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Discriminating Typical and Atypical Cystic Fibrosis-Related Bacteria by Multiplex PNA-FISH

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ABSTRACT: This study aims to report the development of peptide nucleic acid (PNA) probes to specifically detect the cystic fibrosis (CF)-associated traditional and atypical species *Pseudomonas aeruginosa* and *Inquilinus limosus*, respectively. PNA probes were designed in silico, developed and tested in smears prepared in phosphate-buffer saline (PBS), and in artificial sputum medium (ASM). A multiplex fluorescent in situ hybridization (FISH) approach using the designed probes was further validated in artificially contaminated clinical sputum samples and also applied in polymicrobial 24 h-old biofilms involving *P. aeruginosa*, *I. limosus*, and other CF-related bacteria. Both probes showed high predictive and experimental specificities and sensitivities. The multiplex PNA-FISH assay, associated with non-specific staining, was successfully adapted in the clinical samples and in biofilms of CF-related bacteria, allowing differentiating the community members and inferring about microbial-microbial interactions within the consortia. This study revealed the great potential of PNA-FISH as a diagnostic tool to discriminate between classical and less common CF-associated bacteria, being suitable to further describe species-dependent prevention strategies and deliver more effective target control therapeutics.

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KEYWORDS: cystic fibrosis microbiome; fluorescence in situ hybridization; *Inquilinus limosus*; PNA probe; *Pseudomonas aeruginosa*

Introduction

Cystic fibrosis (CF) is a genetic disorder affecting multiple organs, but with a high incidence and frequency in the respiratory system. The CF pulmonary disease is characterized by viscous secretions and airways obstruction, contributing to high rates of mortality in CF individuals due to the respiratory failure. Additionally, the favorable environment that is created in the airways of CF patients (containing dehydrated mucus, products resulting from inflammatory cell death, steep oxygen gradients, etc.) often encourages a complex microbiome to persistently colonize and to cause severe chronic infections since early in the life (Davies and Bilton, 2009; Gomez and Prince, 2007; Lopes et al., 2015; Ratjen, 2009). A limited number of microbial species (*Staphylococcus aureus*, *Haemophilus influenza*, *Pseudomonas aeruginosa*, and *Burkholderia cepacia* complex (BCC) group) has been considered responsible for bacterial lung infections, of which *P. aeruginosa* is recognized as the most significant pathogen and the most commonly isolated organism (Burns et al., 1998; Hoiby, 2011; Lambiase et al., 2006; Lipuma, 2010). However, the respiratory tract of CF patients does not harbor a simple microbiological flora. A wide number of atypical bacteria (e.g., *Inquilinus limosus*, *Stenotrophomonas maltophilia*, *Dolosigranulum pigrum*, *Achromobacter xylosoxidans*, and other species) are now well recognizable, but its identification by routine cultivation methods and biochemical tests remain unreliable (Lopes et al., 2015; Stressmann et al., 2011).

The recent focus has led to the recognition that these microorganisms may co-exist and interact with the traditional species, and also with the host immune system, affecting the disease progression and treatment routes (Magalhães et al., 2016). But while the impact of some of these unusual microorganisms in the CF infection is starting to be elucidated (Costello et al., 2011; Dalboge et al., 2011; Hansen et al., 2010; Lopes et al., 2012; Pompilio

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et al., 2010; Rolain et al., 2009; Ulrich et al., 2010; Waters et al., 2013), the pathogenic potential of others has yet to be fully understood. *I. limosus*, an α-proteobacterium has been constantly emerging from the airways of CF patients (Chiron et al., 2005; Cooke et al., 2007; Pitulle et al., 1999; Salvador-Garcia et al., 2013; Schmoldt et al., 2006; Wellinghausen et al., 2005). Because of the mucoid physiology, the multi-drug resistance character and the ability to persist in the respiratory tract after biofilm formation (Bittar and Rolain, 2010; Chiron et al., 2005), this bacterium is now considered a potential threat for CF pathogenesis, which makes its identification desirable.

The classical identification and characterization of bacterial isolates in many microbiology laboratories and/or hospital centers rely solely on cultivation of pathogens and phenotypic techniques, which carry out a wide number of problems. Firstly, these techniques generally require at least 1–3 or more days for definitive detection of a bacterial species, preventing or delaying effective infection control strategies and therapeutic interventions (Anuj et al., 2009; Hogardt et al., 2000; Saiman and Siegel, 2004). This time-gap is extended for organisms that are fastidious, slow growing, noncultivable, or present as part of polymicrobial infections (Wolk and Dunne, 2011), as often occurs in CF. Additionally, these methods usually lead to the misidentification of unusual bacteria that improperly grow on unsuitable selective media and also fail in detecting microorganisms with compromised growth properties (e.g., after antibiotic therapy), and/or even anaerobic microorganisms, which require specific growth conditions (Bittar and Rolain, 2010; Bousbia et al., 2013; Galluzzi et al., 2007). Also, a range of different unusual species can grow in the same selective media used for the detection of other organisms, leading to misidentification (it is the case of *I. limosus* that grows in the *Burkholderia cepacia* selective medium) when using culturing methods (Bittar et al., 2008a; Kidd et al., 2009; Spilker et al., 2008).

