

Novel strategy to detect and locate periodontal pathogens: The PNA-FISH technique

Luzia Mendes (PhD DMD)^{a,*}, Rui Rocha (MsD)^{b,c,d}, Andreia Sofia Azevedo (PhD)^b, Catarina Ferreira (DMD)^a, Mariana Henriques (PhD)^c, Miguel Gonçalves Pinto (PhD DMD)^a, Nuno Filipe Azevedo (PhD)^b

^a *Department of Periodontology, Faculty of Dental Medicine, University of Porto, Porto, Portugal*

^b *LEPABE, Laboratory for Process Engineering, Environment, Biotechnology and Energy, Department of Chemical Engineering, Faculty of Engineering, University of Porto, Porto, Portugal*

^c *LIBRO, Laboratório de Investigação em Biofilmes Rosário Oliveira, University of Minho, Braga, Portugal*

^d *BIOMODE, Zona Industrial da Gandra, Apartado 4152, 4806-909 Guimarães, Portugal*

Abstract

Purpose: We aim to develop peptic nucleic acid (PNA) probes for the identification and localization of *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* in sub-gingival plaque and gingival biopsies by Fluorescence *in situ* Hybridization (FISH).

Methods: A PNA probe was designed for each microorganism. The PNA-FISH method was optimized to allow simultaneous hybridization of both microorganisms with their probe (PNA-FISH multiplex). After being tested on representative strains of *P. gingivalis* and *A. actinomycetemcomitans*, the PNA-FISH method was then adapted to detect microorganisms in the subgingival plaque and gingival samples, collected from patients with severe periodontitis.

Results: The best hybridization conditions were found to be 59 °C for 150 min for both probes (PgPNA1007 and AaPNA235). The *in silico* sensitivity and specificity was both 100% for PgPNA1007 probe and 100% and 99.9% for AaPNA235 probe, respectively. Results on clinical samples showed that the PNA-FISH method was able to detect and discriminate target bacteria in the mixed microbial population of the subgingival plaque and within periodontal tissues.

Conclusion: This investigation presents a new highly accurate method for *P. gingivalis* and *A. actino- mycetemcomitans* detection and co-location in clinical samples, in just few hours. With this technique we were able to observe spatial distribution of these species within polymicrobial communities in the periodontal pockets and, for the first time with the FISH method, in the organized gingival tissue.

1. Introduction

Periodontitis results from an imbalance between the subgingival microbiota and the host defenses, in susceptible individuals (Sanz and van Winkelhoff, 2011). The study of periodontal biofilms has assumed major importance in the past decades, however, owing to their complex polymicrobial nature and to the difficulties to perform *in vivo* studies, its characterization for research and diagnostic purposes is still challenging. The molecular methods have now supplanted the traditional culture methods, providing new resources to identify not only single microorganisms, but whole communities with potential pathogenic importance (Wade, 2011; Marsh and Devine, 2011).

In this context, fluorescence *in situ* hybridization (FISH) applied to biofilm studies gained importance since it allows *in situ* identification of microorganisms by hybridization of labeled DNA probes with bacterial ribosomal RNA. Some authors have used this technique to observe, *in vivo*, the spatial distribution of periodontal pathogens in the supra and subgingival biofilm (Zijnga et al., 2010), as well as, their ability to invade host epithelial cells (Rudney et al., 2001; Colombo et al., 2007).

However, the added value that this technique offers in the knowledge of three-dimensional structure of biofilms and their interaction with host tissues is strongly influenced by some limitations in the FISH process, such as low cell permeability, hybridization affinity and target site accessibility to the DNA probes. These limitations often cause lack of target site specificity and sensitivity with consequently loss of important information (Amann and Fuchs, 2008; Cerqueira et al., 2008). To overcome these issues, nucleic acid analogues, also known as DNA mimics, have been developed. The peptide nucleic acid (PNA) was the first to be published, in 1991, by Nielsen et al. (1991) and since the late 90s has been used in microbial detection (Guimarães et al., 2007; Cerqueira et al., 2011; Almeida et al., 2011; Alves et al., 2014). In this DNA mimic the negatively charged sugar-phosphate backbone of DNA is replaced by a neutral polyamide backbone composed of N- (2-aminoethyl) glycine units. The lack of charge repulsion between neutral PNA strand and the complementary RNA strand allows a quicker and stronger PNA/RNA binding. As a result, PNA probes can be shorter than its DNA counterparts improving the access to the target sequences. Also, the hydrophobic nature of the PNA molecule facilitates cell penetration and diffusion through the biofilm matrix. The use of PNA probes brought robustness and higher sensitivity and specificity to the conventional FISH technique (Cerqueira et al., 2008).

Accordingly, our group aimed to develop, for the first time, highly specific and highly sensitive PNA probes to enable *in situ* detection of periodontal pathogens. *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* are some of the most relevant putative periodontal

pathogens present in subgingival plaque which have also the machinery to invade oral epithelial cells (Meyer et al., 1996; Lamont et al., 1995). They were, therefore, logical choices for this investigation.

2. Material and methods

2.1. Target species and culture maintenance

Eight *P. gingivalis* strains, clinical isolates and type cultures, were kindly provided by Professor Mike Curtis (Queen Mary University of London) and Professor Koji Nakayama (University of Nagasaki). Three *A. actinomycetemcomitans* strains (type cultures) were kindly provided by Professor Casey Chen (University of Southern California). All strains (Table 2) were maintained on tryptic soy agar (TSA) (VWR, Portugal) supplemented with 5% (vol/vol) defibrinated sheep blood (Probiológica, Portugal). Plates were incubated at 37 °C under anaerobic conditions (AnaeroGen Atmosphere Generation System; Oxoid, United Kingdom). Colonies were streaked onto fresh plates every 5–7 days.

