RESEARCH PAPER

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PNA-based microbial pathogen identification and resistance marker detection

An accurate, isothermal rapid assay based on genome-specific features

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With the rapidly growing availability of the entire genome sequences of microbial pathogens, there is unmet need for increasingly sensitive systems to monitor the gene-specific markers for diagnosis of bacteremia that enables an earlier detection of causative agent and determination of drug resistance. To address these challenges, a novel FISH-type genomic sequence-based molecular technique is proposed that can identify bacteria and simultaneously detect antibiotic resistance markers for rapid and accurate testing of pathogens. The approach is based on a synergistic combination of advanced Peptide Nucleic Acid (PNA)-based technology and signal-enhancing Rolling Circle Amplification (RCA) reaction to achieve a highly specific and sensitive assay. A specific PNA-DNA construct serves as an exceedingly selective and very effective biomarker, while RCA enhances detection sensitivity and provide with a highly multiplexed assay system. Distinct-color fluorescent decorator probes are used to identify about 20-nucleotide-long signature sequences in bacterial genomic DNA and/or key genetic markers of drug resistance in order to identify and characterize various pathogens. The technique's potential and its utility for clinical diagnostics are illustrated by identification of *S. aureus* with simultaneous discrimination of methicillin-sensitive (MSSA) versus methicillin-resistant (MRSA) strains. Overall these promising results hint to the adoption of PNA-based rapid sensitive detection for diagnosis of other clinically relevant organisms. Thereby, new assay enables significantly earlier administration of appropriate antimicrobial therapy and may, thus have a positive impact on the outcome of the patient.

Supplemental Material

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Supplementary Table 1. Signature sites, PNAs, circularizable ODNs, decorator probes and primer used in this work.

