle (n)	Data analysis
Haraszthy et al. (2000) [11]	Conventional qualitative	30	Hybridization
Okuda et al. (2001) [32]		32 or 36	Clonage and sequencing
Taylor-Robinson et al. (2002) [33]		36	Unknown
Ishihara et al. (2004) [19]		36	Sequencing
Kurihara et al. (2004) [26]		36	Agarose gel
Marques da Silva et al. (2005) [27]		32	Agarose gel
Padilla et al. (2006) [8]		30	Agarose gel
Pucar et al. (2007) [20]		35	Polyacrylamide gel
Elkaïm et al. (2008) [31]		30	Hybridization
Nakano et al. (2009) [28]		36	Unknown
Mahendra et al. (2009) [22]		36	Agarose gel and sequencing
Aquino et al. (2011) [18]		36	Agarose gel
Toyofuku et al. (2011) [9]		36	Gel
Fernandes et al. (2014) [16]		40	Unknown
Rath et al. (2014) [23]		40	Agarose gel
Mahalakshmi et al. (2017) [24]		35 or 36	Gel and sequencing
Atarbashi-Moghadam et al. (2018) [25]		35	Agarose gel and sequencing
Fiehn et al. (2005) [13]	Nested and conventional	30 or 35	Agarose gel and sequencing
Aimetti et al. (2007) [14]	Nested	32	Agarose gel
Figuero et al. (2011) [15]	Nested	35	Agarose gel and sequencing
Figuero et al. (2014) [10]	Nested and quantitative	40	Unknown
Cairo et al. (2004) [12]	Quantitative	35	Agarose gel
Kozarov (2005) [2]		40	Melting curve
Kozarov et al. (2006) [17]		40	Melting curve
Gaetti-Jardim et al. (2009) [21]		40 or 45	Melting curve
Delbosc et al. (2011) [29]		50	Agarose gel
Pyysalo et al. (2013) [30]		60	Melting curve
Rangé et al. (2014) [4]		50	Gel and sequencing

atherothrombotic samples is similar to that observed in subgingival samples [34]. However, no periodontal species have been identified by WGS in vascular samples [35] but they have been identified by WGS in other samples [36].

Some studies also identified gut microbiome members within atherothrombotic samples using WGS, however, the results are mixed [37]. WGS is a useful and highly sensitive technique. However, minor changes in laboratory protocols, in the preparation of the samples or in the analysis of the data can modify the results [38]. Thus, given this sensitivity to minor changes, WGS may not be suitable for the identification of periodontal bacterial species in atherothrombotic samples. Consequently, although WGS is a very sensitive technique for characterizing bacterial community diversity in human samples, the analysis of the results must be considered carefully for periodontal species in atherothrombotic tissues.

Proposed protocol for amplification of periodontal bacteria by PCR

Based on the data from the literature reviewed above, and taking into account our experience with the identification of periodontal bacteria in animals [29] and human atherothrombotic plaques [4], we suggest that the presence of periodontal pathogens should be explored by using the protocol discussed below (Figure 1) and described in detail in the supplemental material, for *Porphyromonas gingivalis* amplification. Indeed, we considered each parameters of variability to propose a protocol observing the rules of molecular biology adapted to the specificity of atherothrombotic and higly calcified samples.

Vascular sample collection and preparation

Table 2 summarises vascular sample preparation characteristics across studies exploring periodontal microbiota in atherothrombotic plaques. Besides transit and storage conditions, several other parameters may have a significant impact on DNA yield and quality. Little information is available on the cleaning of surgical specimens to prevent contamination althougt it is important. Given that DNA is denatured at room temperature, most of the studies indicate that samples are rapidly frozen at low temperature (from −20°C to −80°C). Details on the homogenization of the samples are scarce in the literature, although powerful mechanical grinding is necessary to release bacterial DNA. Bead beating [39] and liquid nitrogen electromagnetic methods (cryopulverisation) [29] appear to be those most suitable to give acces to the entire DNA and thus prevent false negative.