Over the last 2 decades, there has been a significant progress on the development of rapid techniques (e.g., quantitative polymerase chain reaction—qPCR, 16S rRNA gene sequencing, denaturing/temperature gradient gel electrophoresis—DGGE/TGGE, terminal restriction fragment length polymorphism—T-RFLP, and matrix-assisted laser desorption ionization-time of flight mass spectrometry—MALDI-TOF MS) to detect, identify, and/or quantify microorganisms within polymicrobial communities (Bittar et al., 2008b; Fernandez-Olmos et al., 2012; Guss et al., 2011; Kirketerp-Moller et al., 2008; Lambiase et al., 2013; Malic et al., 2009; Rogers et al., 2003, 2004; Sibley et al., 2006). These promising molecular approaches have been developed to complement or even replace existing typical microbiological methods and other practices (Amann et al., 1995; Barenfanger et al., 1999; Call et al., 2003; Chadwick et al., 1998; Cleven et al., 2006; Hiyari and Bennett, 2011), highly improving the diagnosis of microbial species, providing more rapid, specific and sensitive systems than culture-dependent methods (Oosterheert et al., 2005), facilitating the identification of microorganisms present in biofilms (Bittar and Rolain, 2010; Galluzzi et al., 2007), and detecting abundant numbers of bacteria that do not grow under culture but are still viable (Hugenholtz, 2002; Oliver, 2005). It has been in this context that PNA FISH has emerged as a valuable tool for the specific and rapid detection of bacteria in polymicrobial communities without the need of cultivation (Almeida et al., 2010). PNA oligonucleotide probes are synthetic DNA analogue molecules, with the main difference residing in the backbone of the molecule. While DNA possesses a negatively charged sugar-phosphate backbone, the PNA molecule consists of repeating *N*-(2-aminoethyl)glycine units, resulting in an achiral and neutral backbone (Nielsen, 2001; Nielsen et al., 1991). This enables PNA molecules to better penetrate through the cell wall and cell membrane of the target microorganism, and specifically hybridize with the complementary rRNA sequence, according with the Watson and Crick base-pairing rules (Stender, 2003). In general, PNA oligonucleotide probes are small (13–18 bp in length) and are covalently labeled with a fluorescent dye molecule (e.g., fluorescein, carbocyanine), becoming easily detected by epifluorescence microscopy or flow cytometry (Almeida et al., 2011; Azevedo et al., 2011; Cerqueira et al., 2008). In comparison with other reference methods currently used in clinical microbiology, FISH has many advantages. For example, it does not require DNA extraction and purification, which are a prerequisite before PCR amplification and may cause sample contamination (Yang and Rothman, 2004). Unlike in PCR, FISH may be carried out in situ (Aslanzadeh, 2004), which makes this technique particularly suitable when applied to biofilms. In fact, as it is not needed to disrupt attached cells, it allows in-depth characterization of microbial composition, hence, deducing about the complex microbial–microbial (Almeida et al., 2011) and microbial-host interplay (Thornton et al., 2011). While MALDI-TOF MS assay is widely used because of its high accuracy, versatility, cost-efficient, and speed of analysis benefits, the method is limited in that it requires subculture before identification. As whole intact cells are typically used in the analysis, abundant number of bacterial cells are needed (which are often low when working with biological fluids) (Wolk and Dunne, 2011). Additionally, MALDI-TOF MS-based sequencing for the detection of antibiotic resistance determinants appears to be labor-intensive and limited by the small size of the DNA fragments that can be sequenced and the technique is often unable to identify antimicrobial resistance determinants (Josten et al., 2013). By the contrary, FISH-based methods have been showing promising results since they can detect intact bacterial cells directly in clinical samples (tissues, blood, sputum, etc.), or even after an enrichment step (Almeida et al., 2010; Mothershed and Whitney, 2006; Trebesius et al., 2000). Moreover, the detection and location of resistant microorganisms directly within biological samples is made possible with FISH methods (Cerqueira et al., 2011). Therefore, FISH appears suitable and easily-to-handle for integration in a clinical microbiology laboratory, only requiring basic technical equipment (an epifluorescent microscope) equipped with an adequate set of filters for fluorochromes. FISH using labeled fluorescent PNA probes has long been used in many fields of microbiology, with some reports being extended to the analysis of pathogens in CF samples (Bjarnsholt et al., 2009; Brown and Govan, 2007; Hogardt et al., 2000; Malic et al., 2009; Rudkjobing et al., 2012; Wellinghausen et al., 2006; Yang et al., 2008). But while a complex microbial diversity is invariably present in CF lungs, reports using PNA-FISH only detect the classical CF-pathogens, preventing an accurate identification of the complex microbial community present within the CF environment and subsequently a full understanding of the bacterial interactions occurring in situ as well as the interplay

of these communities in the host. Moreover, aggregates of bacteria encased in a self-produced extracellular matrix, the so-called biofilms, are found in the CF airways. In these consortia, bacteria become more tolerant to antibiotic treatments and resistant to host immune responses compared to their planktonic, free-living counterparts. Therefore, detection and identification of biofilms in CF are crucial to design better treatment strategies aiming to eradicate these biofilms.

The goal of this study was to develop labeled multiplex PNA-FISH method for the specific identification and localization of the CF-associated traditional organism *P. aeruginosa*, and the emergent species *I. limosus*. Subsequently, the applicability of a multiplex PNA assay was assessed to discriminate the aforementioned bacteria within mixed-species biofilms formed by traditional and atypical bacterial species found in CF.

Material and Methods

Design, Synthesis, and Preparation of the PNA Oligonucleotide Probes

Two novel PNA probes to specifically detect the CF-associated organisms *P. aeruginosa* and *I. limosus* were designed according to the methodology described before (Almeida et al., 2010; Azevedo et al., 2011). In short, the freely accessible PrimRose software (Ashelford et al., 2002) in conjunction with the 16S rRNA databases of Ribosomal Database Project II (RDP II) version 10.0 (Cole et al., 2005) were used to identify useful oligonucleotide sequences for each target organism. After the assessment of a limited number of potential oligonucleotide probes for each organism, the final selection of each PNA probe sequence was based on the criteria described by Azevedo et al. (2011). Nonetheless, for multiplex purposes, both PNA probes should present similar melting temperatures (determined according to the model proposed by Giesen et al. (1998).

The following 15 bp-length PNA oligomer sequences were selected: 5′-GCTGAACCACCTACG-3′ and 5′-CCCGCCCGTATC AAA-3′ for *P. aeruginosa* and *I. limosus*, respectively. The probes were designated as Paer565 and Ilim569, because of the starting position of the target 16S rRNA sequence of the strains *P. aeruginosa* ATCC 27853 (Accession number S0005765) and *I. limosus* AU430 (Accession number S000434854), respectively. Finally, both probe sequences were synthetized by Panagene (Daejeon, South Korea), and each oligonucleotide N-terminus was attached to a fluorochrome (Alexa 594 and Alexa 488 for Paer565 and Ilim569, respectively), via a double 8-amino-3, 6-dioxaoctanoic acid (AEEA) linker. The stock solution (at 100 mM) of each PNA probe was obtained by solubilizing the powder in 10% (vol/vol) of acetonitrile and 1% (vol/vol) of trifluoracetic acid, and stored at −20°C. The working solutions (at 200 nM) were prepared in hybridization solution containing 10% (wt/vol) dextran sulphate, 10 mM NaCl, 30% (vol/vol) formamide, 0.1% (wt/vol) sodium pyrophosphate, 0.2% (wt/vol) polyvinylpyrrolidone, 0.2% (wt/vol) Ficol, 5 mM disodium EDTA, 0.1% (vol/vol) Triton X-100, and 50 mM Tris–HCl (all purchased from Sigma–Aldrich, Sintra, Portugal) and stored at 4°C for further use.

Optimization of the Hybridization Conditions

The hybridization procedure was optimized on pure smears of the CF-associated organisms *P. aeruginosa* (wild type strain UCBPP PA14) and *I. limosus* (strain M53, isolated from CF sputum). Every 2 or 3 days, single colonies of each strain were streaked onto fresh media containing tryptic soy broth (TSB; Liofilchem, Teramo, Italy) supplemented with 1.2% (wt/vol) agar (Liofilchem). The plates were aerobically incubated at 37°C for 18–20 h (for *P. aeruginosa*) or ~48 h (for *I. limosus*). The FISH procedure on smears was previously described by Cerqueira et al. (2011). Since the purpose was to further apply the multiplex assay to CF mixed-species populations, the hybridization conditions should be similar for both probes. Thus, a set of different hybridization temperatures was tested for each probe, with Paer565 and Ilim569 showing the brightest fluorescent signal at 65°C (data not shown).

