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ARTICLE

Rapid detection of urinary tract infections caused by *Proteus* spp. using PNA-FISH

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Abstract We developed a fluorescence in situ hybridization (FISH) method for the rapid detection of *Proteus* spp. in urine, using a novel peptide nucleic acid (PNA) probe. Testing on 137 urine samples from patients with urinary tract infections has shown specificity and sensitivity values of 98 % (95 % CI, 93.2–99.7) and 100 % (95 % CI, 80,8–100), respectively, when compared with CHROMagar Orientation medium. Results indicate that PNA-FISH is a reliable alternative to traditional culture methods and can reduce the diagnosis time to approximately 2 h.

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Introduction

The genus *Proteus* is in the top five causes of urinary tract infections (UTIs) [1, 2] and is related to the emergence of complicated UTI (10–15 % of the cases), mainly for immuno-compromised patients [3]. *Proteus*-associated UTIs can be difficult to treat and are usually associated with bladder and kidney stone formation, that can lead to the obstruction of the urinary tract and catheters [3, 4]. The three species of *Proteus* described as being associated with UTIs are *Proteus mirabilis*, *Proteus vulgaris* and *Proteus penneri*. *P. mirabilis* is considered to be the third most common cause of complicated UTI (responsible for 12 % of infections) and the second most common cause of catheter-associated UTI's (CAUTI's) in long-term catheterized patients (15 %) [4].

In terms of UTI diagnosis, current techniques require 24–48 h to identify pathogenic species in urine [5, 6]. Chromogenic agars, such as CHROMagar Orientation and Candida and MacConkey agar, have been developed to facilitate and accelerate species recognition directly on primary media, and have been widely adopted [7, 8]. However, studies have shown that results obtained using the different chromogenic media are not consistent, because microorganisms are not always detected on media, and the colon counts and species recognized differed [6, 9]. In the absence of expeditious microbiological diagnosis, clinicians must initiate empirical antimicrobial treatment without supportive laboratory data, which has led to the emergence of resistant pathogens. Thus, a rapid molecular approach would be beneficial.

Fluorescence in situ hybridization (FISH) is a molecular method used to identify and quantify microbial populations [10]. The combination of this method with peptide nucleic acid (PNA) probes has shown to have many advantages compared to conventional DNA-FISH [11]. PNA-FISH

has been applied to the detection of several clinical relevant microorganisms on a broad range of samples (for a review see [12]). More recently, some studies have shown that its implementation as a routine method in clinical laboratories allows for a rational use of medicines, and reduces hospitalization times and mortality [13, 14].

In this work we developed and validated a new PNA-FISH method for clinical detection of *Proteus* in urine samples from patients with UTIs.

Material and methods

Culture maintenance

The bacterial strains used in this study are listed in Table 1. *Streptococcus* and *Campylobacter* strains were maintained on Columbia agar (Oxoid) supplemented with 5 % (vol/vol) defibrinated horse blood (Probiológica) and incubated at 37 °C, 10 % CO_2 and 5 % O_2 in a CO_2 incubator (HERAcell 150, Waltham). Single colonies were streaked onto fresh plates every 2–3 days. All remaining bacterial species were maintained on Tryptic Soy Agar (Sigma) at 37 °C and restreaked every 24 h.

PNA probe design and theoretical evaluation

To identify potentially useful oligonucleotides for use as probe, the Primrose program was used coupled with the 16S rRNA databases of the Ribosomal Database Project II (RDPII) (http://rdp.cme.msu.edu/html) [15, 16]. Criteria for the selection of the PNA probe included: high number of *Proteus* detected, low number of non-targets detected, no self-complementary structures, and lower number of non-target sequences with one mismatch. The selected sequence was synthesized (Panagene, South Korea) and the N-terminus attached to Alexa fluor 488 via a double AEEA linker. The theoretical specificity and sensitivity of the probe was evaluated with the updated databases available at RDPII (RDP Release 10, Update 28, January 2012) and NCBI.

Specificity was calculated as nPs/(TnP)x100, where nPs stands for the number of non-*Proteus* strains that did not react with the probe and TnP for total of non-*Proteus* strains examined. Sensitivity was calculated as Ps/(TPs)x100, where Ps stands for the number of *Proteus* strains detected by the probe and TPs for the total number of *Proteus* strains in the databases.

