Detection and Discrimination of Microorganisms Using Locked Nucleic Acid - Fluorescence *In Situ* Hybridization (LNA-FISH)

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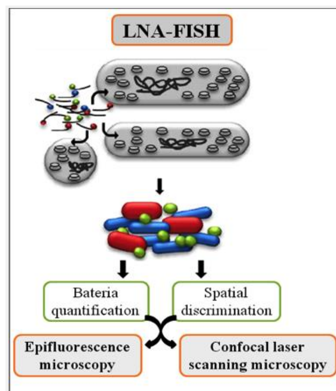

The impact of multispecies biofilms on catheter-associated urinary tract infections outcome is still unclear due to the lack of adequate methodologies to discriminate the populations *in situ*. Employing fluorescence *in situ* hybridization (FISH) to discriminate the populations in a biofilm, can contribute to the understanding of microorganisms interactions in this structures. Consequently, this information might help to develop efficient strategies to prevent this disease.
This work presents the first study that apply the FISH methodology using a set of LNA and/2'-O-Methyl RNA oligonucleotide probes, for the *in situ* detection of microorganisms in biofilms formed under conditions similar to the catheter-associated urinary tract infections.

Introduction

Urinary catheters surface provide an attractive niche for bacterial colonization, leading to the formation and growth of a biofilm [1]. Recent studies involving urinary catheters have shown that catheter-associated urinary tract infections (CAUTIs) are mostly polymicrobial [2]. Nevertheless, there is a lack of knowledge about the impact that multispecies biofilms have on CAUTIs outcome. Most studies related to catheters colonization, have assessed single-species adhesion and biofilm formation, due to the lack of adequate methodologies to discriminate the populations *in situ* [3]. To overcome this problem, researchers have been using different molecular biology approaches, namely fluorescence *in situ* hybridization (FISH), which in combination with epifluorescence microscopy allows the identification and quantification of the bacterial species in multispecies biofilms. On the other hand, FISH in combination with confocal laser scanning microscopy (CLSM) allows the study of spatial organization and changes of specific members of complex microbial populations without disturbing biofilm structure [4]. The combination of the FISH method with nucleic acid mimics, such as locked nucleic acid (LNA) and 2'-O-methyl RNA (2'OMe), has shown to have advantages compared to DNA probes. LNA is an RNA derivative nucleotide analog with a locked C3'-*endo conformation* which has been used recently in microbial detection [5,9]. The 2'OMe presents a C3-*endo conformation* adopted by 2'OMe ribose sugars [6]. The use of LNA residues in oligonucleotides allows the stabilization of the duplex during the hybridization and a sensitive detection [7].

Taking advantage of recent progress in nucleic acid mimics, the main objective of this research project is to develop, optimize and validate a multiplex FISH procedure using LNA/2'OMe oligonucleotides probes, for *in vitro* discrimination and spatial localization of the species in biofilms formed in conditions mimicking the CAUTIs.

As a case study, we selected *E. coli*, the major cause of UTIs, and three other atypical colonizers of urinary catheters (*Delftia tsuruhatensis*, *Achromobacter xylosoxidans* and *Burkholderia fungorum*). These microorganisms present unproven pathogenic potential, but can co-inhabit the catheter surface with disease-causing bacteria (e.g. *E. coli*) [8]. The role that these atypical microorganisms have on the rate at which disease-causing microorganisms adhere and form biofilms, and their affect on CAUTIs outcome, is still unknown.

Methods

Since the probes are intended for a multiplex approach, it is important to assure that all probes will work at the same temperature.

In order to assure this, a set of LNA/2'OMe probes with different sizes (13 bp and 16 bp), were designed to detect specifically the four microorganisms (*E. coli*, *D. tusuruhatensis*, *A. xylosoxidans* and *B. fungorum*). This increases the

chances of finding a combination working at the same temperature (Table 1). For probe design, both an advanced BLAST search and a 16S rRNA sequence match analysis, at the Ribosomal Database Project-II site, were carried out. Then, oligonucleotide synthesis was carried out on an automated nucleic acid synthesizer (PerSpective Biosystems Expedite 8909 instrument) under anhydrous conditions, at 1.0 µmol scale.

In order to determine the optimal hybridization temperature of each oligonucleotide probe, the hybridization procedures were performed in glass slides and in suspension, according to protocols already developed by our group [3, 9]. Afterward, the quantification by ImageJ software was performed in order to determine the average fluorescence intensity of each image obtained by epifluorescence microscopy [9].