After selecting the optimal hybridization temperature, a multiplex (three-species) assay encompassing *P. aeruginosa, I. limosus*, and other CF-related species was assessed. For this, one CF-classical species (*Burkholderia multivorans,* LMG 13010) and two atypical bacteria (*A. xylosoxidans*, a CF clinical isolate, and also *D. pigrum*, CIP 104051) were used. The third organism was identified by counterstaining the samples with 4′, 6-diamidino-2-phenylindole (DAPI; Sigma) at the end of the hybridization procedure. Basically, the mixed bacterial suspensions were prepared in phosphate-buffer saline (PBS) solution by adding the three species (*P. aeruginosa*, *I. limosus*, and each of the other strains) in

Table I. PNA oligonucleotide probe sequences designed for *P. aeruginosa* and *I. limosus* and the respective predicted specificities and sensitivities.

Probe*	Target microorganism	Probe sequence (5′–3′)	No. of targets detected[a]	No. of non-targets detected[b]	Specificity (%)[c]	Sensitivity (%)[d]	Reference of source
Paer565	*P. aeruginosa*	GCTGAACCACCTACG	892	458	99.7	90.3	This study
Ilim569	*I. limosus*	CCCGCCCGTATCAAA	7	11	100.0	87.5	This study
Psaer	*P. aeruginosa*	AACTTGCTGAACCAC	898	447	99.7	90.8	Coull and Hyldig-Nielsen (2003)
Pse16S32	*P. aeruginosa*	CTGAATCCAGGAGCA	805	727	99.5	81.5	Perry-O'Keefe et al. (2001)

*All probes were designed to complement with the 16S rRNA target.
[a]Number of target microorganisms detected by each probe in a total of 988 *P. aeruginosa* and eight *I. limosus* strains sequences presented in the database.
[b]Number of non-target microorganisms detected by each probe in a total of 178996 non-*P. aeruginosa* and 179976 non-*I. limosus* sequences deposited in the database.
[c]Given by (no. of non-targets excluded by the probe)/(total of non-targets in the database) × 100.
[d]Given by (no. of targets detected by the probe)/(total no. of target organisms found in the database) × 100.

equal proportions. Afterward, 20 μL were transferred to each well of 8 mm well glass slides (ThermoScientific, Braunschweig, Germany) and allowed to air dry. Subsequently, 30 μL of each solution of 4% (wt/vol) paraformaldehyde followed by 50% (vol/vol) ethanol was dispensed in the wells for 10 min each and allowed to air dry. This step enables the fixation of the cells and increase the permeabilization of the cell membrane to the subsequent hybridization allowing the labeled oligonucleotide probes to diffuse to their intracellular rRNA target molecules (Amann and Fuchs, 2008). Afterward, 20 μL of hybridization solution containing the probes mixture at 200 nM were dispensed on the smears, which were finally covered with coverslips and incubated in the dark for 1 h at 65°C. After hybridization, the coverslips were carefully removed and the slides were submersed for 30 min in coplin jars containing a prewarmed (65°C) washing solution composed of 5 mM Tris Base, 15 mM NaCl and 1% (vol/vol) Triton X-100 (all from Sigma). Finally, the slides were removed from the coplin jars and allowed to air dry before counterstaining with DAPI at 40 μg/mL. Negative controls were performed for each experiment, with no probes added to the hybridization solution. Next, the same procedure was also tested in the aforementioned mixed smears diluted in artificial sputum medium (ASM), which was prepared as described by Sriramulu et al. (2005). For microscopic visualization, a fluorescence microscope (Olympus BX51, Perafita, Portugal) equipped with the filters sensitive to DAPI (BP 365–370, FT 400, LP 421) and to the signaling molecule of the PNA probes (BP 470–490, FT 500, LP 516, and BP 530–550, FT 570, LP 591, for Alexa 488 and 594, respectively) was used.

Evaluation of Experimental Specificity and Sensitivity of the Probes

In order to test the specificity and sensitivity of both probes, a set of different strains was tested at the optimal hybridization temperature previously determined (65°C). All strains were maintained and incubated as mentioned above, with exception of *Pseudomonas putida*, which was grown at 30°C. The presence of a strong fluorescent signal provided by each probe was assessed by microscopy and the FISH outcome was classified as "−" for a non-fluorescent signal and as "+" for a fluorescent signal.

Application of FISH Method to Clinical Sputum Samples and to Mixed-Species Biofilms

In order to assess the applicability of PNA-FISH on real context situations, sputum clinical samples were obtained from Hospital de Braga (Braga, Portugal) and artificially contaminated with mixed cultures of *P. aeruginosa*, *I. limosus*, and each atypical species (*D. pigrum* and *A. xylosoxidans*). The procedure used in here was similar to that previously described for smears. Equally, for biofilms, a multiplex assay was tested on 24 h-old biofilms of *P. aeruginosa*, *I. limosus*, and the emerging species *D. pigrum* formed on polystyrene (PS) coupons. For this, the material supports were previously submerged 3 min in a commercial detergent solution (Sonasol, Henkel Ibérica Portugal, Bobadela, Portugal), washed three times in sterile ultrapure water and allowed to air dry for 3 h. The biofilms were formed as described before (Lopes et al., 2012), with slight modifications. Briefly, cell suspensions of each organism containing 1×10^7 cells/mL were prepared in TSB. The inoculum used to form the mixed-species biofilms was obtained by adding each pure suspension in equal proportions. The final inoculum was then dispensed in 24-well plates (Orange Scientific, Braine-l'Alleud, Belgium) containing the PS coupons on the bottom of the wells.

Table II. Outcome of the PNA-FISH method obtained for the *P. aeruginosa* (Paer565) and *I. limosus* (Ilim569) probes in the experimental specificity tests.

