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PROTEUS SPP. DETENTION IN ARTIFICIAL URINE SAMPLES BY PNA FISH

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INTRODUCTION

One of the most frequent diseases occurring from the neonate up to geriatric age groups is the urinary tract infection (UTI). The majority of UTIs are uncomplicated infections, caused by *Escherichia coli* (~80%) and by enteric pathogens such as *Enterococcus faecalis, Klebsiella pneumoniae, Proteus* spp., *Staphylococcus* spp. and fungal opportunistic pathogens such as *Candida albicans*. The genus *Proteus* is considered the fourth or fifth cause of UTIs and is related to the emergence of complicated UTI (about 10 to 15% of the cases) mainly for immuno-compromised patients. Catheter-associated UTI's (CAUTI's) are the most common type of nosocomial infections, accounting for over 1 million cases annually in US hospital or over 40% of all nosocomial infections in hospitals and nursing homes and constitute 80% of all nosocomial UTI's. The three species of *Proteus* described as a cause of UTIs are *Proteus mirabilis*, *Proteus vulgaris* and *Proteus penneri*.

In terms of UTI diagnosis, current techniques require 24-48 h to identify pathogenic species in urine midstream specimens. Chromogenic agars, such as CHROMagar Orientation and Candida and MacConkey agar, have been developed to facilitate and to accelerate recognition of species directly on primary media, and have been widely adopted. However, some studies have shown that results obtained using the different chromogenic media are not consistent because microorganisms are not always detected on every type of media, colony counts can differ and the numbers of different species that can be recognized differed. Although screening methods have been studied exhaustively, none has been universally accepted. Some other studies have tried to introduce molecular tests, but microscopy and culture remained the gold standard in every day clinical practice.

Fluorescence in situ hybridization (FISH) is a molecular method used to identify and quantify microbial populations. The combination of this method with probes of peptide nucleic acid (PNA) has been shown to have many advantages compared to the conventional DNA FISH. The characteristics of the PNA molecule allow a stable, fast and more specific hybridization than DNA. These PNA probes have been successfully applied to the detection of several clinical relevant microorganisms on a broad range of samples.

In this work we designed and tested a new PNA probe for the *Proteus* genus, and developed and validated a PNA-FISH method for the clinical detection of this pathogen in urine.

METHODS

The selection of oligonucleotides was based on the 16S rRNA comparison of all Proteus strains in the Ribosomal Database Project II (RDP II).

Once the probe sequence was selected, a search was performed using the National Centre for Biotechnology Information (NCBI) and the Probe Match (RDP II) to further confirm probe specificity. The chosen sequence was synthesized and the attached to Alexa fluor. Following optimization of the hybridization conditions, the specificity of the PNA probe was tested using 22 *Proteus* strains belonging to the different species, and to other 52 strains. After the specificity test, the probe was applied to artificial urine contaminated with *Proteus.* *P. vulgaris* and *P. mirabilis* strains were inoculated in artificial urine and incubated at 37°C, 120 revolutions per minute (rpm). Serial 10-fold dilutions from were made in artificial urine, centrifuged and resuspended in sterile water for evaluation by PNA FISH. The hybridization was performed in suspension or on glass slides, as previously described, and the samples were visualized at the epifluorescence microscope. A 10 µL volume of each serial 10-fold dilution was also spread on CHROMagar Orientation (Becton, Dickinson and Company) to compare with the results obtained by PNA FISH.

RESULTS AND DISCUSSION

Theoretical evaluation

The search on NCBI and ProbeMatch databases showed that the Proteus probe detected 87 of 89 *Proteus* sequences present in the, and thus a sensitivity of 97, 75% was obtained. As we analyzed 495851 non-*Proteus* strains and only two were detected by the probe, a theoretical specificity of 99,99% was obtained.