Hybridization procedure

The hybridization procedures were performed as previously reported [17]. For the protocol optimization the hybridization was repeated but hybridization and washing temperatures (57 to 65 °C) as well as the hybridization time (45 and 90 min) were changed.

After the hybridization, samples were allowed to air-dry, mounted with one drop of non-fluorescent immersion oil (Merck) and covered with coverslips. Samples were visualized in an epifluorescence microscope (Olympus BX51) with an FITC filter. A negative control was performed simultaneously, with hybridization solution without probe.

Proteus probe specificity test

The specificity of the probe was tested using 22 *Proteus* strains of different species and 52 other strains. These latter strains included 32 taxonomically related strains of the same family, and 20 strains of different orders, classes or phyla.

Detection limit in artificial urine

To evaluate the detection limit, artificial urine samples, prepared as reported before [18], were inoculated with *P. vulgaris* ATCC29905 and *P. mirabilis* ATCC21100 (grown overnight in artificial urine at 37 °C, 120 rpm) with concentrations ranging from 1×10^2 to 1×10^7 CFU/mL. As the detection limit reported for PNA-FISH is 2×10^5 cells/mL [17], 20 mL samples (which were 20-fold concentrated by centrifugation at 10,000 g for 5 min) were used before starting the hybridization procedure. This was performed in suspension or on glass slides as described above.

To quantify the number of cells lost during the PNA-FISH method, samples were taken during the different procedure steps (inoculum in water, paraformaldehyde, ethanol, washing solution 1 and washing solution 2), stained with DAPI and counted under the microscope.

Quantification of cells by DAPI or PNA-FISH was obtained by counting a total of 15 fields with an area of 0.0158 mm^2. The average was used to calculate total cells per mL of sample. These experiments were performed three times.

Detection in urine samples

To evaluate method performance on real samples, 78 urine samples from outpatients with symptoms of UTI, were collected in sterile flasks at Hospital de S. João (Porto, Portugal) and Laboratório de Análises Clínicas S. Lázaro (Braga, Portugal) and simultaneously analyzed by PNA-FISH and CHROMagar Orientation medium. For PNA-FISH analyses, hybridization was performed as described above, but for turbid urine samples only 1 ml was pelleted by centrifugation.

Table 1 Results of the *Proteus* probe (ProPNA190) specificity and sensitivity test

Microoganisms	PNA FISH outcome
Proteus mirabilis ATCC 29906[a], ATCC 14153[a], ATCC 21100[a], 414[b], 417[b], 933[b], M13[b], PL050596[b], PL050835[b], PL49417[b], PL49905[b] and PL50359[b]	+
Proteus vulgaris ATCC 6380[a], ATCC 6896[a], ATCC 9484[a], ATCC 29905[a], M12[b], 404[b] and 43[b]	+
Proteus penneri ATCC 33519[a], CDC2518-74[a]	+
Proteus hauseri ATCC 13315[a]	+
Cronobacter sakazakii ATCC 29544[a]	−
Cronobacer malonaticus[b]	−
Enterobacter aerogenes CECT 684[a]	−
Enterobacter amnigenus CECT 4078[a]	−
Enterobacter asburiae[b]	−
Enterobacter cloacae[b]	−
Enterobacter helveticus[b]	−
Enterobacter hormaechei[b]	−
Escherichia coli K12[a], ATCC 25922[a], N5[b] and N9[b]	−
Escherichia hermannii ATCC 33650[a]	−
Escherichia vulneris ATCC 29943[a]	−
Klebsiella oxytoca ATCC 13182[a]	−
Klebsiella pneumoniae ATCC 11296[a]	−
Citrobacter freundii[b]	−
Serratia plymuthica F4[b]	−
Morganella morganii CDC4195-69[a]	−
Salmonella bongori SGSC 3100[a], SGSC 3103[a]	−
Salmonella enterica subp. *enterica* serovar Enteritidis SGSC 2474[a]	−
Salmonella enterica subp. *enterica* serovar Newport SGSC 2493[a]	−
Salmonella enterica subp. *enterica* serovar Panama SGSC 2497[a]	−
Salmonella enterica subp. *enterica* serovar Paratyphi C SGSC 2506[a]	−
Salmonella enterica subsp. enterica serovar Indiana SGSC 2482[a]	−
Salmonella enterica subp. *enterica* serovar Dublin SGSC 2470[a]	−
Shigella boydii ATCC 9207[a]	−
Shigella dysenteriae ATCC 11835[a]	−
Shigella sonnei ATCC 25931[a]	−
Yersinia kritensenii PL115185[b]	−
Yersinia enterocolitica subsp. *enterocolitica* PL129178[b]	−
Pseudomonas aeuroginosa ATCC 10145[a]	−
Pseudomonas fluorescens ATCC 13525[a]	−
Campylobacter coli[b]	
Campylobacter jejuni[b]	
Staphylococcus aureus subsp. *aureus* ATCC 13565[a], ATCC 12600[a], ATCC 6538[a] and 239[b]	−
Staphylococcus sciuri[b]	−
Staphylococcus epidermidis ATCC 35983[a]; ATCC 35984[a]; ATCC 1798[a]; ATCC 14990[a]	−
Streptococcus mutans UC[b], UP[b]	
Enterococcus faecalis ATCC 27285[a]	
Enterococcus faecium ATCC 19434[a]	
Listeria monocytogenes CECT 4031T[a], 747[b], 924[b]	−