Strain	Paer565	Ilim569
Achromobacter xylosoxidans (CF isolate 1)	−	−
Achromobacter xylosoxidans (CF isolate 2)	−	−
Achromobacter xylosoxidans (CF isolate 3)	−	−
Achromobacter xylosoxidans (CF isolate 4)	−	−
Achromobacter xylosoxidans (CF isolate 5)	−	−
Achromobacter xylosoxidans (CF isolate 6)	−	−
Achromobacter xylosoxidans (CF isolate 7)	−	−
Achromobacter xylosoxidans (CF isolate 8)	−	−
Achromobacter xylosoxidans (CF isolate 9)	−	−
Achromobacter xylosoxidans (CF isolate 10)	−	−
Acinetobacter baumannii (clinical isolate)[a]	−	−
Burkholderia cenocepacia (LMG 16656)	−	−
Burkholderia cenocepacia (LMG 18863)	−	−
Burkholderia dolosa (LMG 18943)	−	−
Burkholderia multivorans (LMG 13010)	−	−
Burkholderia stabilis (LMG 14294)	−	−
Burkholderia sp. (clinical isolate)[b]	−	−
D. pigrum (CIP 104051)	−	−
*Escherichia coli (*ATCC 25922)	−	−
Inquilinus limosus (CF isolate M53)[c]	−	+
Inquilinus limosus (CF isolate 2)	−	+
Klebsiella oxytoca (ATCC 13182)	−	−
Klebsiella pneumoniae (clinical isolate)[a]	−	−
P. aeruginosa (ATCC 10145)	+	−
P. aeruginosa (ATCC 27853)	+	−
P. aeruginosa (ATCC 39324)	+	−
P. aeruginosa (clinical isolate 1)[d]	+	−
P. aeruginosa (clinical isolate 2)[b]	+	−
P. aeruginosa (clinical isolate 3)[a]	+	−
P. aeruginosa (clinical isolate 4)[a]	+	−
P. aeruginosa (clinical isolate 5)[e]	+	−
P. aeruginosa (clinical isolate 6)[e]	+	−
P. aeruginosa (clinical isolate 7)[e]	+	−
P. aeruginosa (clinical isolate 8)[e]	+	−
P. aeruginosa (UCBPP-PA14)	+	−
P. aeruginosa (PAO1)	+	−
P. fluorescencens (ATCC 13525)	−	−
P. fragi (food isolate)[f]	−	−
P. putida (environmental isolate)[f]	−	−
Staphylococcus aureus (ATCC 25923)	−	−
Staphylococcus aureus (JKD 6008)	−	−
Staphylococcus aureus (MRSA)[b]	−	−
Stenotrophomonas maltophilia (CF isolate 1)	−	−
Stenotrophomonas maltophilia (CF isolate 2)	−	−
Stenotrophomonas maltophilia (CF isolate 3)	−	−
Stenotrophomonas maltophilia (CF isolate 4)	−	−
Stenotrophomonas maltophilia (CF isolate 5)	−	−
Stenotrophomonas maltophilia (CF isolate 6)	−	−
Stenotrophomonas maltophilia (CF isolate 7)	−	−
Stenotrophomonas maltophilia (CF isolate 8)	−	−

[a]Isolated from expectoration (Hospital de Braga, Portugal).
[b]Isolated from urinary tract, kindly provided by Margarida Martins (University of Minho, Braga, Portugal).
[c]Isolated from CF sputum, kindly provided by Dr. Michael G. Surette (University of Calgary, Alberta, Canada).
[d]Isolated from an endoscope (Paris, France).
[e]Isolated from the urinary tract (Clínica de São Lázaro, Braga, Portugal).
[f]Kindly provided by Hugo Oliveira (University of Minho, Braga, Portugal).

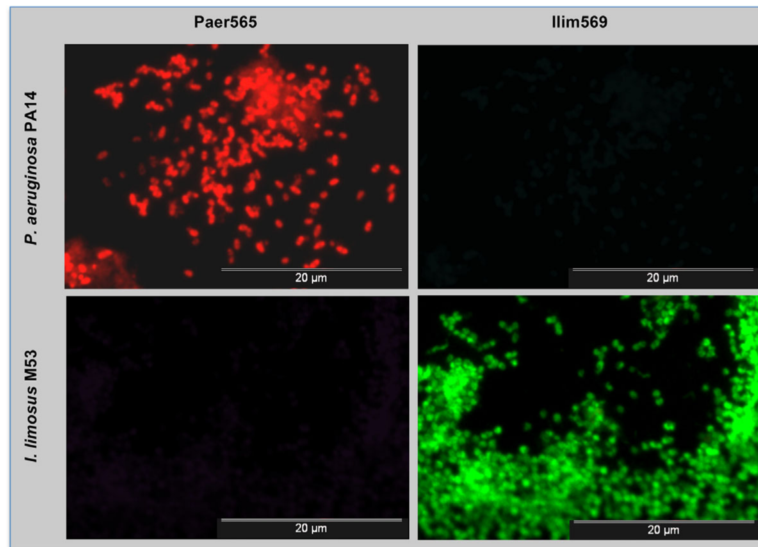


Figure 1. Detection of *P. aeruginosa* PA14 and *I. limosus* M53 by the red and green fluorescent probes Paer565 and Ilim569 in pure smears of each organism. The microscopy filters used to visualize each fluorochrome are represented on each column (left: Alexa 594; right: Alexa 488). No cross-hybridization was observed between the two PNA probes. Both images were obtained using the same exposure time.

Plates were incubated aerobically for 24 h, at 37°C and 120 rpm. Afterward, biofilms adhered to the coupons were washed with sterile distilled-water and dried at ~60°C for 15 min. Biofilms were finally fixed with methanol (100% vol/vol) for 20 min. The initial step of fixing the biofilm with methanol is crucial to avoid the detachment of bacterial cells during the hybridization procedure (Almeida et al., 2011). The FISH protocol was identical to the one applied for slides. Fixed biofilms were stored at 4°C for a maximum of 48 h before the multiplex PNA procedure.

Results and Discussion

Probes Design

In the present study, two novel PNA probes to specifically detect and localize the CF-classical opportunist *P. aeruginosa* and the atypical species *I. limosus* within multi-species populations were developed. Although a suitable number of studies have employed PNA probes for targeting *P. aeruginosa* (Peleg et al., 2009; Perry-O'Keefe et al., 2001; Rudkjobing et al., 2012), to the author's knowledge, no probes have been developed that specifically target *I. limosus*. A possible reason for this dissimilarity in the number of reports is because *P. aeruginosa* is often the dominant organism in lungs with advanced chronic stage disease. By contrary, other bacteria are found in small amounts, with researchers considering them as contaminants (Rudkjobing et al., 2011, 2012). Nonetheless, this scenario might not represent the other stages of the infection in the CF lungs, as shifts from clinically stability to episodes of exacerbations may lead to alterations in the airway microbiome composition (Carmody et al., 2013). Additionally, disease-derived factors (e.g., antibiotic selective pressure) and/or other perturbations (e.g., changes in pH, temperature oxygen) may lead to a "natural selection" of the community present in the CF lungs (Conrad et al., 2013; Lynch and Bruce, 2013).