Bacterium	Signature sites*	PNAs**	Decorator Probe/ Circularizable Oligonucleotides / Primer***
S.aureus (MSSA and MRSA)	**SA-1:** AAAGAAAAAGCAACAAGAGGAA **SA-2:** AGAGGAAGCAGAGCGCAAGGGAAA **SA-3:** AAAAGAAGAAAGATTCAGAGGAAG	**PNA1**: H-Lys$_2$-TTTJTTTT-(eg1)$_3$-TTTTCT-Lys-NH$_2$; **PNA2**: H-TTJTJJTT-(eg1)$_3$-TTCCTCTT-Lys-NH$_2$ **PNA3**: H-Lys$_2$-TJTJJTTJ-(eg1)$_3$-CTTCCTCT-Lys-NH$_2$ **PNA4**: H-TTJJJTTT-(eg1)$_3$-TTTCCCTT-Lys-NH$_2$ **PNA5**: H-TTJTTJTTTT-(eg1)$_3$-TTTTCTTCTT-Lys-NH$_2$ **PNA3**	**RED decorator: 5'-CY3-TCACGGAATGGTTACTTGCACAGC-BIOTIN-3'** **ODN_SA1:** 5'-**p**-aacAAGAGGAA(tcacggaatggttacttgcaCAGC) CAGCAGCC(TCACggaatggttacttgcacagc)AAAGAAAAagc-3' **ODN_SA2:** 5'-**p**-gcgcAAGGGAAA(tcacggaatggttacttgcCAGC) CAGCAGCC(TCACggaatggttacttgcacagc)AGAGGAAGcaga-3' **ODN_SA3:** 5'-**p**-attcAGAGGAAG(tcacggaatggttacttgcCAGC) CAGCAGCC(TCACggaatggttacttgcacagc)AAAAGAAGAAag-3' **Primer: 5'-GTGAGGCTGCTGGCTG-3'**
S.aureus (MRSA)	**MR-1:** AAGGAGGATATTGATGAAAAAGA **MR-2:** GGAAGAAAAATATTATTTCCAAAGAAAA	**PNA6**: H-Lys$_2$-TTJJTJJT-(eg1)$_3$-TCCTCCTT-Lys-NH$_2$; **PNA7**: H-Lys-TCTTTTTC-(eg1)$_3$-JTTTTTJT-Lys$_2$-NH$_2$ **PNA10**: H-Lys$_2$-TTTJTTJJ-(eg1)$_3$-CCTTCTTT-Lys-NH$_2$; **PNA1**	**GREEN decorator: 5'-FITC-CCTCAATCGTCGTCGTGTACTAC- FITC-3'** **ODN_MR1:** 5'-**p**-gatGAAAAAGAttatCAGCCAGCAGCCTCA (Cctcaatcgtcgtcgtgtactac)tattAAGGAGGAtatt-3' **ODN_MR2:** 5'-**p**-atttccAAAGAAAAttatCAGCCAGCAGCCTCA (Cctcaatcgtcgtcgtgtactac)tattGGAAGAAAaatatta-3'
E. coli *vs* *B. subtilis*	***E. coli* rpoN:** **(PNA polymerase sigma N factor region)** GAAAGAAGATGTGCTGAAAGAAG	**PNA8**: H-Lys$_2$-JTTTJTTJ-(eg1)$_3$-CTTCTTTC-Lys-NH$_2$	**ODN_rpoN1:** 5'-**p**-gctGAAAGAAG(tcacggaatggttacttgcCAGC) CAGCAGCC(TCACggaatggttacttgcacagc)GAAAGAAGatgt-3' **ODN_rpoN2:** 5'-**p**-gctAAAAGAAG(tcacggaatggttacttgcCAGC) CAGCAGCC(TCACggaatggttacttgcacagc)GAAAGAAGatgt-3' **RED**
	***B. subtilis*:** GAAAGAAGATGTaCTaAAAGAAG	**PNA8** **PNA9**: H-Lys$_2$-TTTTJTTJ-(eg1)$_3$-CTTCTTTT-Lys-NH$_2$	**ODN_rpoN3:** 5'-**p-a**CT**a**AAAGAAGttatCAGCCAGCAGCCTCA (Cctcaatcgtcgtcgtgtactac)tattGAAAGAAGatgt-3' **GREEN**
B. subtilis	**serA:** GAAAAGAAACCCTTCAGAGGAAG **yxjA:** GGAAGAAGCGCACTAAAGAAAA	**PNA11**: H-Lys$_3$-JTTTTJTT-(eg1)$_3$-TTCTTTTC-Lys-NH$_2$ **PNA3** **PNA12**: H-Lys$_2$-JJTTJTTJ-(eg1)$_3$-CTTCTTCC-Lys-NH$_2$ **PNA1**	**ODN_serA:** 5'-**p**-ttcAGAGGAAGttatCAGCCAGCAGCCTCA (Cctcaatcgtcgtcgtgtactac)tattGAAAAGAAAccc-3' **ODN_yxjA:** 5'-**p**-cactAAAGAAAAagtCAGCCAGCAGCCTCA (Cctcaatcgtcgtcgtgtactac)taattGGAAGAAGcg-3' **GREEN**
P.aeruginosa	**PA-popB:** GGAAGAAGCCGCGAAAGAAG **(translocator protein popB gene)** **PA-clp:** AAAGAAAAGCGAGCGAGAAGAG **(clp protease gene region)** **PA-MgtC:** GAGGGAAGGGACACGAAGGGAAA **(MgtC magnesium transporter gene)**	**PNA12** **PNA8** **PNA1** **PNA13**: H-Lys$_2$-JTJTTJTJ-(eg1)$_3$-CTCTTCTC-Lys-NH$_2$ **PNA14**: H-Lys$_2$-JTJJJTTJ-(eg1)$_3$-CTTCCCTC-Lys-NH$_2$ **PNA4**	**ODN_popB:** 5'-**p**-gcGAAAGAAG(tcacggaatggttacttgcaCAGC) CAGCAGCC(TCACggaatggttacttgcacagc) GGAAGAAGcc -3' **ODN_clp:** 5'-**p**-agcGAGAAGAG(tcacggaatggttacttgcCAGC) CAGCAGCC(TCACggaatggttacttgcacagc)AAAGAAAAgcg-3' **ODN_MgtC:** 5'-**p**-acgAAGGGAAA(tcacggaatggttacttgcCAGC) CAGCAGCC(TCACggaatggttacttgcacagc)GAGGGAAGggac-3' **RED**

* underlined are PNA binding site; **for PNA notations see ref.11;

*** In circularizable probe sequences: capitalized and underlined are primer annealing sites; capitalized not underlined are sequences identical to PNA binding sites; in (...) are sites for decorator probe's hybridization.