[a]Type strains
[b]Isolates

For culture analyses, a 10-μL volume of each sample was spread on CHROMagar Orientation and incubated for 24 or 48 h at 37 °C. Only samples with *Proteus* concentrations higher than 1×10^4 CFU/ml were considered positive for UTI.

Results

Probe design

The selection of oligonucleotides was based on the 16S rRNA comparison of all *Proteus* spp. strains in the RDP II database. Several potentially useful oligonucleotides were identified using the PRIMROSE software. From the possible probes identified, and based on criteria described in "Material and Methods", we selected the following PNA oligomer sequence: 5′-GCCCCTGCTTTGGTC- 3′. This probe hybridizes between positions 190 and 204 of the *P. mirabilis* strain HI4320 16S rRNA (accession number: NC_010554.1) and was thus designated ProPNA190.

The search showed that the ProPNA190 detected 135 of 140 *Proteus* sequences present in the database (last accession, March 2012), and thus a sensitivity of 96.4 % was obtained. The five non-detected *Proteus* sequences were three *P. mirabilis* (accession number: S000806301, S002305608 and S002409155), one *P. vulgaris* (S000414235) and one *Proteus spp.* (S002950968). To confirm this result the five sequences of rDNA were aligned with the probe reverse-complement. We observed that sequence S002950968 was incomplete and did not include the region where the probe hybridizes. Sequences S000414235, S000806302, S002305608, and S002409155 present one mismatch of base pairs compared to ProPNA190 (Supplemental material, Fig. S1). For the *P. mirabilis* S000806301 strain the mismatch corresponds to an R (A/G) in the rRNA sequence, meaning that this strain might also be detectable with the probe. From the 495940 16S rRNA sequences analyzed, ProPNA190 also detected four non-*Proteus* sequences: one *Orientia tsutsugamushi* (S000266975), two *Idiomarina sediminum* (S000842774 and S002446946) and one *Pseudidiomarina* sp. S001098108 (*Pseudidiomarina* spp. inclusion in *Idiomarina* genus have been recently proposed [19]). However, none of these non-targets is relevant to the diagnosis of urinary infection [20, 21]. As we analyzed 495,851 non-*Proteus* strains and only two were detected by the probe, a theoretical specificity of 99.99 % was obtained.

Probe specificity and sensitivity testing

The hybridization conditions for ProPNA190 were optimized and a strong signal-to-noise ratio was obtained from 55 °C to 65 °C for 45 min of hybridization. However, to achieve an appropriate specificity, 65 °C was used for all subsequent tests. After optimization, the specificity and sensitivity of the PNA probe were tested on 22 representative *Proteus* strains and another 52 strains, including common urinary pathogens, such as *E. coli*, *E. faecalis*, *K. pneumonia* and *Staphylococcus* [22].