2.2. Probe development

A PNA probe was designed for each microorganism. Firstly, potentially useful oligonucleotides with 15 base pairs (bp) were identified using the freely available Primrose program ([http:// www.cf.ac.uk/biosi/research/biosoft/Primrose/index.html](http://www.cf.ac.uk/biosi/research/biosoft/Primrose/index.html)) coupled to the 16S rRNA databases of Ribosomal Database Project II (RDP-II) (<http://rdp8.cme.msu.edu/html/>, last access August 2014). The sequence selection was based on the 16S rRNA comparison of five randomly chosen strains. To avoid missing possible sequences of interest several sets of five random strains were tested. Secondly, several criteria were applied in order to select the best PNA-FISH probes for our purpose, namely: high number of target microorganisms detection and low number of non-targets detection; no self-complementary structures within the probe; similar predicted melting temperature for both probes and high guanine and cytosine content. Finally, the selected sequences were synthesized (Panagene, South Korea) being the N-terminus of *P. gingivalis* and *A. actinomycetemcomitans* PNA probes attached to Alexa Fluor 488 and 594, respectively, via a double AEEA linker (-8-amino-3,6-dioxo octanoic acid).

2.3. Theoretical sensitivity, specificity and binding affinity evaluation

Theoretical sensitivity and specificity were evaluated with the updated databases available at RDPII and confirmed by a search on the National Center for Biotechnology Information (NCBI) available at

<http://www.ncbi.nlm.nih.gov/BLAST/>. Only target sequences with at least 1200 bp and good quality were included (Almeida et al., 2010). In short, theoretical sensitivity and specificity were calculated according to the formulas $(\text{target})\text{s}/\text{total}(\text{target})\text{s} \times 100$ and $(\text{non-target})\text{s}/\text{total}(\text{non-target})\text{s} \times 100$, where $(\text{target})\text{s}$ stands for the number of strains detected by the oligonucleotide probe, $'\text{total}(\text{target})\text{s}'$ for the total number of target strains present in the database, $'(\text{non-target})\text{s}'$ for the number of non-target strains that did not react with the oligonucleotide probe and $'\text{total}(\text{non-target})\text{s}'$ for the total of non-target strains found in the database. The binding affinity was estimated based on the accessibility of 16S rRNA target sites map to fluorescent oligonucleotide probes established by Fuchs et al. (1998).

2.4. Development of the PNA-FISH multiplex protocol

Each of the designed probes was previously tested and optimized before being tested in clinical samples. The hybridization method, in slide, was based on the procedure reported in Almeida et al. (2010), with some modifications. Hybridization time and temperature were adjusted to achieve the highest signal for both microorganisms simultaneously (multiplex PNA-FISH).

Firstly, we evaluated the PNA-FISH protocol in pure cultures of each target species. In each case, cells from 3-days-old cultures were harvested from TSA plates, suspended in sterile water and homogenized by vortexing for 1 min. Using 3-wells glass slides, 30 μL of each strain suspension was dried up at 55 $^{\circ}\text{C}$ for about 15 min and subsequently immersed in 4% (wt/vol) paraformaldehyde followed by 50% (vol/vol) ethanol, for 10 min each, at room temperature. The fixated smears were then covered with 20 μL of hybridization solution containing 10% (wt/vol) dextran sulfate (Fisher), 10 mM NaCl (Panreac), 0.2% (wt/vol) polyvinylpyrrolidone (Sigma), 0.2% (wt/vol) Ficoll (Fisher), 5 mM disodium EDTA (Pan-reac), 0.1% (vol/vol) Triton X-100 (Panreac), 50 mM Tris-HCl (pH 7.5; Fisher) and 200 nM PNA probe. With coverslips on, the slides were placed in moist chambers and incubated for 150 min at 59 $^{\circ}\text{C}$. After the hybridization step, the coverslips were removed, the slides submerged and maintained in a pre-warmed (59 $^{\circ}\text{C}$) washing solution containing 5 mM Tris base (Fisher), 15 mM NaCl (Panreac) and 0.1% (vol/vol) Triton X (pH 10; panreac) for 30 min and then allowed to air dry in a dark place for a maximum of 24 h before microscopy. For each experiment a negative control was carried out with hybridization solution without probe. Secondly, both probes were tested against taxonomically related microorganisms and/or possible oral colonizers.

Finally, to ensure that each species-specific probe maintained its behavior in a multiplex procedure, a mix of two PNA probes was applied simultaneously in a mixed smear of the two corresponding species (*A. actinomycetecomitans* and *P.*

gingivalis). For this, 10 µL from each specie-specific suspension was mixed and spread on slides. Hybridization was performed as described above.

Discrimination and co-localization of *A. actinomycetecomitans* and *P. gingivalis* in clinical samples by PNA-FISH

2.5. Collection of clinical samples

Clinical specimens were collected from a convenient sample of 5 adult patients attending the clinic of Faculty of Dental Medicine of Oporto. All patients were diagnosed with severe periodontitis, were otherwise healthy and haven't been submitted to any antimicrobial therapy in the past 3 months. This convenience and non-probabilistic sample was selected based only on the critical judgment of the investigator face to high probability of detection of both microorganisms in subjects with severe periodontitis. Informed consent was obtained prior to sample collection, in accordance with the study protocol approved by the Ethical Committee of the Faculty of Dentistry of Oporto.

Subgingival plaque samples were collected using sterile Gracey curettes (Hu-Friedy®), after tooth isolation with cotton rolls and supragingival plaque removal with sterile cotton pellets. Samples were suspended in a sterile saline solution (NaCl 0.9%) and processed immediately.

Gingival samples were collected during periodontal surgery or during extraction of hopeless teeth. Gingival samples were individually placed in 4% (wt/vol) paraformaldehyde and stored at 4 °C until further usage.