Specificity and sensitivity test

After optimization the specificity and sensitivity of the PNA probe was tested. For this, the procedure was applied to 22 representative *Proteus* strains. Four *Proteus* species: *P. vulgaris, P. mirabilis, P. penneri* and *P. hauseri*, even though only *P. vulgaris, P. mirabilis and P. penneri* species are reported as causing UTIs. The *P. hauseri* usually is not reported as a cause of UTI's, because it is considered the genomospecie 3 of *P. vulgaris* species. The other species, P. myxofaciens, is not associated to humans.

Another 52 strains, including common urinary pathogens, such as *E. coli, E. faecalis, K. pneumonia* and *Staphylococcus*, were also included. In addition to the strains expected to be present in urine, the non-*Proteus* strains also included 32 taxonomically related strains and 20 strains belonging to different orders, classes or even phyla.

As shown in Table 1, all of the 22 Proteus strains were detected, whereas no hybridization was observed for the other species used. Therefore, experimental specificity and sensitivity were both of 100% (sensitivity, 95% CI, 81.5 – 100 and specificity, 95% CI, 91.4 - 100).

Table 1- *Proteus* probe specificity and sensitivity test.

Microoganisms (nº of strains tested)	**PNA FISH outcome**
Proteus mirabilis (12)	+
Proteus vulgaris (7)	+
Proteus penneri (2)	+
Proteus hauseri (1)	+
Cronobacter spp (2)	-
Enterobacter spp. (6)	-
Escherichia spp. (6)	-
Klebsiella spp. (2)	-
Citrobacter freundii (1)	-
Serratia plymuthica (1)	-
Morganella morganii (1)	-
Salmonella spp (8)	-
Shigella spp (3)	-
Yersinia spp. (2)	-
Pseudomonas spp (2)	-
Campylobacter spp. (2)	
Staphylococcus spp. (9)	-
Streptococcus mutans (2)	
Enterococcus spp. (2)	
Listeria monocytogenes (3)	-

Detection in urine

In this work, we describe the development of a new PNA FISH method able to detect *Proteus* in urine samples in, approximately, 2 hours. For this, we artificially contaminated urine with Proteus spp., with cell concentrations ranging from $1x10^2$ to $1x10^7$ CFU/mL. A urine culture of at least 10^5 CFU/mL of any single type of bacterium has traditionally been believed to provide conclusive evidence of UTI; although, it was reported that for catheterized individuals this value can be lower ($\sim10^4$). Therefore, as *Proteus* is associated with the infection of catheterized patients and as CAUTI's are the most common nosocomial infection, we defined a threshold of 1×10^4 CFU/mL. The hybridization was performed in suspension to accurately quantify the number of cells present in the sample. In Figure 1 we can observe the results obtained for each bacterial concentration.

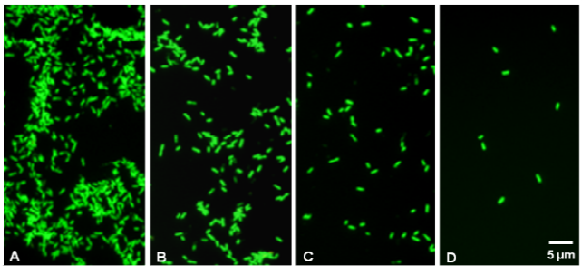

Figure 1 – Epifluorescence detection of *P. vulgaris* ATCC 29905 using PNA probe in artificial urine with 1×107 (A), 1×10^6 (B), 1×10^5 (C) and 1×10^4 (D) CFU per mL of urine.

The results showed that this procedure was able to detect 1×104 CFU/mL, while concentrations lower than this, which were considered non-significant bacteriuria, were not detected. As such, a positive PNA FISH out-come will always be indicative of infection.

AUTHOR BIOGRAPHY

Carina Almeida was born in Vila Verde, Braga. She did her degree at the University of Minho (2001-2005) in Applied Biology. In 2006 she had a research grant in the European SAFER project (Surveillance and control of microbiological

stability in drinking water distribution networks) under the supervision of Prof. Maria João.
In the present she is doing her PhD on Biomedical Engineering, subjected to the theme “Development and application of PNA probes for the detection of specific bacteria by FISH”, under the supervision of Prof. Charles William keevil (University of Southampton) and Prof. Maria João Vieira.