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

Detection limit in artificial urine

To achieve the desired sensitivity, and considering the reported detection limit for PNA-FISH of 2×10^5 cells/mL [17], samples were 20-fold concentrated by centrifugation before the hybridization procedure.

As the PNA-FISH method also involves centrifugation steps, the number of cells lost during the procedure was determined to assure that it did not interfere with the PNA-FISH result. We observed that the percentage of cells lost during the procedure was 7–8 % (Table S1), and the total cells lost from the initial step till the end of the PNA-FISH method was 26.39 % (±7.46). Therefore, and considering that we are measuring total cells that are even higher than CFU counts (for *Proteus* in artificial urine, the number of cells detected by cultivability was 85.75± 3.65 %), we were assured that this loss does not interfere with the outcome. In Fig. 1 we can observe the results obtained for each bacterial concentration. This procedure was able to detect 1×10^4 CFU/mL (corresponding to ~10 cells/microcopy field; Supplemental material Table S2), while concentrations lower than this were not detected. However, if a concentration of 1×10^5 CFU/mL is desired as indicative of infection, a maximum initial urine volume of 2 mL should be used.

Detection of *Proteus* in clinical urine specimens

To evaluate method performance on real samples, urine from outpatients with symptoms of UTI were collected and simultaneously analyzed by PNA-FISH and CHROMagar Orientation medium. For CHROMagar Orientation, only samples with *Proteus* concentrations higher than 1×10^4 CFU/ml were considered positive for UTI. For PNA-FISH a positive outcome was always considered indicative of UTI (Fig. 2 shows a positive result for *Proteus* presence in a real urine sample). As shown in Table 2, from the 137 samples analyzed, only 23 were positive for *Proteus* presence using the CHROMagar Orientation medium. Using this PNA-FISH method, no false negative results were observed, but two false positive results were obtained. As such, the observed value of sensitivity was 100 %, with a confidence interval (CI) of 95 %, from 80.8 to 100 %, and for specificity a value of 98 % was determined, with a CI from 93.2 to 99.7 %. Results were quite consistent between the two techniques, with the main difference being the time required to achieve the final result.

Discussion

The *Proteus* genus includes five species, *P. vulgaris*, *P. mirabilis*, *P. penneri*, *P. hauseri* and *P. myxofaciens*, which

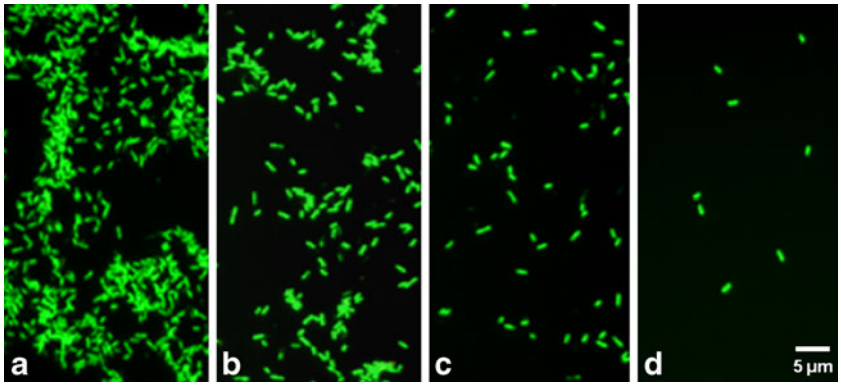

Fig. 1 Epifluorescence detection of *P. vulgaris* ATCC 29905 using the ProPNA190 probe in artificial urine with 1×10^7 (**a**), 1×10^6 (**b**), 1×10^5 (**c**) and 1×10^4 (**d**) CFU per mL of urine

exist in soil, polluted water, and intestines of humans and a wide variety of animals [23, 24]. For the probe specificity test, we included four *Proteus* species: *P. vulgaris*, *P. mirabilis*, *P. penneri* and *P. hauseri*, though only *P. vulgaris*, *P. mirabilis* and *P. penneri* species are reported as causing UTIs [22, 23]. *P. hauseri* is not usually reported as a cause of UTI's, because it is considered the genomospecies 3 of *P. vulgaris* species [25]. Additionally, 32 taxonomically related strains of the same family (*Shigella*, *Klebsiella, Citrobacter, Pantoea, Yersinia, Enterobacter*, *Escherichia* and *Serratia, Morganella*) and 20 strains of different orders (*Pseudomonas*), classes (*Campylobacter*) or phyla (*Listeria, Streptococcus, Enterococcus* and *Staphylococcus*) were also tested, and no cross-hybridization was observed for the non-*Proteus* strains tested.