Table I summarizes the predicted theoretical specificities and sensitivities obtained for both designed probes and calculated as previously described by Guimarães et al. (2007), with some modifications. Specificity and sensitivity are two of the most important parameters to take into account during probe design. Specificity is expressed as $NT_{excluded}/(NT_{database}) \times 100$, where $NT_{excluded}$ stands for the number of non-targets (i.e., non-microorganism of interest strains) that did not react with the probe and $NT_{database}$ is the total of non-microorganism of interest strains examined. Sensitivity is calculated as $T_{detected}/(T_{database}) \times 100$, where $T_{detected}$ stands for the number of targets (i.e., microorganism of interest strains) detected by the probe and $T_{database}$ is the total number of targets present in the databases (that did not react with the probe) (Almeida et al., 2010; Azevedo et al., 2007). For the estimation of the probe parameters, a search on the Probe Match program (from the RDP II software) was performed, where only sequences with high quality and more than 1200 bp were considered. For comparison purposes with Paer565, other probes earlier developed for *P. aeruginosa* (Coull and Hyldig-Nielsen, 2003; Perry-O'Keefe et al., 2001) were also evaluated with the Probe Match (Table I). The search confirmed high theoretical specificity (~99.7%) and sensitivity (~90.7%) for Paer565. Both other probes already described for *P. aeruginosa* detection presented similar predicted specificities between each other and Paer565. However, the sensitivity of Paer565 probe was significantly higher than that obtained for Pse16S32 (81.5%). The evolution dynamics of the databases since these probes were designed, with the constant introduction of new RNA sequences that may also be

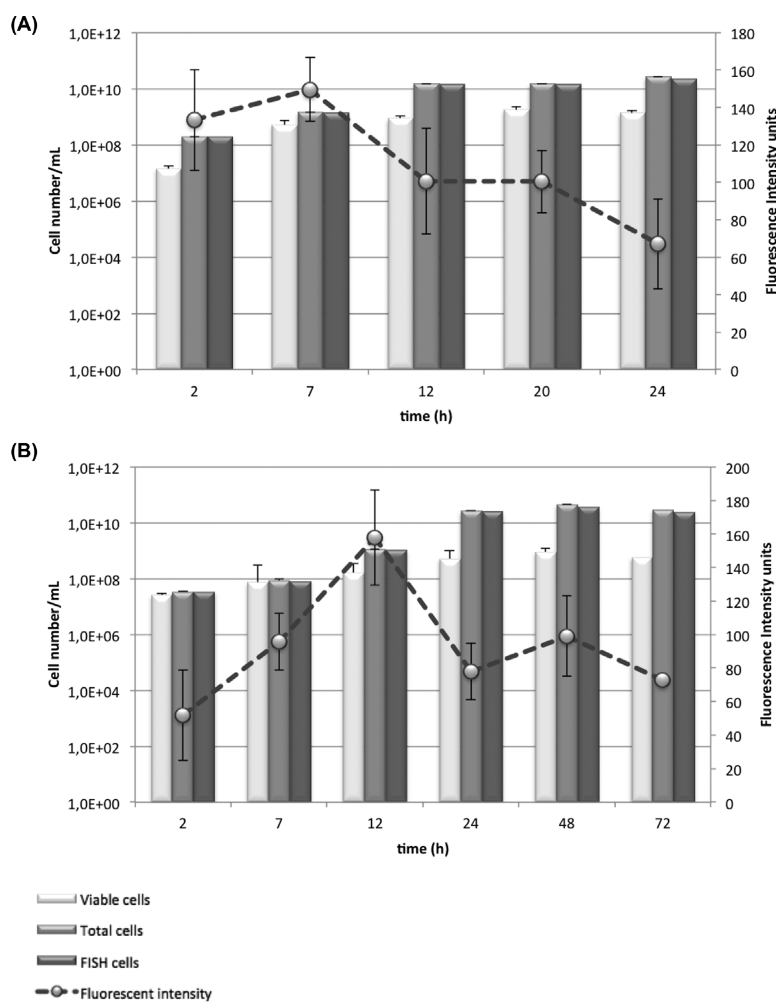


Figure 2. Correlation between cultivable (light gray columns), total (medium gray columns), FISH cells (dark gray columns), and mean intensity fluorescent signal (dashed line) in planktonic suspensions of *P. aeruginosa* (**A**) and *I. limosus* (**B**). Cultivable cells were quantified by conventional culture techniques and total cells were determined by DAPI. Data were expressed as cell number/mL. The intensity of FISH signal (expressed as arbitrary fluorescence intensity units) was monitored throughput the time by an adapted version of the MacBiophotonics ImageJ software.

complementary with the sequences of those probes, help to explain the decreasing sensitivities to detect *P. aeruginosa*. The probe designed by Perry-O'Keefe et al. (2001) presents a reasonable theoretical sensitivity, but it is able to hybridize with other bacterial species frequently isolated from CF samples, *Burkholderia cepacia* and *Klebsiella* sp., thus, invalidating its use in CF settings. The theoretical sensitivity obtained for Paer565 was similar to that obtained for the patented probe Psaer (Coull and Hyldig-Nielsen, 2003). Within the 988 *P. aeruginosa* sequences deposited in the database, Paer565 is specific for 892 whereas, Psaer detects 898 *P. aeruginosa* strains. Paer565 is able to detect other bacteria (in a total of 458), namely bacteria from different filo and genera, and even other species of *Pseudomonas* (such as *P. fluorescens, P. indica*, *P. alcaligenes*, *P. otidis*, *P. putida*, etc.). In any case, apart from *P. aeruginosa*, neither of these microorganisms is known to be associated to CF pulmonary infections. The probe Ilim569 presents an elevated theoretical specificity (100%), being sensitive to detect seven from a total of only eight *I. limosus* strains deposited in the database, and thus, showing a sensitivity of 87.5%. Indeed, the low number of *I. limosus* rRNA sequences deposited in the database reflects the well-recognized difficulty in the isolation of this recently characterized microorganism (Bittar and Rolain, 2010; Coenye et al., 2002). This suggests the need to continuously reassess the sequences that are in use for this microorganism, as the databases are continuously being updated. By checking the Probe Match program for this probe, it was possible to observe that Ilim569 could also detect other but non-related CF organisms, such as *alpha proteobacterium*, *agrobacterium* sp., and *Inquilinus ginsengisoli.* The remaining eight non-targets were non-identified species from the genus *Inquilinus*, which may possibly also belong to the *limosus* species.

Probes Specificity and Sensitivity Testing

To assess the experimental specificity and sensitivity of each probe, the hybridization procedure was carried out at the optimal