2.6. PNA-FISH validation in clinical samples

Subgingival plaque suspensions were centrifuged at 10,000 × *g* for 5 min. The pellet was resuspended in 400 µL of 4% (wt/vol) paraformaldehyde for 1 h, before being centrifuged again. The fixed cells were then resuspended in 500 µL of 50% (vol/vol) ethanol and storage at 20 °C until further usage. For hybridization, a 30 µL aliquot of fixed cells was spotted onto 3-wells glass slides and allowed to air dry.

Gingival tissue biopsies were embedded in paraffin, cut to 3 µm thickness, and mounted on microscope glass slides. Prior to hybridization, slides were immersed twice in xylol for 15 min each time, then rehydrated by decreasing concentrations of ethanol (100%, 95%, 80%, 70% and 50%) for 5 min each time and finally, washed up with distilled water for 10 min and allowed to air dry. A 20 µL of hybridization solution with 200 nM of each specie-specific probe was added to both clinical samples and hybridization proceeded as previously described. An additional staining with 60 µL of 4',6-diamidino-2-phenylindole (DAPI; 100 µg/ml) for 10 min in the dark was performed. Slides were then

washed with 10 mL of water and allowed to air dry before microscopic visualization.

2.7. Microscopic visualization

Prior to microscopy visualization samples were mounted with 1 drop of nonfluorescent immersion oil (Leica) and covered with coverslips. Image was acquired using a Leica DM LB2 epifluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany) connected with Leica DFC300 FX camera (Leica Microsystems GmbH, Germany) equipped with sensitive filters to the selected Alexa Fluor molecules. Filters that were not able to detect the probe were used to confirm the absence of autofluorescence. All images were acquired using Leica IM50 Image Manager, Image Processing and Archiving software using a magnification of 1000. For multiplex purposes same field images were obtained with each one of the filters for the corresponding PNA probe and overlapped.

3. Results

3.1. Probe design and theoretical assessment of probe specificity, sensitivity and binding affinity

The initial purpose of this work was to find highly sensitive and specific PNA probes for *A. actinomycetemcomitans* and *P. gingivalis* which allowed a multiplex PNA-FISH hybridization. For *A. actinomycetemcomitans* the following 15-bp sequence was obtained: 5'-CTAATCACACTTGGG-3'. This probe hybridizes between positions 235 and 250 of the 16S rRNA *A. actinomycetemcomitans* (T) strain and was designated AgPNA235. For *P. gingivalis* the following 14-bp sequence was obtained: 5'-AGACGGTTTTTCACC-3'. It hybridizes between positions 1007 and 1022 of the 16S rRNA *P. gingivalis* (T) strain and was thus designated PgPNA1007. Based on the hybridization positions, the binding affinity of the designed probes to the targets will presumably result in a fluorescence intensity of Class V for the PgPNA1007 probe and Class IV for the AaPNA235 probe.

The 1-bp reduction was needed to lower the melting temperature and achieve similar hybridization conditions for both probes. This decrease in probe length did not affect the theoretical sensitivity and specificity of the original 15-bp probe (data not shown). According to the RDP-II database, the AgPNA235 probe is fully complementary with all the 73 target sequences of *A. actinomycetemcomitans*. Similarly, the PgPNA1007 was fully complementary with all 43 target sequences but also matched one non-target strain: *Porphyromonas gulae* (S000390658). Accordingly, 100% of sensitivity and

specificity was determined for the AgPNA235 probe, while the PgPNA1007 probe achieved 100% of sensitivity and 99.9% of specificity (Table 1).

Multiple sequence alignments using the BLASTn program revealed the presence of 1-bp mismatches with other bacterial species that compose the human microbiota or are known pathogens in humans (Fig. 1). However, for none of these species the human oral cavity is described as a recognized place of colonization. *P. gulae* has been exclusively associated to the oral cavity colonization of various animals (Fournier et al., 2001), but a recent study identified *P. gulae* in the oral cavity of native South Americans with a traditional diet (Gaetti-Jardim et al., 2015).

3.2. PNA-FISH protocol optimization

Multiple tests were conducted to optimize the PNA-FISH protocol in several strains of *P. gingivalis* and *A. actinomycetemcomitans*. From temperatures between 57 and 61 °C and a range of hybridization times between 60 and 180 min (data not shown) we found optimum conditions for simultaneous hybridization of both probes with their target microorganism at 59 °C for 150 min (Fig. 2). Between 57–61 °C the hybridization of the AaPNA235 probe showed a strong signal-to-noise ratio from 90 up to 150 min, but the same was only observed for the PgPNA1007 probe at 59 °C for 150 min. As expected, by the *in silico* analysis, both probes hybridized with its target microorganism whereas no cross-hybridization was observed (Table 2). Additionally, it was observed that, under the established conditions, both probes retained their behavior in a sample containing the two target microorganisms, providing an exact discrimination between the two species involved, as shown in Fig. 3. The green filter image presents a slightly visible red background due to a small overlap between the Alexa Fluor 594 (red) absorption spectrum and the excitation filter used to visualize the PgPNA1007 probe.

3.3. PNA-FISH validation in clinical samples

Being the multiplex PNA-FISH protocol optimized the ability of the designed probes to hybridize in polymicrobial clinical samples was then tested. The application of this technique to subgingival plaque samples revealed the absence of *A. actinomycetemcomitans* in our convenience sample. By contrast, *P. gingivalis* was present and occasionally exhibited an organization in microcolonies (Fig. 4).

The results in gingival tissue biopsies showed that AaPNA235 and PgPNA1007 probes were able to detect, discriminate and co-locate both species

(Fig. 5). *P. gingivalis* was detected in greater numbers and more frequently than *A. actinomycetemcomitans*. Furthermore, it was interesting to observe the existence of super- invaded cells by *P. gingivalis* contrasting with poorly or non-invaded cells (Fig. 4).