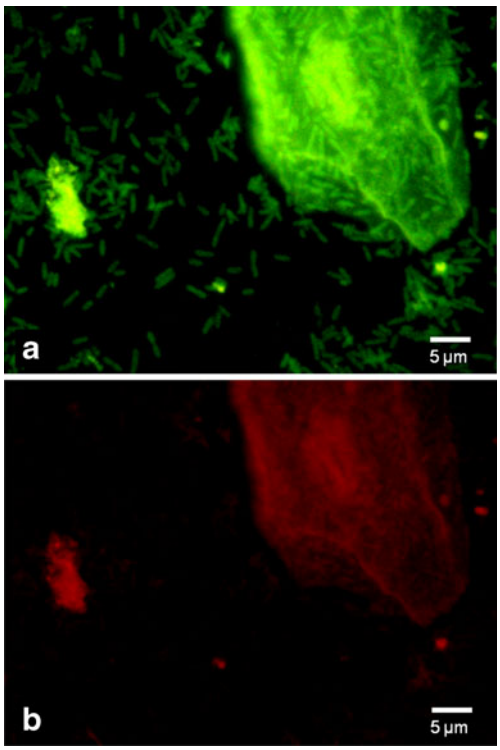

Fig. 2 Detection of *Proteus* in a real urine sample using the ProPNA190 probe. **a** Image at the green filter capable of detecting the Alexa 488 (BP 470–490, FT 500, LP 516 for LmPNA1253). **b** Image at the red filter (BP 530–550, FT 570, LP 591)

After the probe testing the PNA FISH was adapted for the detection of UTIs caused by *Proteus* spp. in urine samples. Currently, urinalysis and the detection of nitrites in urine are the most used UTI diagnosis assays [26]. However, they are not able to identify the pathogen. The only assay capable of this and of establishing a definitive diagnosis of UTI is urine culture, a method pioneered by Koch and Petri in the 1880s, and refined during the last several decades. The latest urine culture technology allows the identification of various organisms in a single test since various pathogens grown on a chromogenic medium produce a color change that is species specific. However these tests usually require 24–48 h, meaning that clinicians need to start the empirical treatment before having urine culture result [5, 6]. An 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 [26, 27]; although, for catheterized individuals this value can be lower (~10^4 CFU/ml) [28]. Therefore, as *Proteus* is associated with the infection of catheterized patients [3, 4], a threshold of 1×10^4 CFU/mL was defined. Using the PNA-FISH method described here, we were able to detect as low as 1×10^4 CFU/mL in approximately 2 h, with similar accuracy (sensitivity of 100 % and specificity of 98 %) to the standard culture method (Chromagar Orientation).

Several researchers have been working on the development of new techniques to reduce the time and cost of urine culture processing. However, while some methods, such as PCR, are unable to distinguish between clinically significant or non-significant bacteriuria, others like the use of chromogenic

Table 2 PNA-FISH and CHROMagar results for *Proteus* presence in real urine samples. The presence of *Proteus* in CHROMagar was considered only for concentrations higher than 1×10^4 CFU/ml of urine

Measurement		CHROMagar		
		Absence	Presence	Total
PNA-FISH	Positive	2	21	23
	Negative	114	0	114
	Total	116	21	137

media are limited by the slow growth, or inability to grow, of some microorganisms [6, 9, 29].

The protocol presented in this work has been demonstrated to be a reliable alternative to the currently used culture-based techniques. It also presented an appropriate level of sensitivity for detection of clinically relevant concentrations of bacteria in urine. The method is also likely to be adapted for a multiplex detection of other common urinary pathogens, as previously performed for foodborne pathogens [30].

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Conflict of interest The authors declare that they have no conflict of interest.

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