Microbiological Research 192 (2016) 185–191

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

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

ARTICLE INFO

Article history:
Received 28 January 2016
Received in revised form 1 May 2016
Accepted 10 July 2016
Available online 14 July 2016

Keywords:
PNA-FISH
Tissue invasion
Aggregatibacter actinomycetemcomitans
Porphyromonas gingivalis
Periodontal disease

ABSTRACT

Purpose: We aim to develop peptic nucleic acid (PNA) probes for the identification and localization of *Aggregatibacter actinomycetemcomintans* 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. actinomycetemcomitans* 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 (Zijnge 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,

* Corresponding author at: Rua Dr. Manuel Pereira da Silva, 4200-393 Porto, Portugal.
E-mail address: lgoncalves@fmd.up.pt (L. Mendes).

http://dx.doi.org/10.1016/j.micres.2016.07.002
0944-5013/

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; Oxioid, 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) 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-dioxa 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 (target)s/total(target)s × 100 and (non-target)s/total(non-target)sx100, where (target)s stands for the number of strains detected by the oligonucleotide probe, 'total(target)s' for the total number of target strains present in the database, '(non-target)s for the number of non-target strains that did not react with the oligonucleotide probe and 'total(non-target)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 °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 (Panreac), 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 °C. After the hybridization step, the coverslips were removed, the slides submerged and maintained in a pre-warmed (59 °C) washing solution containing 5 mMTris 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