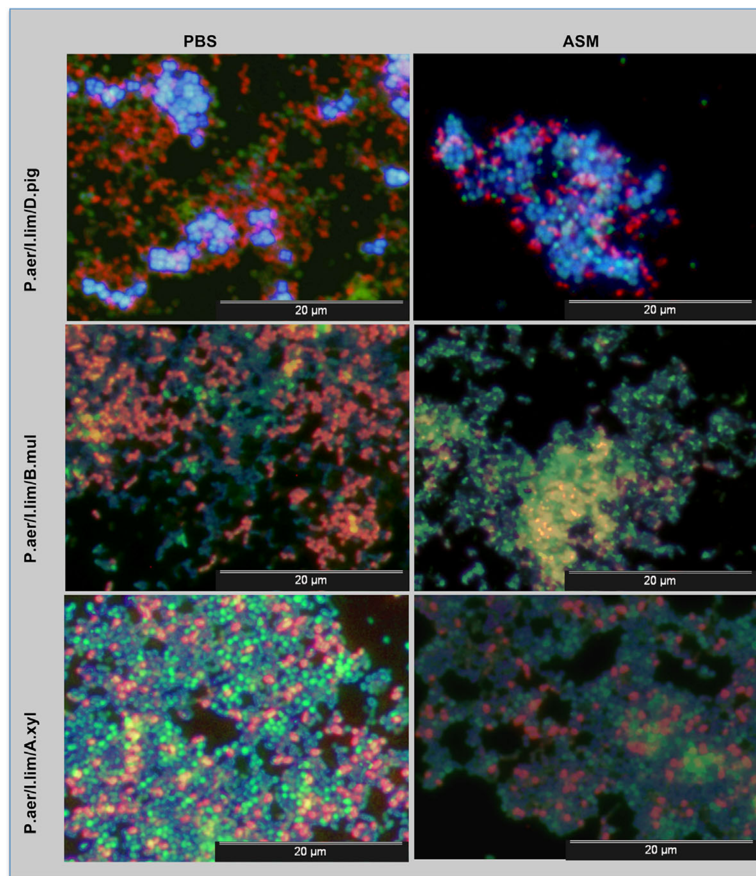
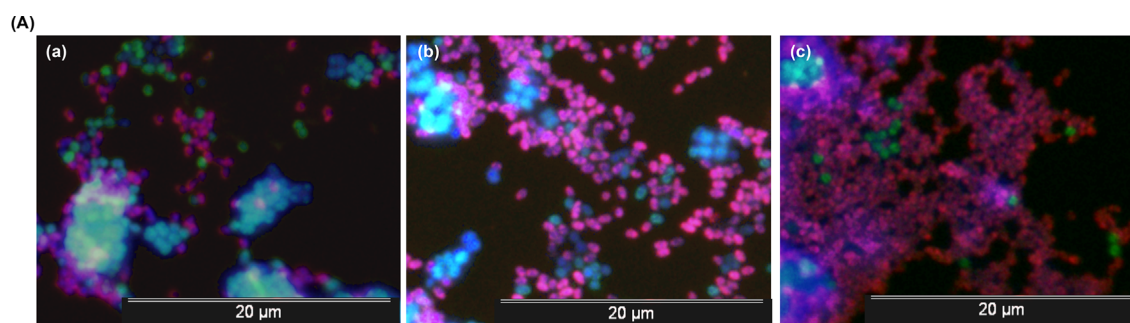


Figure 3. Multiplex FISH assay for three-species smears in phosphate-buffer solution (PBS, left column) and in artificial sputum medium (ASM, right column), using the two PNA probes (Paer565 and Ilim569) and DAPI staining. Each row corresponds to mixed-species populations encompassing *P. aeruginosa* (P.aer, red cells), *I. limosus* (I.lim, green cells), and a third species (*D. pigrum*—D.pig; *B. multivorans*—B.mul; and *A. xylosoxidans*—A.xyl), which were stained blue.

temperature previously determined (65°C) against a panel of distinct bacterial strains, including taxonomically related organisms and even CF-associated bacteria (Table II).

All *P. aeruginosa* strains, including the clinical isolates and also a mucoid strain (*P. aeruginosa* ATCC 39324), could be easily detected by the Paer565 probe, showing a strong red fluorescent signal under microscopy. No hybridization outcome was found for the closely related species *P. fluorescens, P. putida*, and *P. fragi* although some of those species (eventually not the same strains) were shown to be a target for Paer565. Finally, no cross-reactivity was observed for all the other tested organisms, demonstrating the high specificity/sensitivity found by the sequence analysis described before. Concerning the probe Ilim569, a positive FISH result was obtained for both *I. limosus* isolates, with the species emitting a bright green fluorescent signal after the application of the method. As an example, Figure 1 shows the identification of *P. aeruginosa* PA14 (red cells) and *I. limosus* M53 (green cells) when using the probes Paer565 and Ilim569, respectively, under fluorescence microscopy. The strength of the fluorescent signal provided by the fluorochromes coupled to each probe and obtained at the same exposure times, provide a precise identification of both species, with a clear observation of the rod-shaped morphology of both species. No other organisms that are frequently detected in CF samples (*S. aureus*, *Burkholderia* spp., and *D. pigrum*) as well as any of the CF isolates could hybridize with the probes (Table II), suggesting that both probes may be effectively applied for further identification of *P. aeruginosa* and *I. limosus* within the polymicrobial populations recovered from CF airways.

In order to evaluate the specificity and sensitivity of the FISH probes for detection of bacteria with respect to the results of conventional microbiological culture and by DAPI counting, we performed an experiment on planktonic suspensions (in TSB) of *P. aeruginosa* and *I. limosus*. At certain time points, samples of each microorganism were analyzed for CFUs, total cells, and FISH counts. After microscope acquisition, FISH photographs were also analyzed using an adaptation to the image-processing software MacBiophotonics ImageJ version 1.43 m (Collins, 2007) for fluorescence intensity signal monitoring. For this, an automatic approach able to run in a batch-mode fashion was designed. Herein, the method was based in developing a binary mask in which the regions of interest (ROIs) (i.e., the cells) were highlighted from the background by means of a Laplacian of Gaussian (LoG) filter (9×9 kernel). Subsequently, the binary mask was overlapped with the



(B)

Corresponding fluorescence microscopy picture	Inoculum concentration (CFU/mL)			FISH Cells/mL	
	P. aeruginosa	*I. limosus*	*D. pigrum*	*P. aeruginosa* (detected by Paer565)	*I. limosus* (detected by Ilim569)
(a)	6.15×10^8 ($\pm1.29\times10^8$)	5.63×10^8 ($\pm5.68\times10^7$)	4.98×10^8 ($\pm3.12\times10^8$)	1.95×10^8 ($\pm4.52\times10^7$)	1.83×10^8 ($\pm5.60\times10^7$)
(b)	7.40×10^8 ($\pm1.28\times10^8$)	1.22×10^8 ($\pm4.24\times10^6$)	1.49×10^8 ($\pm2.49\times10^8$)	4.73×10^8 ($\pm1.23\times10^8$)	1.23×10^8 ($\pm8.29\times10^7$)
(c)	9.97×10^8 ($\pm1.82\times10^8$)	6.20×10^7 (±0)	8.28×10^7 ($\pm2.76\times10^8$)	4.30×10^8 ($\pm1.61\times10^8$)	5.14×10^7 ($\pm1.28\times10^7$)

Figure 4. (**A**) Fluorescence microscopy photographs of three-species smears of *P. aeruginosa* (red cells), *I. limosus* (green cells), and *D. pigrum* (blue cells) visualized by specific PNA probes Paer565, and Ilim569 and DAPI staining, respectively. Each picture represents different *P. aeruginosa* cells proportions in the whole population: 33% (**a**), 75% (**b**), and 90% (**c**); (**B**) Bacterial composition of the inoculum, determined by culture and expressed by CFU/mL, and estimated number of *P. aeruginosa* and *I. limosus* detected by each PNA probe after hybridization.

original red channel and then the mean fluorescence intensity was calculated as the average of each ROI mean intensity.