Sequence specificity of detection (single-nucleotide mismatch discrimination).

Sequence-specificity of strand-invading homopyrimidine PNAs has been extensively studied *in vitro*, which made it possible to design optimal PD-loop sites (13). Here we report the data on PNA invasion into dsDNA within cells and the data on PNA ability of sequence discrimination while targeting dsDNA *in situ*. This process is essential because the choice of optimal signature sites and conditions for *in situ* applications is crucial to achieve high sequence-specificity necessary in order to avoid false positives and enable accurate detection of bacteria directly in clinical specimens.

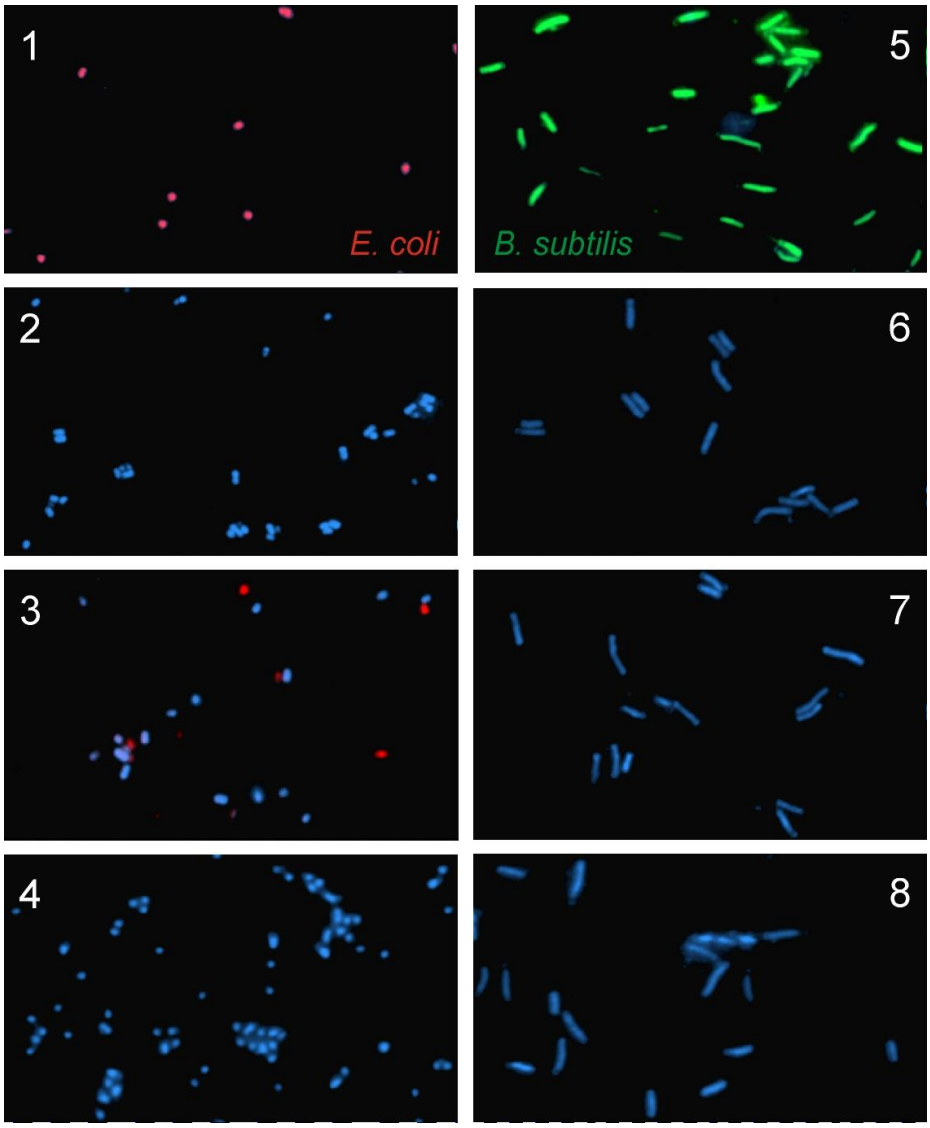
To check the tolerance of our *in situ* approach to variously positioned mismatches, we analyzed genome sequences in order to find a clear-cut case of single-mismatch difference between two strains within the PD-loop site. Based on PNA openers available in our collection, we selected a signature site within the *rpo*N gene, which differs for two genomes: *E. coli* and *B. subtilis* in two positions (see Table 1, *E. coli* vs. *B. subtilis*).