4. Discussion

Over the years efforts have been made to clarify the etiopathogenesis of periodontal diseases and the triggering mechanism that disrupts the homeostasis between the subgingival microbiota and the host defenses. Bacterial invasion of the periodontal tissues has been pointed as such mechanism. However, the difficulties to visualize, discriminate, ascertain position and quantify an intragingival polymicrobial flora in a reproducible, time and cost-effective way precludes a definitive association (Tribble and Lamont, 2010). A systematic review conducted by our study group showed that there is no scientific evidence to support the invasion of periodontal pathogens as a key step in the pathogenesis of periodontitis due to the reduced number of studies and the strong limitations and heterogeneity seen in the available studies (Mendes et al., 2015).

Our work intended to contribute for improving the knowledge about this possible pathogenic mechanism by developing highly sensitive and specific PNA probes for simultaneous detection of *P. gingivalis* and *A. actinomycetemcomitans*.

The theoretical evaluation of sensitivity and specificity allowed the comparison of both PNA probes with previously developed probes for the *in situ* detection of these microorganisms (Table 1). The PgPNA1007 probe has a higher theoretical performance in *P. gingivalis* detection compared with the DNA probes used in several previous studies (Rudney et al., 2001; Colombo et al., 2007; Stoltenberg et al., 1993; Sunde et al., 2003; Rudney et al., 2005a,b; Johnson et al., 2008). Its theoretical performance is only comparable to the DNA probe previously developed by Sunde et al. (2003). However, and according to the map developed by Fuchs et al. (1998), PgPNA1007 probe hybridizes in an area with greater hybridization affinity (class V) compared to Pg477 probe (class VI). In addition, the PNA probes can hybridize with surrounding low salt concentration which destabilizes the secondary structures of the rRNA. Thus, the access of the probes to the target sequences is improved enhancing the expected signal. Even so, 150 min are required to achieve the best signal-to-noise ratio which can be explained by the fact that the PgPNA1007 probe is still hybridizing in an area of difficult access. Additionally, the complex structure of the *P. gingivalis* outer layers with two LPS molecules (O-LPS and A-LPS) as cell wall constituents and the presence of a thick polysaccharide matrix encapsulating various strains could be the reason why hybridization times were not significantly improved compared to the DNA probe (Arndt and Davey,

2010). The AgPNA235 probe shows clearly higher sensitivity compared with all DNA probes previously used in the above mentioned studies and therefore a better discrimination is expected. Additionally, the developed PNA probes have a shorter oligonucleotide sequence which tend to make them less tolerant to mismatches (Fontenete et al., 2015). Moreover, the hydrophobic nature of PNA molecules enhances cell penetration and diffusion through the biofilm, and, as DNA mimics, they are inherently more resistant to enzyme activity from the bacterial cell or from the surrounding microenvironment. Therefore, a higher efficiency of the PNA-FISH technique in the detection of *A. actinomycetemcomitans* and *P. gingivalis* is expected (Almeida et al., 2011; Drobniewski et al., 2000).

The best hybridization conditions were found to be at a temperature of 59 °C for 150 min, which make our FISH protocol as fast as the previously reported for DNA-FISH (Zijngel et al., 2010; Rudney et al., 2001; Colombo et al., 2007; Stoltenberg et al., 1993; Sunde et al., 2003; Rudney et al., 2005a,b; Johnson et al., 2008) and suitable both for clinical, if it proves useful (Colombo et al., 2009; Sayehmiri et al., 2015; Hong et al., 2015), and research purposes.

The results showed that our probes were able to simultaneously detect *P. gingivalis* and *A. actinomycetemcomitans* in a two-species suspension. In addition, we observed no cross-hybridization with several other bacterial species, corroborating the theoretical prediction.

The experiments in clinical samples demonstrate the ability to discriminate both species, *in situ*, supporting the added value of this technique. Our clinical samples had an overall scarce quantity of the targeted species in our convenience sample, despite the severity of the periodontal disease. We observed *P. gingivalis* organized in micro-colonies in subgingival plaque. This was an interesting observation since it was expected that the centrifugation force used to separate debris from bacteria, and facilitate microscopic observation, would probably destroy any remaining tridimensional architecture of the biofilm collected from the tooth surface by Gracey curettes. We also observe *P. gingivalis* internalized in gingival tissues where overloaded rounded cells coexisted with poorly or non-invaded cells. Studies from Colombo et al. (2007) and Rudney et al. (2005a, b), in epithelial cells scraped from periodontal pockets and oral mucosa, also reported similar observations. The exposure of host cells to *P. gingivalis* invasion resulted in an apparent cell rounding, probably due to cleavage of cadherins and integrins by gingipains, as reported by Sheets et al. (2005). These observations support the notion that *P. gingivalis* is present in small numbers when compared with the total microbiota of the periodontal pockets (Kumar et al., 2006) and, as it remains in the intracellular environment, can multiply and spread to neighboring cells (Tribble and Lamont, 2010), obtaining not only a privileged position to modulate the immune response, causing a 'dysbiosis' between the host and the subgingival microbiota (Darveu

et al., 2012), but also a location that allows *P. gingivalis* to escape from the host eliminating mechanisms and from mechanical periodontal treatment, perpetuating the chronicity of the periodontitis. Furthermore, the existence of overloaded cells supports the ability of the host cells to withstand an extensive *P. gingivalis* colonization without losing viability, due to the induction of anti-apoptotic mechanisms by *P. gingivalis* as a survival strategy (Nakhjiri et al., 2001).

We were unable to detect *A. actinomycetemcomitans* in our subgingival plaque samples. Nevertheless, we observed *A. actino- mycetemcomitans* internalized in periodontal tissues. The absence of super-invaded cells by *A. actinomycetemcomitans* is in agreement with prior studies claiming that *A. actinomycetemcomitans* seems to move from cell to cell (Meyer et al., 1999), not overcolonizing them in order to minimize cell apoptosis.