In our laboratory, the samples are stored at −80°C before being pulverized, using a freezer mill (Spex Certiprep Ltd) ensuring perfect homogenization of the samples without destroying the DNA by heating, which is not discussed in the reported studies. Briefly, the samples are placed one by one in a previously cooled tube containing a magnetic bar and then introduced into the grinding machine filled with liquid nitrogen. Importantly, tissue samples must be cleaned up under flow to avoid external contamination, then strongly homogenized before being processed. Ideally, to maintain optimal DNA integrity and yield and prevent its compromise due to thawing and tissue lysis, DNA extraction and the amplification of the targeted sequence by PCR must be performed just

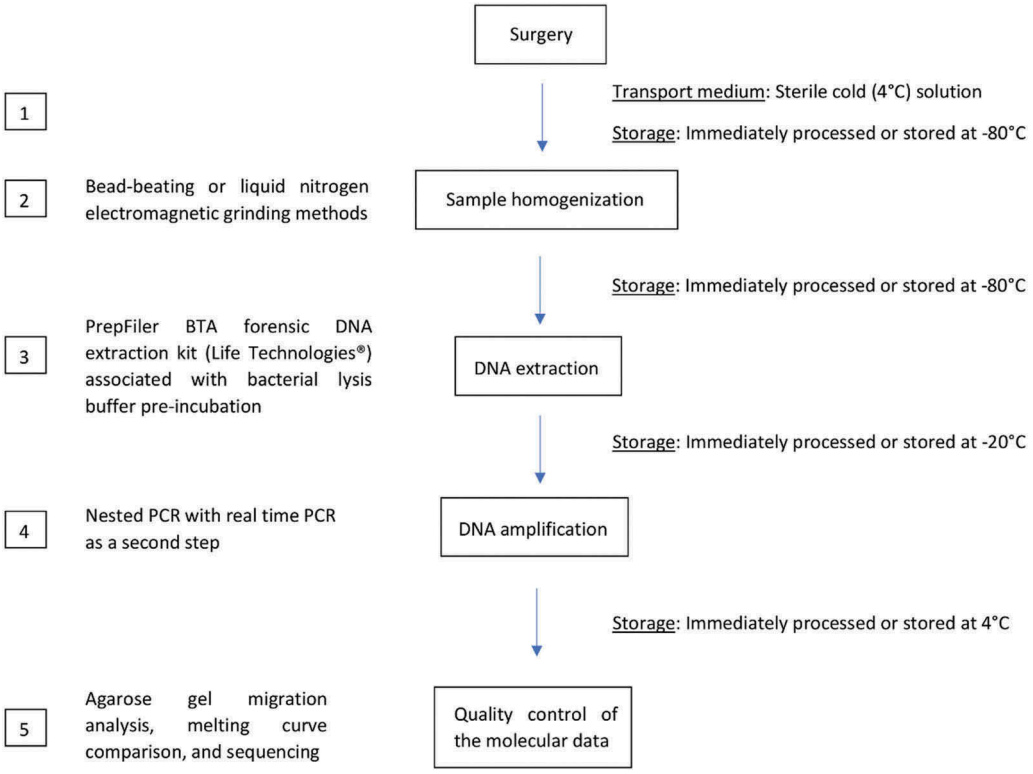

Figure 1. Proposed protocol for *Porphyromonas gingivalis* 16S rDNA amplification in human calcified atherothrombotic samples in 5 steps. Sterile equipment must be used for all the following steps. (**1.**) Samples must be transported to the laboratory in a cold (4°C) sterile solution within a few hours after harvesting. (**2.**) Then, samples must be processed with homogenization. A liquid nitrogen electromagnetic grinding method is preferred over a bead-beating method to maximize nucleic acid preservation. Samples can be stored at −80°C while waiting for homogenization, but freezing and thawing steps should be minimized. (**3.**) DNA extraction is carried out after pre-incubation with a bacterial lysis buffer using the PrepFiler BTA forensic DNA extraction kit (Life Technologies®) to remove calcifications. (**4.**) Then, nested PCR is carried out, with conventional PCR as the first step and real-time PCR as the second step (≤40 cycles). (**5.**) For quality control, the molecular data is obtained using agarose gel migration, melting curve comparison, and sequencing.

after sampling. However, this is not always possible in standard laboratory practice. Immediate storage at −80°C after freezing on dry ice or in liquid nitrogen is usally considered appropriate. However, freezing and thawing cycles must be kept to a minimum.