As expected, PNA-FISH counts were higher than CFU, which most probably was due to the presence of non-cultivable cells, but very close to total cells as determined by DAPI (Fig. 2). Therefore, determination of CFU numbers may not reflect the true concentration of microbes within the CF samples (there is a great possibility of most species within the whole community stop growing after antibiotic treatment or even existing bacterial aggregates that are very difficult to disintegrate) (Hogardt et al., 2000). Regarding the mean intensity of the fluorescent signal, faster-growing or highly active cells (showing the highest CFU before the stationary phase—at 7 and 12 h for *P. aeruginosa* and *I. limosus*, respectively) tend to have more ribosomes, and hence bind proportionately more probe molecules, resulting in cells hybridized with a stronger fluorescent signal ($\sim$160 arbitrary fluorescence units for both cases).

Hybridization in Smears

To assess if *P. aeruginosa* and *I. limosus* could be easily discriminated within mixed-species populations, a multiplex PNA assay was applied to smears of both species mixed together with other CF-related organisms such as *Burkholderia multivorans*, *A. xylosoxidans*, and *D. pigrum*. *B. multivorans* is a prominent and one of the predominant bacteria from all currently defined BCC species that have been cultured from CF sputum (Mahenthiralingam et al., 2001; Reik et al., 2005). It poses a serious health threat to CF patients, causing a form of septicemia known as "cepacia syndrome" (Zahariadis et al., 2003) and presenting innate, and acquired multiple resistance face to the selected pressure by antibiotics (Stokell et al., 2013). *A. xylosoxidans* and *D. pigrum* have been detected in the airways of CF in the last few years. In patients with CF, an increasing detection of *A. xylosoxidans* and *D. pigrum* has recently been reported (Bittar et al., 2008b; Kanellopoulou et al., 2004; Raso et al., 2008; Saiman et al., 2001), but the clinical significance of both species infection is still not clarified due to the scarcity of data on its impact and transmissibility. However, several lines of investigation have supported signals for their adaptation and their possible contributions to the lung disease chronicity (Hansen et al., 2010; Lopes et al., 2012, 2015).

In this study, the identification of the third organism within the mixed-species smears was possible by counterstaining the samples with the non-specific dye DAPI at the end of the hybridization process. In Figure 3, it is possible to accurately differentiate between the different species when the bacteria were suspended either in PBS (left column) or in ASM (right column), with the species presenting similar proportions within the whole population. The clear-cut discrimination in smears of *P. aeruginosa* and *I. limosus*, provided by the application of the multicolor FISH method described in here, highlights the practicability and reliability of this technique in rapidly detecting a broad spectrum of microorganisms (including classical and emergent) in specimens of CF patients.

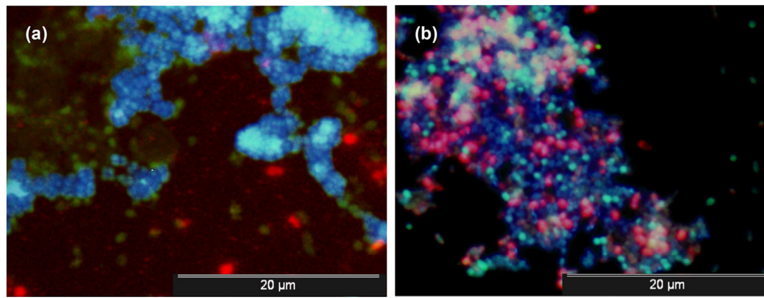


Figure 5. PNA-FISH applied to sputum clinical samples artificially contaminated with three-species encompassing: *P. aeruginosa*, *I. limosus*, and *D. pigrum* (**a**) and *P. aeruginosa*, *I. limosus*, and *A. xylosoxidans* (**b**). *P. aeruginosa* cells are stained red by Paer565 PNA probe; *I. limosus* cells are stained green by Ilim569 PNA probe, and the third organism (*D. pigrum* or *A. xylosoxidans*) are both stained blue by DAPI staining.

After employing the FISH technique in PBS and ASM samples of the three-species and to evaluate the practicability of the method, we examined the limit in the detection in the presence of a large excess of one species (*P. aeruginosa)*. For this, we performed a FISH experiment with a mixture of *P. aeruginosa*, *I. limosus*, and *D. pigrum* in equal (∼33%) and in different proportions, with *P. aeruginosa* ranging between ∼75% and 90%. The concentration of inoculated bacteria was determined by conventional plating techniques (as CFU/mL) and a quantitative analysis was further performed for *P. aeruginosa* and *I. limosus* cells after hybridization