The PNA-FISH technique was shown to be able to discriminate and locate periodontal pathogens in subgingival biofilm and internalized in periodontal tissues. Using this technique, future studies should focus on identifying colonization patterns of the periodontal tissues in patients with different periodontal diagnosis and identifying changes in these patterns in response to different periodontal treatment strategies.

5. Conclusion

This investigation presents a new highly accurate method for *P. gingivalis* and *A. actinomycetemcomitans* detection in clinical samples in just few hours. With this technique we were able to observe spatial distribution of these species within polymicrobial communities in the periodontal pockets and, for the first time with the FISH method, in the organized gingival tissue. By opening new perspectives this technique has the potential to clarify the role of bacterial invasion in the etiopathogenesis of periodontal diseases.

Acknowledgements

The authors report no conflicts of interest. This work was financially supported by: Project POCI-01-0145-FEDER-006939 Laboratory for Process Engineering, Environment, Biotechnology and Energy – LEPABE funded by FEDER funds through COMPETE2020 – Programa Operacional Competitividade e Internacionalização (POCI) – and by national funds through FCT – Fundação para a Ciência e a Tecnologia; PIC/IC/82815/2007, SFRH/BDE/51910/2012, SFRH/BD/82663/2011].

References

- Almeida, C., Azevedo, N.F., Fernandes, R.M., Keevil, C.W., Vieira, M.J., 2010. Fluorescence in situ hybridization method using a peptide nucleic acid probe for identification of *Salmonella* spp. in a broad spectrum of samples. *Appl. Environ. Microbiol.* 76, 4476–4485.
- Almeida, C., Azevedo, N.F., Santos, S., Keevil, C.W., Vieira, M.J., 2011. Discriminating multi-Species populations in biofilms with peptide nucleic acid fluorescence in situ hybridization (PNA FISH). *PLoS One* 6 (3), e14786, <http://dx.doi.org/10.1371/journal.pone.0014786>.
- Alves, C.T., Wei, X.Q., Silva, S., Azeredo, J., Henriques, M., Williams, D.W., 2014. *Candida albicans* promotes invasion and colonisation of *Candida glabrata* in a reconstituted human vaginal epithelium. *J. Infect.*, S0163–S4453, <http://dx.doi.org/10.1016/j.jinf.2014.06.002> (Epub ahead of print, 00161-00163).
- Amann, R., Fuchs, B.M., 2008. Single-cell identification in microbial communities by improved fluorescence *in situ* hybridization techniques. *Nat. Rev. Microbiol.* 6 (5), 339–348.
- Arndt, A., Davey, M.E., 2010. *Porphyromonas gingivalis*: surface polysaccharides as virulence determinants. In: Sasano, O., Suzuki, T. (Eds.), *Interface Oral Health Science 2009*. Springer, Tokyo, pp. 382–387.
- Cerqueira, L., Azevedo, N.F., Almeida, C., Jardim, T., Keevil, C.W., Vieira, M.J., 2008. DNA mimics for the rapid identification of microorganisms by fluorescence in situ hybridization (FISH). *Int. J. Mol. Sci.* 9, 1944–1944.
- Cerqueira, L., Fernandes, R.M., Ferreira, R.M., et al., 2011. PNA-FISH as a new diagnostic method for the determination of clarithromycin resistance of *Helicobacter pylori*. *BMC Microbiol.* 11, 101, <http://dx.doi.org/10.1186/1471-2180-11-101>.
- Colombo, A.V., da Silva, C.M., Haffajee, A., Colombo, A.P.V., 2007. Identification of intracellular oral species within human crevicular epithelial cells from subjects with chronic periodontitis by fluorescence in situ hybridization. *J. Periodontal Res.* 42 (3), 236–243.
- Colombo, A.P., Boches, S.K., Cotton, S.L., Goodson, J.M., Kent, R., Haffajee, A.D., Socransky, S.S., Hasturk, H., Van Dyke, T.E., Dewhirst, F., Paster, B.J., 2009. Comparisons of subgingival microbial profiles of refractory periodontitis, severe periodontitis, and periodontal health using the human oral microbe identification microarray. *J. Periodontol.* 80, 1421–1432.
- Darveu, R.P., Hajishengallis, G., Curtis, M.A., 2012. *Porphyromonas gingivalis* as a potential community activist for disease. *J. Dent. Res.* 91, 816.
- Drobniowski, F.A., More, P.G., Harris, G.S., 2000. Differentiation of *Mycobacterium tuberculosis* complex and nontuberculous mycobacterial liquid cultures by using peptide nucleic acid fluorescence *in situ* hybridization probes. *J. Clin. Microbiol.* 38, 444–447.
- Fontenete, S., Barros, J., Madureira, P., Figueiredo, C., Wengel, J., Azevedo, N.F., 2015. Mismatch discrimination in fluorescent *in situ* hybridization using different types of nucleic acids. *Appl. Microbiol. Biotechnol.* 99, 3961–3969.
- Fournier, D., Mouton, C., Lapiere, P., Kato, T., Okuda, K., Ménard, C., 2001. *Porphyromonas gulae* sp. nov., an anaerobic, gram-negative coccobacillus from