DNA extraction from atherothrombotic tissues

The DNA extraction method selected must be adapted for bacterial DNA extraction and to the specificity of the human tissue. Calcifications are a common macroscopic feature of atherothrombotic samples (Figure S1), which prevent optimal DNA extraction because they saturate and block the extraction column filter or interfere with free DNA [40] or extraction reagents. This technical point is not addressed in the literature, and details are lacking on how to adjust for this limitation when using the DNA extraction kits (Table 3), while it is very important to limit false negative due to low yield. DNA yield and quality may be improved with commercial kits specifically designed for calcified tissues, like teeth or bone. For example, the PrepFiler BTA forensic DNA extraction kit (Life Technologies®) is meant for this type of application. In addition, considering human atherothrombotic samples, the bacterial DNA/human DNA ratio is low to very low, and this factor impairs the detection of bacterial DNA. Techniques for separating human DNA from bacterial DNA have been proposed and warrant more attention [41]. This would make bacterial DNA amplification easier and more reliable, but (1) the yield would be low, (2) and the cost would be high. Therefore, some authors recommended the use of MoIYsis Complete5 kit (Molzym®) for bacterial DNA extraction from human tissues [42]. In addition, bacterial characteristics of the sample should be considered, such as the presence of Gram + or Gram − bacteria. This consideration will help improve the isolation of the bacterial DNA from the tissue [43]. For example, Gram-positive bacteria require pre-incubation with specific enzymes, such as lysozyme, to lyse the rigid multilayered cell wall. This can also be used with Gram-negative bacteria to ensure efficient DNA extraction.

Periodontal bacterial DNA amplification

Most studies implement conventional qualitative PCR to identify bacterial DNA, whereas others use real-time quantitative PCR or nested PCR (Table 3). All studies used previously published references but they often

slightly modify the methodology, for example by customizing primers and/or PCR conditions.

Targeting specific *16s* rRNA gene sequences for periodontal pathogens can be implemented with primers previously used in the literature or designed by dedicated software like Primer-BLAST. This tool is easy to use and it helps select custom primers that meet the specific requirements of the selected PCR protocol. However, these newly generated primers will not have been tested, unlike those published in the literature. Without a consensus on optimal primers, scientists must first systematically test the selected primers on known bacterial culture samples and on clinical subgingival bacterial samples. Once primers have been positively confirmed on standard samples, they can then be used for sequencing and comparison with a *16s* rRNA gene database, like the one from the Ribosomal Database Project (RDP) [44].

To improve bacterial detection, some studies increase the number of PCR cyles (>40) to increase the DNA copy number [4,21,29,30]. This approach is not recommended because it increases the risk of false positives, which decreases specificity. Some authors pooled duplicate or triplicate samples to increase the likelihood of detection [34]. However, comparing results from duplicate or even triplicate samples instead of pooling, improves the sensitivity of bacterial identification. Based on our experience, we recommend using 3 dilutions of DNA extracts because human atherothrombotic plaque samples contain variable concentrations of bacterial DNA. This approach has been useful because we have found that at least one dilution is often positive for a given sample. This approach has not been described previously, although it impacts the PCR detection rate for periodontal pathogens, preventing false negative. In addition, since each Taq DNA polymerase and master mix have their own characteristics, testing at least 2 different commercial mixes is a good way to optimize the PCR conditions for the atherothrombotic plaque samples. Lastly, according to the primers and the PCR machine used, multiple PCR programs that call for different times and temperatures have been described in the literature (Table 3). For genomic DNA amplification, including bacterial DNA amplification, manufacturers recommend using standard protocols and not the fast programs present in newer devices.

Direct real-time PCR and nested PCR

Direct real-time PCR is a good technique for amplifying DNA. However, in our study, it showed poor results for the amplification of a specific fragment of the 16S rRNA gene of *P. gingivalis* in human atherosclerotic calcified samples. Indeed, only one sample out of 45 was positive (Figure 2(a)). When we tried to improve the sensitivity, by increasing the number of cycles of amplification from 40 to 50, we lost specificity (Figure 2(b)).