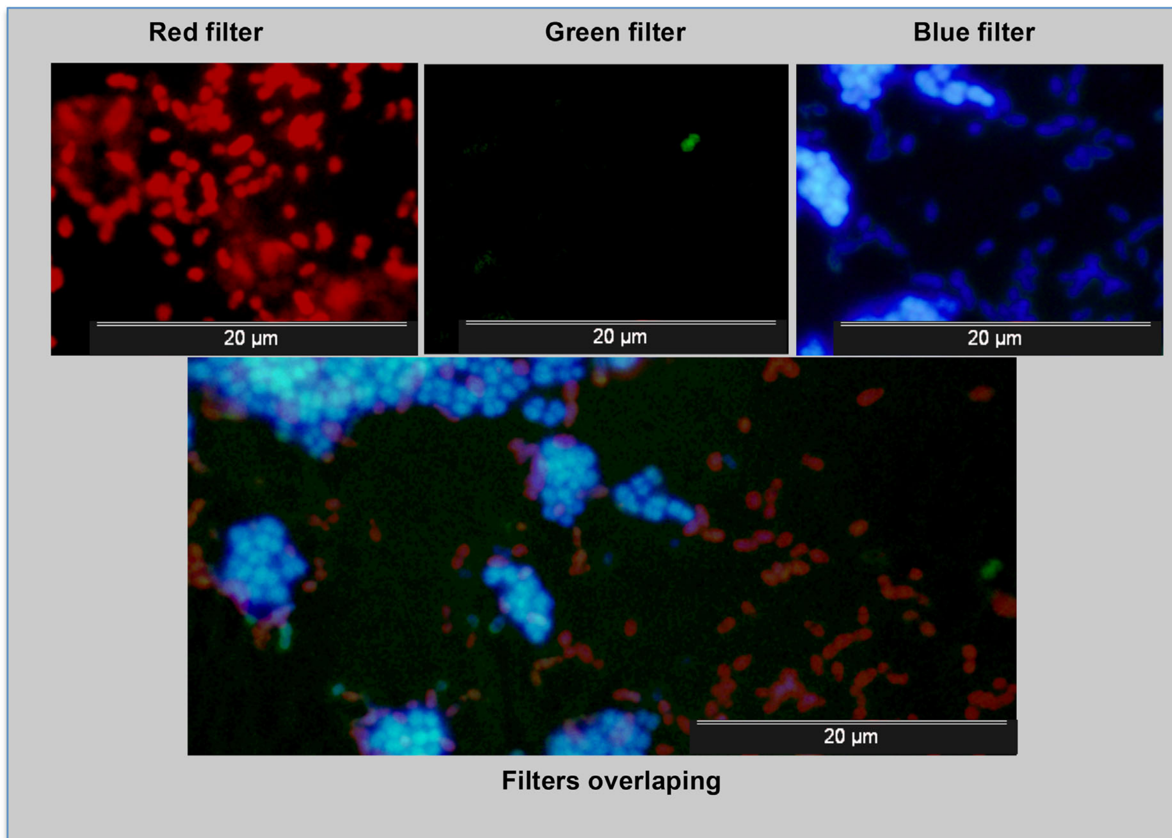


Figure 6. Multiplex PNA assay applied to the three-species 24 h-old biofilms formed by *P. aeruginosa*, *I. limosus*, and *D. pigrum* on PS coupons. The different channels enable to visualize the species involved in the consortium, according with the fluorochromes used (Alexa fluor 594, red: *P. aeruginosa*; Alexa Fluor 488, green: *I. limosus*; DAPI, blue: all species). The bands superposition discriminating the different species within the consortium is (bottom row) allows observing that the biofilm is mainly composed by *P. aeruginosa* and *D. pigrum*, with *I. limosus* as the lowest representative population.

with the Paer565 and Ilim569 probes, respectively. It was possible to conclude the estimated number of each bacterium within the whole populations was not entirely in accordance with the number of bacterial cells present in the inoculum (likely because of the standard deviations), but cell numbers in the same magnitude orders were detected by both PNA probes, allowing us to ensure about the potential of PNA-FISH as an effective quantitative tool (Fig. 4).

FISH Applied to Clinical Sputum Samples and to Biofilms

FISH using labeled fluorescent PNA probes is a methodology that has been successfully applied in many fields of microbiology, but while few studies have focused on its application to CF (Brown and Govan, 2007; Kirketerp-Moller et al., 2008; Malic et al., 2009; Rudkjobing et al., 2012; Wellinghausen et al., 2006; Yang et al., 2008) most only report the identification of traditional CF-pathogens, preventing an accurate identification of the complex microbial ecology present within the CF environment and subsequently a full understanding of the bacterial interactions occurring in situ as well as the interplay of these communities in the host. Considering the application of PNA-FISH method in clinical settings, we successfully validated the developed PNA probes to identify and differentiate mixed-species cultures of *P. aeruginosa*, *I. limosus*, and *D. pigrum* or *A. xylosoxidans* in artificially contaminated clinical sputum samples (Fig. 5), with results clearly showing a strong discrimination between all species in the mixed-cultures. Although, these results need to be confirmed in a larger series of cases, they indicate that PNA-FISH is applicable to clinical samples and may be useful, for instance, for the selection of the most adequate antibiotic treatment to be used for bacterial eradication, depending on the infecting species.

The establishment of bacterial biofilms in the CF lungs is becoming an increasing problematic (Bjarnsholt et al., 2009; Worlitzsch et al., 2002), as the bacteria are well adapted to the environment and protected from the host immune system, developing persistent chronic infections due to the ineffectiveness of antibiotic treatment (Hogardt and Heesemann, 2010; Moskowitz et al., 2004). It is believed that FISH may be useful for the rapid detection of microorganisms that cause acute or even chronic pulmonary infections, supporting an adequate pathogen-directed antibiotic therapy for patients with exacerbations and eventually improving strategies for a better control of these consortia. When applied to biofilms, FISH allows providing information not only about the specific detection and discrimination between the biofilm-forming bacterial species, but also concerning cell count and cell morphology. Moreover, it is possible to observe the in situ spatial distribution and arrangement of the bacterial cells within the consortia without biofilm disruption, leading to a better understanding of the real bacterial interactions occurring in these polymicrobial consortia (Almeida et al., 2011).

As for mixed-species smears, a multiplex PNA probing assay was addressed in three-species biofilms developed on PS surfaces and encompassing *P. aeruginosa*, *I. limosus*, and *D. pigrum*. In Figure 6, it can be seen that all microorganisms could be easily distinguished with distinct cell wall characteristics in the multiplex experiment. The strength of each fluorescent signal enabled to visualize and discriminate *P. aeruginosa* (red cells, image on top left), *I. limosus* (green cells, image on top center) within the consortia, while *D. pigrum* can be identified by DAPI (blue cells, image on top right). The superposition of the filters (bottom image) allowed observing the distribution of the microorganisms within the consortium, resulting in an accurate identification and distinction of each species. Interestingly, the consortium was dominated not only by *P. aeruginosa* but also by *D. pigrum*, with *I. limosus* representing the smallest population. These results were corroborated by culture methods, using non- and selective agar media (average CFU numbers, per cm^2, were 8.3×10^6 ($\pm 3.6 \times 10^5$) for *P. aeruginosa*, 4.2×10^7 ($\pm 1.7 \times 10^7$) for *D. pigrum*, and 2.9×10^5 ($\pm 1.2 \times 10^5$) for *I. limosus*). Therefore, the low proportion of *I. limosus* within the biofilm seems to indicate that this species was outcompeted by the *P. aeruginosa* and/or *D. pigrum*. It is suggested that the preponderance of *D. pigrum* in the biofilm could be decisive to decline *I. limosus*, with the first species also triggering sensitivity to a large number of antibiotics to the whole biofilm (Lopes et al., unpublished data). The ability of these emergent species to develop multidrug-resistant biofilms alone and in modulating social interactions with *P. aeruginosa* was addressed before (Lopes et al., 2012, 2014). Altogether, these results highlight the significance of emergent species in CF infection and therapeutics, and should not be therefore, disregarded.

Certainly that these and other interactions occurring between bacterial species involved in CF mixed infections could be fully understood with the applicability of FISH in biofilms formed in the conditions found in CF lungs.

Conclusions

The use of molecular methods, such as the PNA-FISH, for the identification of bacteria in CF samples, has revealed that the complexity and microbial diversity of CF airways is greater than that found by culturing. In here, the PNA probes designed to detect *P. aeruginosa* and *I. limosus* yielded high specificities and sensitivities to identify the corresponding target microorganisms. The versatility and the fast procedure, taking no longer that 3 h, make FISH a valuable tool for the rapid diagnosis of bacteria in CF samples. The multiplexed PNA-FISH assay, counterstained with DAPI, was successfully applied directly in mixed-species biofilms, enabling an accurate identification of the bacteria involved in the consortia. The method also allows the in situ microscopic visualization of the biofilm structure, which might facilitate the understanding of the real complex interactions between the species. While this work gives awareness on the potentialities of a multiplex approach of PNA-FISH, it is of extreme importance the employment of PNA-FISH in clinical specimens. This will be certainly useful to examine/confirm several important CF-old enigmas, namely: (i) the spatial distribution and arrangement of bacterial species inhabiting CF airways; (ii) the prevailing and non-dominant bacterial species within the consortium; (iii) possible synergism or even the competition interactions provided by the bacterial species; and (iv) eventual alterations in the structure and composition of the mixed-species communities.

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