- the gingival sulcus of various animal hosts. *Int. J. Syst. Evol. Microbiol.* 51, 1179–1189.
- Fuchs, B.M., Wallner, G., Beisker, W., Schwiippl, I., Ludwig, W., Amann, R., 1998. Flow cytometric analysis of the *in situ* accessibility of escherichia coli 16S rRNA for fluorescently labeled oligonucleotide probes. *Appl. Environ. Microbiol.* 64, 4973–4982.
- Gaetti-Jardim Jr., E., Pereira, M.F., Vieira, E.M., Schweitzer, C.M., Okamoto, A.C., Ávila-Campos, M.J., 2015. Occurrence of periodontal pathogens in ethnic groups from a native Brazilian reservation. *Arch. Oral Biol.* 60, 959–665.
- Guimarães, N., Azevedo, N.F., Figueiredo, C., Keevil, C.W., Vieira, M.J., 2007. Development and application of a novel peptide nucleic acid probe for the specific detection of helicobacter pylori in gastric biopsy specimens. *J. Clin. Microbiol.* 45 (9), 3089.
- Hong, B.Y., Furtado Araujo, M.V., Strausbaugh, L.D., Terzi, E., Ioannidou, E., Diaz, P.I., 2015. Microbiome profiles in periodontitis in relation to host and disease characteristics. *PLoS One* 10, e0127077.
- Johnson, J.D., Chen, R., Lenton, P.A., Zhang, G., Hinrichs, J.E., Rudney, J.D., 2008. Persistence of extracrevicular bacterial reservoirs after treatment of aggressive periodontitis. *J. Periodontol.* 79 (12), 2305–2312.
- Kumar, P.S., Leys, E.J., Bryk, J.M., Martinez, F.J., Moeschberger, M.L., Griffen, A.L., 2006. Changes in periodontal health status are associated with bacterialcommunity shifts as assessed by quantitative 16S cloning andsequencing. *J. Clin. Microbiol.* 44, 3665–3673.
- Lamont, R.J., Chan, A., Belton, C.M., Izutsu, K.T., Vasel, D., Weinberg, A., 1995. Porphyromonas gingivalis invasion of gingival epithelial cells. *Infect. Immun.* 63, 3878–3885.
- Marsh, P.D., Devine, D.A., 2011. How is the development of dental biofilms influenced by the host? *J. Clin. Periodontol.* 38 (Suppl. 11), 28–35.
- Mendes, L., Azevedo, N.F., Pinto, M.G., 2015. Relationship between invasion of the *periodontium* by periodontal pathogens and periodontal disease: a systematic review. *Virulence* 6, 208–215.
- Meyer, D.H., Lippmann, J.E., Fives-Taylor, P.M., 1996. Invasion of epithelial cells by Actinobacillus actinomycetemcomitans: a dynamic, multistep process. *Infect. Immun.* 64, 2988–2997.
- Meyer, D.H., Rose, J.E., Lippmann, J.E., Fives-Taylor, P.M., 1999. Microtubules are associated with intracellular movement and spread of the periodontopathogen *Actinobacillus actinomycetemcomitans*. *Infect. Immun.* 67, 6518–6525.
- Nakhjiri, S.F., Park, Y., Yilmaz, O., Chung, W.O., Watanabe, K., El-Sabaeny, A., Park, K., Lamont, R.J., 2001. Inhibition of epithelial cell apoptosis by *Porphyromonas gingivalis*. *FEMS Microbiol. Lett.* 200, 145–149.
- Nielsen, P.E., Egholm, M., Berg, R.H., Buchardt, O., 1991. Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science* 254, 1497–1500.
- Rudney, J.D., Chen, R., Sedgewick, G.J., 2001. Intracellular *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in buccal epithelial cells collected from human subjects. *Infect. Immun.* 69, 2700–2707.

- Rudney, J.D., Chen, R., Sedgewick, G.J., 2005a. *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Tannerell forsythensis* are components of a polymicrobial intracellular flora within human buccal cells. J. Dent. Res. 84, 59–63.
- Rudney, J.D., Chen, R., Zhang, G., 2005b. Streptococci dominate the diverse flora within buccal cells. J. Dent. Res. 84 (12), 1165–1171.
- Sanz, M., van Winkelhoff, A.J., 2011. Periodontal infections: understanding the complexity—consensus of the Seventh European Workshop on Periodontology. J. Clin. Periodontol. 38 (Suppl. 11), 3–6.
- Sayehmiri, F., Sayehmiri, K., Asadollahi, K., Soroush, S., Bogdanovic, L., Jalilian, F.A., Emaneini, M., Taherikalani, M., 2015. The prevalence rate of *Porphyromonas gingivalis* and its association with cancer: a systematic review and meta-analysis. Int. J. Immunopathol. Pharmacol. 28, 160–167.
- Sheets, S.M., Potempa, J., Travis, J., Casiano, C.A., Fletcher, H.M., 2005. Gingipains from *Porphyromonas gingivalis* W83 induce cell adhesion molecule cleavage and apoptosis in endothelial cells. Infect. Immun. 73, 1543–1552.
- Stoltenberg, J.L., Osborn, J.B., Pihlstrom, B.L., et al., 1993. Association between cigarette smoking, bacterial pathogens, and periodontal status. J. Periodontol. 64, 1225–1230.
- Sunde, P.T., Olsen, I., Göbel, U.B., 2003. Fluorescence in situ hybridization (FISH) for direct visualization of bacteria in periapical lesions of asymptomatic root-filled teeth. Microbiology 149 (pt. 5), 1095–1102.
- Tribble, G.D., Lamont, R.J., 2010. Bacterial invasion of epithelial cells and spreading in periodontal tissue. Periodontol. 2000 52 (1), 68–83.
- Wade, W.G., 2011. Has the use of molecular methods for the characterization of the human oral microbiome changed our understanding of the role of bacteria in the pathogenesis of periodontal disease? J. Clin. Periodontol. 38 (Suppl. 11), 7–16.
- Zijnga, V., van Leeuwen, M.B.M., Degener, J.E., et al., 2010. Oral biofilm architecture on natural teeth. PLoS One 5 (2), e9321, <http://dx.doi.org/10.1371/journal.pone.000932>.