Results and Conclusions

For the selection of useful oligonucleotides, conserved regions for the 23S rRNA (*E. coli*) and 16S rRNA (*D. tsusruhatensis*, *A. xylosoxidans* and *B. fungorum*) sequences, were identified using ClustalW. Based on the GC percentage, presence or absence of self-complementary structures, theoretical specificity and sensitivity values, the probes were selected (Table 1). The sequences selected hybridize between the position 410 and 425 on *E. coli* 23S rRNA sequence, 404 and 419 on *D. tsuruhatensis* 16S rRNA sequence, 590-605 on *A. xylosoxidans* 16S rRNA sequence and 411-426 on *B. fungorum* 16S rRNA sequence. Then, the theoretical specificity and sensitivity of each selected probe were evaluated, using the ProbeCheck program (for *E. coli* probes) coupled to the LSU database; or ProbeMatch from RDP II database (for the other microorganisms). The theoretical sensitivity was calculated as Bs/(TBs)×100, where Bs stands for the number of bacteria strains detected by the probe and TBs for the total number of bacteria strains present in the database. Theoretical specificity was calculated as nBs/(TnB)×100, where nBs stands for the number of non-bacteria strains that did not react with the probe and TnB for total of non-bacteria strains examined. The *E. coli* probes showed a theoretical specificity and sensitivity of 98% and 43.13%, respectively. For *D. tsuruhatensis*, *A. xylosoxidans* and *B. fungorum* a theoretical sensitivity of 92%, 88% and 90% was obtained, respectively. On the other hand, a theoretical specificity of 99.9% was obtained for *D. tsuruhatensis*, *A. xylosoxidans* and *B. fungorum*. The hybridization procedures in glass slides were optimized by testing different temperatures and the hybridization temperature that provided the best signals was 55ºC for most of the probes (Figure 1a). However, when tested for specificity against the non-target strains, a slight cross-hybridization was observed for some of the probes at this temperature. This problem was solved by increasing the hybridization temperature in 2ºC. To achieve an appropriate specificity, 57ºC was used for all subsequent experiments. As such, for each microorganism, the LNA probes that presented the best signal at 57ºC, were selected. For *E. coli*, *D. tsusruhatensis* and *A. xylosoxidans*, the oligonucleotides probes with 16 bp (NAC6120, NAC6122 and NAC6119, respectively) were selected; and for *B. fungorum* the oligonucleotide probe with 13 bp (NAC6117), was chosen. After this initial optimization, the hybridization of the LNA probes in suspension was evaluated. The hybridization in suspension is the best way to quantify the bacteria population in a sample by flow cytometry or by epifluorescence microscope, so it is important to confirm that the signals on a standard glass slide test are maintained on suspension. Results have shown that the signals obtained in hybridizations performed in suspension at 57ºC, were similar or higher than those obtained in glass slides (Figure 1b).

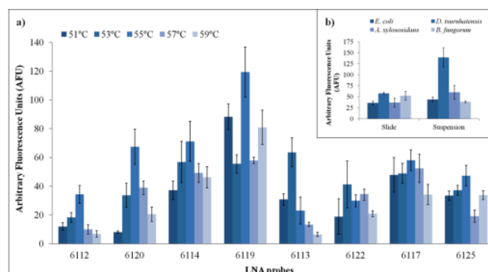

Figure 1. Figure 1. Average fluorescence intensity from each LNA probe obtained in the hybridization procedures performed in a) glass slides and b) suspension. Fluorescence signal intensity is expressed in arbitrary fluorescence units (AFU) and was quantified using ImageJ software. All images were acquired at equal exposure conditions.

After selecting a set of LNA probes working at same temperature (57°C), we proceeded with the discrimination in mixed population Afterward, it was observed that a mix of the probes is able to discriminate the populations at 57ºC in a smear of two species (Figure 2) in a 1:1 proportion.

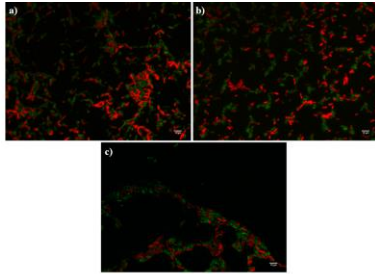

Figure 2. Fluorescence microscopy pictures of dual-species samples. a) *E. coli/ D. tsuruhatensis*; b) *E. coli/ A. xylosoxidans*; c) *E. coli/ B. fungorum*.

As a result, FISH methodology using LNA/2'OMe probes might be used in combination with epifluorescence microscopy for in situ spatially discrimination. Further studies will focus on the application to multispecies catheter associated biofilms, and on CLSM analysis.

Table 1. Sequence of oligonucleotide probes used for detection of *E. coli* and atypical species.

Bacteria	Name	Sequence (5'Label-3')
E. coli	NAC6112	5' FAM - lGmCmClTmCmAlGmCmClTmUmGlA - 3'
	NAC6120	5' FAM - lCmAmClGmCmClTmCmAlGmCmClTmUmGlA - 3'
D. tsuruhatensis	NAC6114	5' CY3 - lGmAmGlCmUmUlTmUmUlCmGmUlT - 3'
	NAC6119	5' CY3 - lGmAmGlCmUmUlTmUmUlCmGmUlTmCmClG - 3'
B. fungorum	NAC6117	5' CY3 - lTmAmUlTmAmAlCmCmAlCmGmGlC - 3'
	NAC6125	5´CY3 - lGmGmUlAmUmUlAmAmClCmAmClGmGmClG - 3´
A. xylosoxidans	NAC6113	5' CY3 - lAmAmAlTmGmClAmGmUlTmCmClA - 3'
	NAC6122	5' CY3 - lAmAmAlTmGmClAmGmUlTmCmClAmAmAlG - 3'

LNA nucleotide monomers are represented with 'l'; 2'-OMe-RNA monomers are represented with 'm'; FAM- Fluorescein; CY3-Cyanine 3.

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