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Rapid time-resolved luminescence based screening of bacteria in urine with luminescence modulating biosensing phages

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A B S T R A C T

Urinary tract infections (UTIs) are a common problem worldwide. The most prevalent causative pathogen of UTI is *Escherichia coli*, focus of this study. The current golden standard for detecting UTI is bacterial culture, creating a major workload for hospital laboratories - cost-effective and rapid mass screening of patient samples is needed. Here we present an alternative approach to screen patient samples with a single-step assay utilising time-resolved luminescence and luminescence modulating biosensing phages. Filamentous phage M13 was biopanned for binding luminescence quenching metal (copper) and further *E. coli*. The screening assay luminescence modulation was further enhanced by selecting right chemical environment for the functioning phage clones. Semi-specific interaction between phage, target bacteria and metal was detected by modulation in the signal of a weakly chelating, easily quenchable lanthanide complex. In the presence of the target pathogen, the phages collected quenching metal from solution to the bacterial surface changing the quenching effect on the lanthanide label and thus modulating the signal. Our method was compared with the bacterial culture data obtained from 70 patient samples. The developed proof-of-principle screening assay showed sensitivity and a specificity at the 90% mark when compared to culture method although some samples had high turbidity and even blood. The detection limit of *E. coli* was in the range of 1000–10 000 colony forming units/mL. Untreated urine sample was screened and time-resolved luminescence signal result was achieved within 10 min in a single incubation step.

1. Introduction

The diagnosis of urinary tract infection (UTI) is based on the clinical symptoms and determination of a causative pathogen. The golden standard for detection and identification of urinary tract pathogen is culture, which enables estimation of the amount of known uropathogenic bacteria in urine [1]. The most common UTI causing pathogen is *Escherichia coli* [2]. Other commonly found pathogen species include *Klebsiella* species, *Proteus mirabilis*, other *Enterobacteriaceae, Enterococcus* species, *Pseudomonas aeruginosa*, and *Staphylococcus saprophyticus* [3]. Growth of 10^5 colony forming units (cfu)/mL is the most commonly used cutoff for significant bacteriuria, but depending on the isolated bacteria, patient's symptoms and sampling technique it may be significantly lower [4]. Even with modern media and techniques, urinary cultures create a considerable workload for hospital laboratories. UTIs are very common and therefore also lead to substantial amount of antibiotic prescriptions, some of which could be avoided by faster diagnosis [5–7].

Rapid urine tests for UTIs have been developed with aim to reduce laboratory workload. Many of them are based on detecting chemical changes in urine. Leukocyte esterase and nitrite dipstick tests are the most commonly used. Leukocyte esterase leaks from white blood cells to the urine and nitrite is produced mainly from gram-negative bacteria. These two parameters are relatively good indicators when used at the same time. It is recommended that dipstick test results should be considered positive if either parameter was positive [8]. However, current diagnostic guidelines state that nitrite and leukocyte esterase tests are unable to eliminate the possibility of bacterial infection [4].

Flow cytometry is a convenient method for separating particles in different liquids. In case of urine samples it can detect bacteria and different blood cell types, most importantly leucocytes [9]. Cytometry alone or used with results of dipsticks does not, however, give accurate prediction of UTI With lower cut-offs (10^4 or even 10^3 CFU/mL) which are applied on primary uropathogens, (especially *E. coli*) and certain symptomatic patient groups, the percentage of false-negatives may become high with this method, especially if only bacterial counts are considered [10]. However, with locally validated cutoffs flow cytometry can be used to rule out UTI and reduce the number of samples to be cultivated by up to 50% and thus workload in the lab, as well as shorten the time to a negative result [10,12].

Compared to culture methods, PCR based pathogen identification may be considerably faster. PCR methods detecting several different targets (multiplex) can reach very high sensitivity [13,14]. For certain pathogens the method is also capable of detecting antibiotic resistance genes which is important for administration of the right treatment for

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the disease. Due to the high variety of possible pathogens and antimicrobial resistance patterns and lack of a suitable high-throughput instruments, PCR has thus far not become a practical alternative to bacterial culture in clinical laboratories [15,16].