	5' CTAATCACACTTGGG 3' AaPNA235	
<i>Haemophilus influenzae</i> strain 680	GATTAGG GTGAACCC	246
<i>Aggregatibacter aphrophilus</i> NJ8700 strain NJ8700	GATTAGG GTGAACCC	240
<i>Haemophilus influenzae</i> Rd KW20	GATTAGG GTGAACCC	246
<i>Haemophilus ducreyi</i> 35000HP strain 35000HP	GATTAGG GTGAACCC	246
<i>Haemophilus sputorum</i> strain CCUG 13788	GATTAGG GTGAACCC	222
<i>Haemophilus haemolyticus</i> strain CIP 103290	GATTAGG GTGAACCC	226
<i>Haemophilus paraphrohaemolyticus</i> strain NCTC 10670	GATTAGG GTGAACCC	220
	5' AGACGGTTTTTCACC 3' PgPNA1007	
<i>Porphyromonas gulae</i> strain JCM 13865	TCTGCCG AAAGTGG	1004

Fig. 1. Partial alignment of 16S rRNA sequences with one base pair mismatch with AaPNA235 and PgPNA1007. Hybridization position is shown on the right.

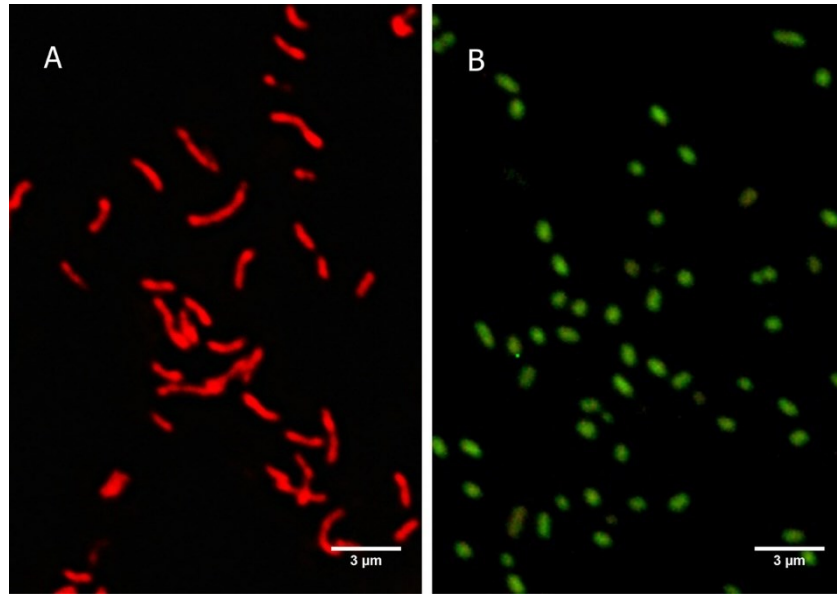


Fig. 2. Pure cultures of (A) *Aggregatibacter actinomycetemcomitans* (red) and (B) *Porphyromonas gingivalis* (green) after PNA- FISH and epifluorescence microscopy. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

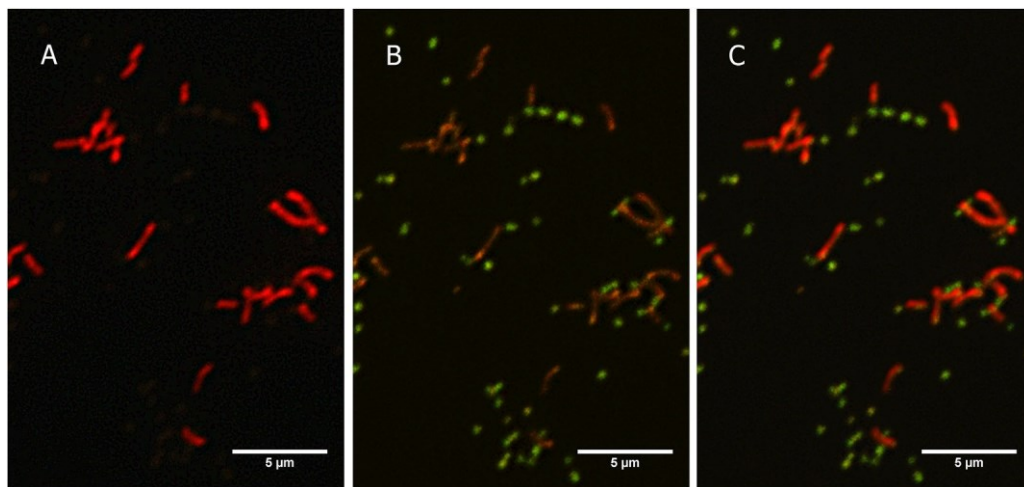


Fig. 3. Same field images of a multiplex PNA-FISH in a double-specie suspension. Panel (A) shows *Aggregatibacter actinomycetemcomitans* (red filter), panel (B) shows *Porphyromonas gingivalis* (green filter) and panel (C) shows same field overlapped images. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