Using two bacteria and different combinations of probes shown in Table 1, we performed a series of experiments on mismatch discrimination. The results are shown on Supplementary Figure 4 and summarized in Supplementary Table 2. The observed single nucleotide mismatch discrimination demonstrates high specificity of our approach. Nearly all experiments showed no signal when mismatched probes (with mismatches in PNA or circularizable probe binding site) were used. Only in one experimental design did we observe a discordant signal, from about 20% of cells; this was when a single-nucleotide mismatch was in 4th position from the ligation point at the circularizable probe binding site (case 3) No signal was observed with two single-nucleotide mismatches (case 4). Therefore, in case a single mismatch happens farther from the ligation point, discrimination may be insufficient under our standard conditions and a small modification of the protocol may be necessary. The current protocol can be still applied in case the potential signature site is carefully checked to avoid single-nucleotide replacement located at 4th position or farther from the ligation point. Another possibility to enhance selectivity of detection may be found in simultaneous targeting of two signature sites within the chosen bacterial genome. Different sites can be detected using different fluorophores with resolvable emission bands. Such double-coincidence detection will eliminate the possibility of false positives.

Supplementary Table 2. Results of experiments on mismatch discrimination

Figure	Bacterium	PNAs	Circularizable Oligonucleotide	Type of Mismatch	Signal
1	*E. coli*	PNA8	ODNrpoN1	None (positive control)	+
2	*E. coli*	PNA9	ODNrpoN1	mismatches in both PNAs binding sites	-
3	*E. coli*	PNA8	ODNrpoN2	circular probe binding site (internal) mismatch	+/-
4	*E. coli*	PNA8	ODNrpoN3	circular probe binding site (two) mismatches	-
5	*B. subtilis*	PNA8 & PNA9	ODNrpoN3	None (positive control)	+
6	*B. subtilis*	PNA8	ODNrpoN3	mismatch in PNA binding site	-
7	*B. subtilis*	PNA9	ODNrpoN3	mismatch in PNA binding site	-
8	*B. subtilis*	PNA8 & PNA9	ODNrpoN2	circular probe binding site (end) mismatch	-

Supplementary Figure 4. Sequence specificity of detection. Images of bacterial cells observed by fluorescent microscope in experiments on mismatch discrimination, which results are summarized in Supplementary Table 2. The fluorescent signals were acquired separately using three filter sets (DAPI for DNA and Cy3 or FITC for the RCA product). Each image is a superposition of two separate images, with DAPI and Cy3 or DAPI and FITC. Signals for Cy3, DAPI and FITC were pseudocolored in blue red, blue and green, respectively.