Nested PCR is a modification of the conventional PCR method, which consists of a succession of two PCRs, the second PCR uses the product of the first PCR as sample. Indeed, two sets of primers are used in the nested PCR protocol. The first set of primers (first PCR) amplifies large fragments by binding outside of the target DNA. The second set of primers (second PCR) binds specifically at the target DNA. It is intended to reduce non-specific binding by reducing the amplification of unexpected primer binding sites. Different methods have been described for purifying the PCR products from the first universal PCR (using 16S rRNA gene universal primers). One option is the Denaturing Gradient Gel Electrophoresis (DGGE) technique, which involves using a gel extraction kit after the amplified products have migrated on an agarose gel. Alternative options include using PCR purification kits or incubation with an enzyme that removes unincorporated primers and dNTPs. Use of the Illustra ExoProStar® (Dutcher) product for

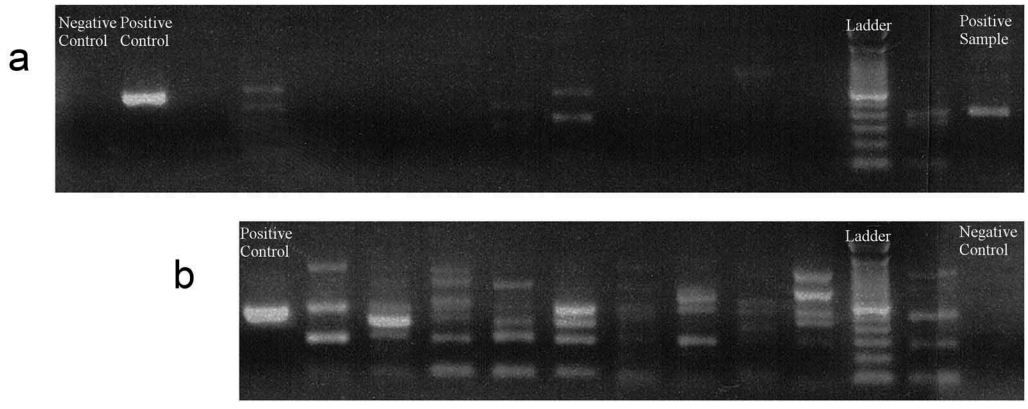

Figure 2. Images of agarose gels showing the migration of amplicons from an amplification by direct real-time PCR using primers designed for *Porphromonas gingivalis*. (**a**) PCR protocol using 40 cycles. (**Lane 1:**) Negative control. (**Lane 2:**) Positive control. (**Lanes 3–13 and 15, 16:**) Samples. (**Lane 14:**) Ladder. Only the last sample (**lane 16**) had an amplicon with the same molecular weight as the positive control. (**b**) PCR protocol using 50 cycles. (**Lane 1:**) Positive control. (**Lanes 2–10 and 12:**) Samples. (**Lane 11:**) Ladder. (**Lane 13:**) Negative control. All the samples present multiple amplicons.

removing unincorporated primers and dNTPs is easier to implement compared to other methods, and was highly effective in our study.

The implementation of this protocol based on nested PCR has increased the sensitivity, without losing specificity (Figure 3). Extra cost and time were moderate compared to the gain.

Based on our experience, nested PCR is a good approach for identifying periodontal pathogens in highly challenging samples, like atherothrombotic plaques, preventing false negative by increasing the sensitivity via a double amplification and false positive by reducing non-specific binding.

Quality control considerations

Evaluation of the migration of PCR products on agarose gels is a widely used method [45]. However, this approach is inaccurate and is not reliable for identification of periodontal pathogens. Therefore, recent studies have added a sequencing phase to confirm their results (Table 3). Admittedly, this step represents additional cost and preparation time. An alternative option for greater quality control is using a comparative analysis of the melting curve. However, this technique may introduce error, since a slight variation in the profile of the melting curve may correspond to an aberrant PCR product (Figures S2 and S3). In our study, the 3 approaches were used to strengthen the identification of the bacteria. First, we compared the melting curves, then positive samples were loaded on agarose gel and, finally, positive samples were sequenced, preventing false positive, which is not the case for most of the reported studies. In summary, for reliable identification of periodontal pathogens in atherothrombotic plaques, PCR products must be sequenced.