Phages have been applied as biosensors in several application areas. In detection of microbes they offer several potential advantages: 1) They can be affinity selected against myriad of different receptors and targets [17] - phage biorecognition layers can bind biological targets with high affinity and specificity. 2) Phage-derived probes are readily applicable to many detection platforms [18]. 3) They are inexpensive and resistant to harsh conditions in which antibodies start losing function [19].

Especially filamentous phages are widely used in biosensor applications. For example, phages were immobilized onto magnetoelastic resonator that lengthens and shortens under an external magnetic field. This system was first reported successful of detecting *Bacillus anthracis* spores and then *S. typhimurium* bacteria [20,21]. Surface plasmon resonance (SPR)-based biosensors have also been used as phages can be immobilized on SPR sensor chips or the target bacteria for phage is immobilized on the chip [22–24]. Metals and phages have been used in other systems including Raman applications [25]. Srivastava et al. reported a Surface-enhanced Raman spectroscopy (SERS) nanobiosensor that utilizes thin silver films on a silicon platform along with phages. The T4 phage was immobilized on the film and used to recognize *E. coli* cells. The system was reported to sense as low concentrations as 150 cfu/mL [26]. Another group of phage detection systems relies on infecting and lysing the target bacteria. The lysis is then measured for instance by bioluminescence that is enhanced by released ATP from the bacterial cell [27,28]. Highly specific lysis releases also intracellular enzymes that can be detected amperometrically [29].

In our previous work, we developed a rapid assay for detecting *E. coli*. It was based on lytic phage and bacterial lysis which can be detected via environmentally sensitive lanthanide label. The sensitive label interacts with released molecules from lysed cells [30]. However, specific infection and lysis is a time consuming process and phage has to be specifically lytic for each bacterial species. As a broader concept, sensitive lanthanide labels have shown to be effective in liquid fingerprinting technology. The method is based on different nonspecific interactions of the liquid sample and its components [31].

In this article we show that affinity selected filamentous phages and their peptides can be readily used to assemble a biosensing system that can be harnessed to detect pathogenic *E. coli* from urine samples. In this system the phage binding to target microbe does not involve process of infection and lysis of the bacterial cell but faster semi-specific binding provided by phages bearing peptides. In this assay a signal in response to the concentration of *E. coli* present is generated by the use of bifunctional phage, environmentally sensitive luminescent lanthanide label, quenching metal and controlled chemical environment to modulate the reaction/luminescence.

2. Materials and methods

2.1. Samples

Seventy clinical samples were analyzed and cultured at Clinical Microbiology Laboratory of Turku University Central Hospital. Samples were collected in vacutainer Plus C&S Boric Acid Sodium Borate/Formate tubes (Becton Dickinson). Clearly bloody or smear samples were not excluded from analysis. The urine samples were stored at 4 °C. Ethical approval for using the patient samples was not required for the reason that the study was considered as a basic laboratory screening method development and no additional patient information was collected (Table 1).

Table 1
Microorganisms identified in 70 urine cultures.

Microorganism	CFU Count/mL	No. Of Cases
Escherichia coli	$\geq 10^3$	29
negative	–	14
Klebsiella pneumoniae	$\geq 10^3$	5
Enterococcus faecalis	$\geq 10^5$	4
Citrobacter freundii	$\geq 10^5$	3
Proteus mirabilis	$\geq 10^4$	2
Citrobacter koseri	$\geq 10^3$	2
Pseudomonas aeruginosa	$\geq 10^4$	2
enterococcus (non-faecalis species)	$\geq 10^3$	2
Hafnia alvei	$\geq 10^5$	1
Streptococcus agalactiae	$\geq 10^5$	1
Pseudomonas putida	$\geq 10^5$	1
Staphylococcus saprophyticus	$\geq 10^5$	1
Staphylococcus hominis	$\geq 10^5$	1
Raoultella ornithinolytica	$\geq 10^3$	1