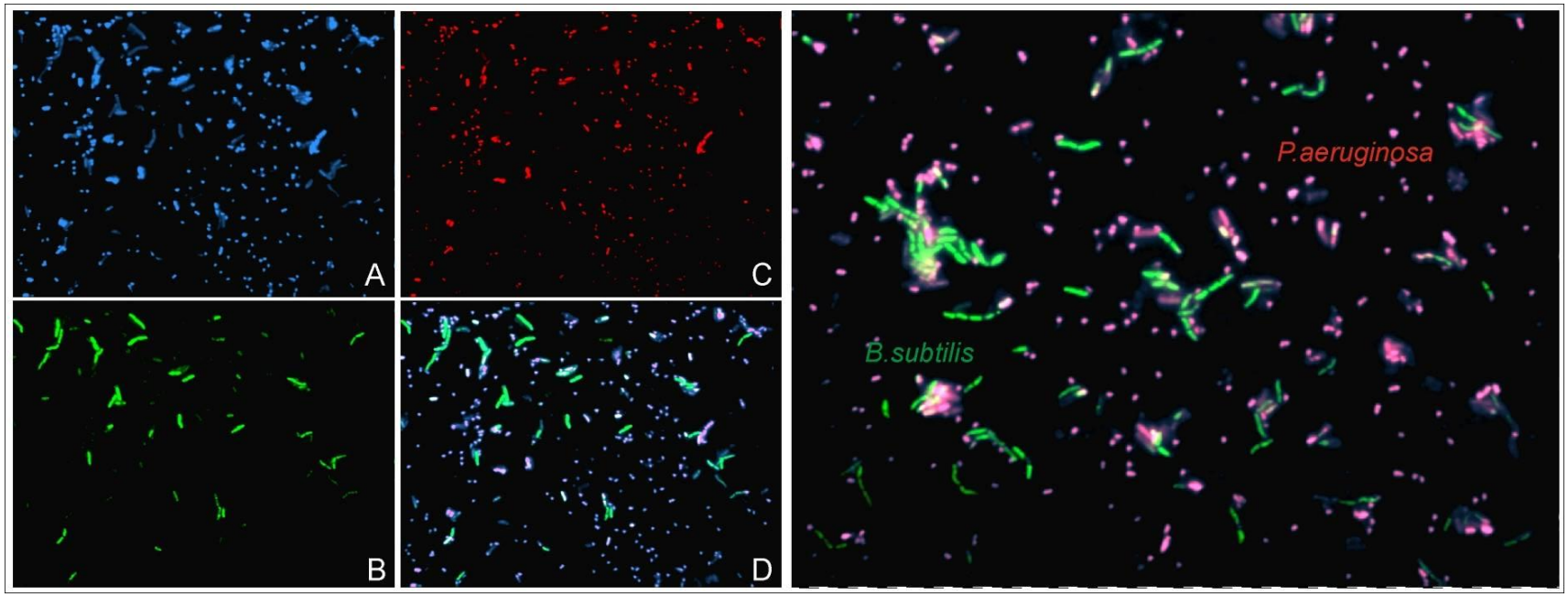


Detection and discrimination of a common, often multidrug-resistant pathogen: *P. aeruginosa*

We further validated our approach by detecting a well-known, often multidrug-resistant pathogen, *P. aeruginosa*. *P. aeruginosa* is a major cause of morbidity and mortality due to healthcare-associated infections. Since the outer membrane of *P. aeruginosa* is less permeable than of other Gram-negative bacteria, it is intrinsically resistant to many antimicrobials. This outer membrane therefore presents a potential technical challenge since our protocol depends on PNA and RCA molecules being able to easily enter bacterial cells. Based on genomic sequences of two *P. aeruginosa* strains that were available in Bacteria Genomes Database and the availability of PNAs in the laboratory, we chose three unique target sites specific for different gene regions (Table 1). . Then we examined whether we are able to reliably distinguish *P. aeruginosa* from other species using the selected signature sites. For reference species we used bacteria and signature sites previously studied while developing our approach (6, 7).

Supplementary Figure 5 shows typical results obtained with *P. aeruginosa* and *B. subtilis* when any combination of chosen sites for each bacterium was used for detection. *P. aeruginosa* and *B. subtilis* are morphologically distinguishable and nearly all cells in the mixture displayed red or green specific signals only. No signal was observed in control experiments when probes specific for *S. aureus* were applied (not shown). These data demonstrate high specificity of detection by our approach and they support expectations that our approach can be developed for simultaneous detection of various bacterial pathogens in clinical samples as long as appropriate sets of probes are applied.

Supplementary Figure 5. Detection and discrimination of a multidrug-resistant pathogen: *P. aeruginosa.* Images of mixture of *P.aeruginosa* and *B.subtilis* bacterial cells observed by fluorescent microscope in experiments performed directly in blood culture when mixture of probes popB specific for *P. aeruginosa* and yxjA specific for *B. subtilis* were applied. The fluorescent signals were acquired separately using three filter sets. Images are superposition of two/three separate images, with: **(A)** DAPI; **(B)** FITC; **(C)** CY3; **(D)** DAPI, CY3 and FITC. Signals are pseudocolored in blue for DAPI, red for CY3 and green for FITC, respectively. *Image in right*: Simultaneous detection of two bacteria in blood by the proposed approach.


A
C
B
D
P.aeruginosa
B.subtilis