Conclusion

Identification of periodontal microbiota in human atherothrombotic plaques is very important. Indeed, pathogens are central to the relationships that bind periodontitis to atherothrombosis [46]. The detection of the 16 rRNA gene is a signature of the pathogen that colonized the atheromatous plaque at some point in the patient's life, which means that the pathogen had been alive at one point and may be still be alive. This may result in oxidation and proteolysis, which further leads to plaque vulnerability [2]. However, it remains highly challenging despite technical improvements. Heterogeneity in the published data may be linked to differences in sample characteristics, in sample collection and preparation, and the molecular-based techniques used for identification. False negatives and false positives may occur when inadequate methodological approaches and quality control measures are implemented, leading to an inacturate estimation of the presence of periodontal bacteria in atherothrombotic plaques. Identification of periodontal bacteria in human atherothrombotic samples should be performed carefully to avoid significant pitfalls. Standardization of the molecular techniques and protocols across laboratories and clinical teams are needed to improve the quality of reported data in the field of periodontitis and atherothrombotic cardiovascular disease research. Furthermore, WGS may not be suitable for species identification of periodontal bacteria in atherothrombotic samples. The PCR protocol described in this

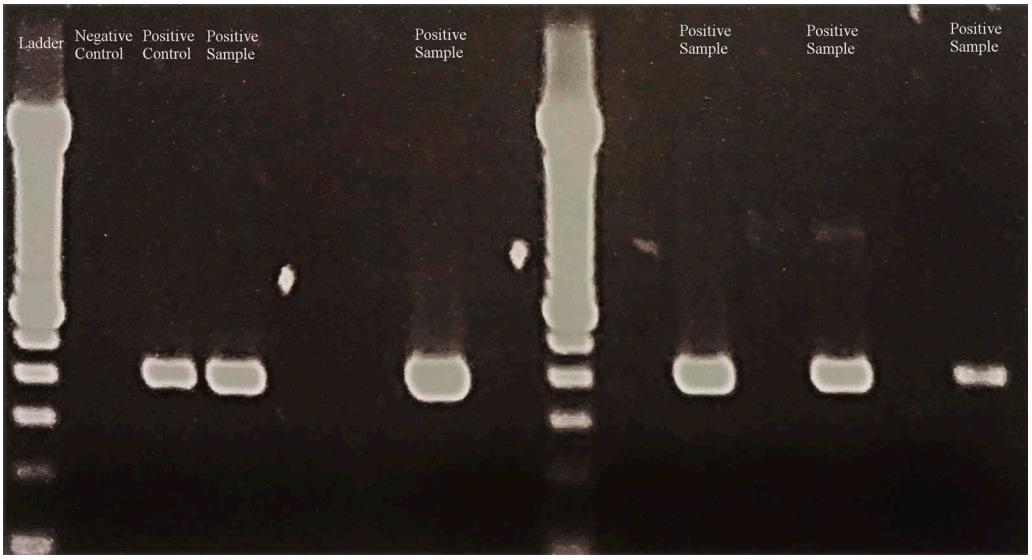

Figure 3. Images of agarose gel showing the migration of amplicons from an amplification by nested PCR (conventional PCR as the first step and real-time PCR as the second step) using primers designed for *Porphromonas gingivalis*. (**Lane 1 and 9:**) Ladder. (**Lane 2:**) Negative control. (**Lane 3:**) Positive control. (**Lanes 4–8 and 10–15:**) Samples. Samples from (**lanes 4, 7, 11, 13, and 15**) had an amplicon with the same molecular weight as the positive control. They were confirmed to be positive by comparison of the melting curves and by sequencing.

report may be a first step in standardizing these approaches, and thereby contributing to a consensus in the identification of periodontal pathogens in atherothrombotic samples, especially in highly calcified ones, preventing false positive and false negative biais.

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