2.2. Materials and reagents

Yeast-Tryptone (YT) medium was made of mixing 16 g of tryptone, 10 g of yeast extract and 5 g of NaCl to 1 L of MQ water. Diethyl malonate, Bovine Serum Albumin (BSA), Europium (III) chloride hexahydrate, 2,3-Dichloro-5,6-dicyano-*p*-benzoquinone and (TOPO) tri-*n*-octyl-phosphine oxide were purchased from Sigma-Aldrich. Triisopropylsilane was purchased from Fluka, Buchs, Switzerland and (NTA) 4,4,4-trifluoro-1-(2-naphthalenyl)-1,3-butanedione from Acros Organics. Dimethyl sulfoxide, analytical reagent grade (DMSO) was purchased from Thermo Fisher Scientific, MA, USA. The bacterial reference strains used in the development of the assay were: *Staphylococcus aureus* ATCC 25 923 and *E. coli* strain B ATCC 11 303 as a reference of wild type *E. coli* because it has no F plasmids that M13 phage requires for infection process.

2.3. Library screening

2.3.1. First affinity screening procedure for copper

Copper beads (Sigma Aldrich 254 177, 2–8 mm) were rinsed with distilled water and autoclaved 120 °C for 60 min before the biopanning experiments. The selected biopanning system, The Ph.D.-12 phage display peptide library (E8110S) was supplied by New England Biolabs (NEB). The library contains 1.5×10^{13} plaque-forming units (pfu)/ml and it has complexity of 2×10^9 independent peptide sequences. Each phage contains five copies of the minor coat protein pIII, and each copy of pIII has a single peptide displayed at its *N*-terminus. Suitable strain *E. coli* ER2738 containing the F + Δ(lacZ)M15plasmid (New England Biolabs) was used to amplify the eluted phage. LB medium was used to culture *E. coli*. To prevent contaminating phage from the environment, eluted phage were plated on LB agar plates containing 60 μg/ml isopropylβ-D-thiogalactoside (IPTG) and 40 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal). Biopanning was performed following a modified protocol from New England Biolabs. An 10 μl aliquot of the random peptide library was incubated with a copper beads at RT for 30 min with gentle shaking in a microcentrifuge tube containing 1 mL of physiological saline. Unbound phage from metal beads was washed serially with 4 mL of TBSTbuffer [0.1% (v/v) Tween-20 in TBS (50 mM Tris-HCl, pH7.5, 150 mM NaCl)]. After the 15 washes, the bound phage was eluted with 1 mL of 0.2 M glycine-HCl (pH 2.2). In each round, the bound phages were rescued and amplified using *E. coli*ER2738 to make more copies. These were used in a second round of biopanning. After three rounds of biopanning (three rounds of 15 washes) the bound phages were harvested for binding analysis.

2.3.2. Second affinity screening procedure for E.coli B

Enriched phages from previous screening were used for biopanning

experiments against *E. coli* B. The panning procedure was performed according to the following protocol: *E. coli* B cells were grown in tryptic soy broth (TSB; Sigma-Aldrich) medium at 37 °C. The growth medium was centrifuged and the pellet washed twice with 1 mL of 4 °C PBST (Phosphate Buffered Saline with Tween 20). The random peptide library (1.5×10^{11} plaque-forming units) was mixed with washed cells and incubated in 1% BSA (Bovine serum albumin) with the washed infectious *E. coli* B cells and left on ice for 1 h. After this phage bound cells were washed twice with 1 mL PBST and three times 1 mL PBS. Washed cells were mixed with 1 mL of phage propagating *E. coli* ER2738 (1.0×10^9 cells/mL) in 2 x YT medium. Before mixing *E. coli* ER2738 cells were grown in intense shaking (250 rpm) at 37 °C. The mixed culture of *E. coli* ER2738 and *E. coli B* was incubated for 30 min at 37 °C without shaking and following an incubation period of 30 min with gentle shaking (100 rpm). The enrichment of phages was made according to the manufacturer's protocol (NEB). After third affinity selection phages were ready for to be used in assays.