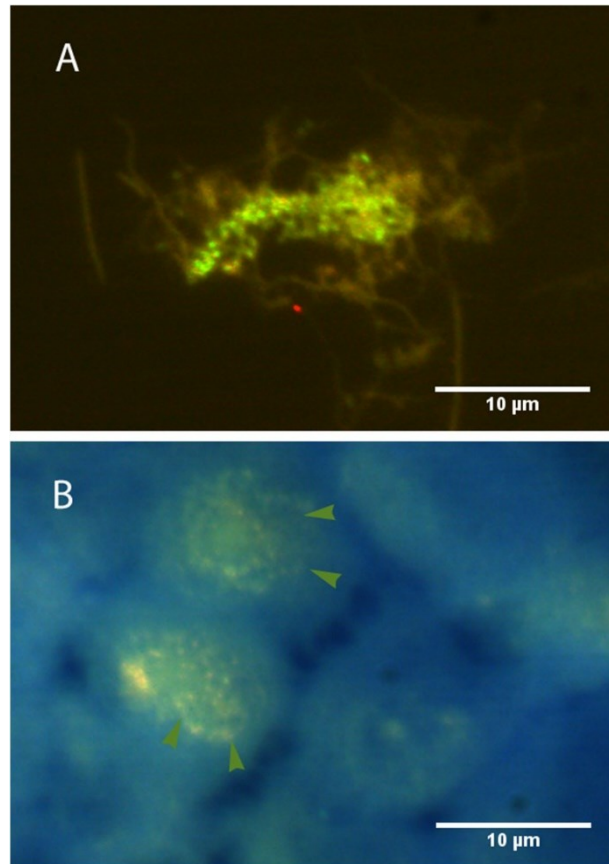


Fig. 4. Detection of *Porphyromonas gingivalis* after PNA-FISH and epifluorescence microscopy in a sample of subgingival plaque (A) and in a gingival tissue biopsy (B). In panel (B) we can observe invaded epithelial cells by *P. gingivalis* (green arrows). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

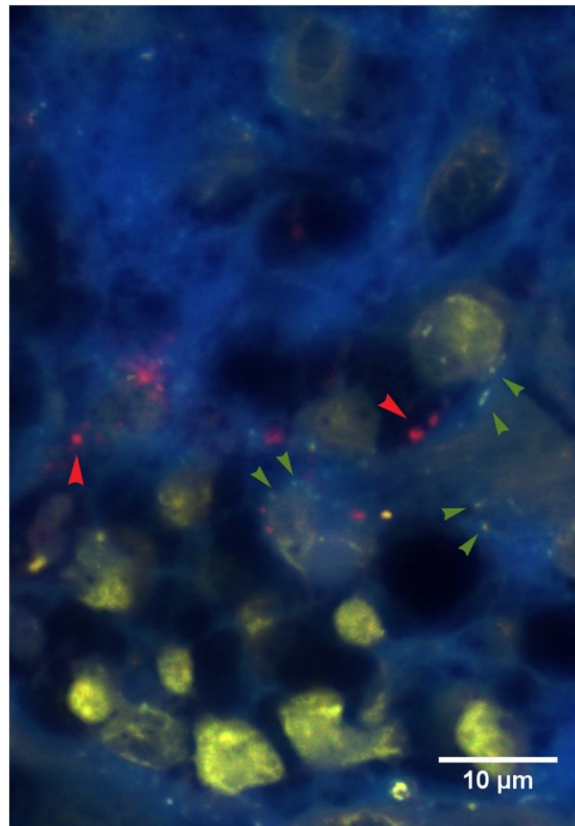


Fig. 5. Images of multiplex PNA-FISH in a gingival tissue biopsy sample. This image from connective gingival tissue shows *P. gingivalis* (green arrows) and *A. actinomycetemcomitans* (red arrows) invasion in close relationship with the polymorphonuclear neutrophil infiltrate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1. Theoretical sensitivity and specificity of PNA or DNA probes for *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* detection.

Bacteria	Probe	Sequence (5' → 3')	No. of target strains detected ^a	No. of non-target strains detected ^a	Sensitivity (%) ^b	Specificity (%) ^c	Reference
<i>A. actinomycetemcomitans</i>	DNA Aa889	CACCAGGGCTAAACCCCAAT	50	0	68.5	100	Rudney et al. (2001), Colombo et al. (2007), Stoltenberg et al. (1993), Sunde et al. (2003), Rudney et al. (2005a,b), Johnson et al. (2008)
	DNA Aa829	GGGCTAAACCCCAATCCC	50	0	68.5	100	Zijnga et al. (2010)
	PNA Ag235	CTAATCACACTTGGG	73	0	100	100	This study
<i>P. gingivalis</i>	DNA Pg1054	GGTTTTCACCATCAGTCATCTACA	16	0	37.2	100	Rudney et al. (2001), Colombo et al. (2007), Stoltenberg et al. (1993), Rudney et al. (2005a,b), Johnson et al. (2008)
	DNA Pg477	CAATACTCGTATCGCCCGTTATTC	43	1	100	99.9	Zijnga et al. (2010), Sunde et al. (2003)
	PNA Pg1007	AGACGGTTTTCACC	43	1	100	99.9	This study

^a Calculated through Probe Match (<http://rdp8.cme.msu.edu/html/>, last access, August 2014) with the following data set options: Strain – Both; Source – Both; Size →1200 bp; Quality–Both.

^b Sensitivity was calculated as (target)s/T(target)sx100.

^c Specificity was calculated as (non-target)s/T(non-target)sx100.

Table2 Results of the hybridization tests for the *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* PNA probes developed in this study

Microorganisms	AgPNA235	PorPNA1007
<i>Aggregatibacter actinomycetemcomitans</i> ANH	+	—
<i>Aggregatibacter actinomycetemcomitans</i> D115	+	—
<i>Aggregatibacter actinomycetemcomitans</i> D75	+	—
<i>Porphyromonas gingivalis</i> GAI ^c	—	+
<i>Porphyromonas gingivalis</i> TDC 60 ^c	—	+
<i>Porphyromonas gingivalis</i> 13.3.3 ^{a,b}	—	+
<i>Porphyromonas gingivalis</i> w86 ^c	—	+
<i>Porphyromonas gingivalis</i> ATCC 33277 ^b	—	+
<i>Porphyromonas gingivalis</i> w50 ^b	—	+
<i>Porphyromonas gingivalis</i> ATCC 33277 ^c	—	+
<i>Porphyromonas gingivalis</i> 34.2.2 ^{a,b}	—	+
<i>Porphyromonas endodontalis</i> ^a	—	—
<i>Escherichia coli</i> CECT 434	—	—
<i>Staphylococcus epidermidis</i> RP61A	—	—

^a Clinical isolate.

^b From Prof. M. Curtis.

^c From Prof. K. Nakayama.