2.3.3. Copper binding experiments

Tested copper chloride concentration (0–200 mM) in MQ was added in 100 µL volume to 96 well plate. Then each well was added 10^{12} pfu/mL M13 wild-type (wt-m13) phage or copper selected phage in volume of 10 µl. Finally 4 µl of 0.1 mM europium chloride, 0.06 mM NTA and 0.06 mM TOPO was added to the microtiter wells. After 10 min of incubation, delayed luminescence emission intensities were measured in a 400 µs window after a 400 µs delay time using a Victor 2 multilabel counter (Wallac, Perkin-Elmer Life and Analytical Sciences).

2.3.4. Assay for E.coli B and comparison with S.aureus

A 96 well plate was filled with 100 µL of varying concentrations (0-10^6 cfu/mL) of *E. coli* in physiological saline and after this 8 µL of 20 µM copper chloride in MQ was added. Next each well was added 10^{13} pfu/mL wt-m13 phage or copper/*E. coli* B selected phage in volume of 10 µl. Finally 4 µl of 0.1 mM europium chloride, 0.06 mM NTA and 0.06 mM TOPO was added to the microtiter wells. After 10 min of incubation, modulated delayed europium luminescence emission intensities were measured again in a 400 µs window after a 400 µs delay time using a Victor 2 multilabel counter. Comparison with *S. aureus* and *E. coli* was done with the same protocol but only one concentration of 1 µM copper chloride was used. Finally 4 µl of 0.1 mM europium chloride, 0.06 mM NTA and 0.06 mM TOPO was added to the microtiter wells. After 10 min of incubation, luminescence emission intensities were measured in a 400 µs window after a 400 µs delay time using a Victor 2 multilabel counter.

2.3.5. Screening clinical urine samples

A 96 well plate was filled with 100 µL of urine sample and right after this, 10 µl of 10^{13} pfu/mL M13-wt phages or copper/*E. coli* B selected phages and 8 µL of 20 µM copper chloride in MQ was added. Next 4 µl one of three additional chemicals in DMSO were added (600 mM of Diethyl malonate, 50 mM of 2,3-Dichloro-5,6-dicyano-*p*-benzoquinone or 600 mM Triisopropylsilane). Finally 4 µl of 0.1 mM Europium chloride, 0.06 mM NTA and 0.06 mM TOPO was added to the microtiter wells. After 10 min of incubation, luminescence emission intensities were measured in a 400 µs window after a 400 µs delay time using a Victor 2 multilabel counter.

2.4. Statistical analysis

The samples were analyzed in three chemical environments and with two different phage types: *E. coli* B/copper specific phage and reference phage. In the analysis the signal from time point 0 min was compared with that of 10 min. The aim was to obtain and evaluate the statistical difference in signal between *E. coli* and other samples. These include 14 other bacterial species representing both known uropathogens and normal microbiota of the urogenital area, and negative samples. High signal difference between specific and reference phage indicated positive result for *E. coli* in urine sample, whereas small signal difference indicated the negative result for *E. coli*. For screening assay method demonstration, all selected three chemistries were used as a "fingerprint" of the sample. The used classification method was K-Nearest Neighbor (KNN), and the averages from each of the class, contaminated with random noise of amplitude equivalent to the noise in the real data, were used in teaching of the algorithm. The KNN analysis was performed with Molegro Data Modeler (Version 2.1), and all plotting and statistics with Prism 6.0 g.

3. Results and discussion

In this study, we presented the development of a phage-based biosensing assay capable of detecting *E. coli* from urine samples. The detection is based on unstable lanthanide label that is sensitive to chemical environment and especially to metals that are able to quench the label. The phage in turn interacts both with the metal and the target bacteria. The phage acts as a modulating factor to the achieved time-resolved fluorescence signal. We chose copper as it is well known to quench the fluorescence of lanthanide complexes like Eu^{3+}:TTA:TOPO [32].

First step of developing biosensing system consists of selecting copper binders from phage library (Fig. 2.). Phages from previous screening were used in the second screening against *E. coli* B. The used filamentous phage M13 is not capable of infecting this *E. coli* strain. Phage clones were tested with sensitive lanthanide label in the presence of *E. coli* B and copper chloride. From tested 24 clones the best interacting phage clone was enriched. The function of the chosen clone was further tested with 80 different chemical environments to screen

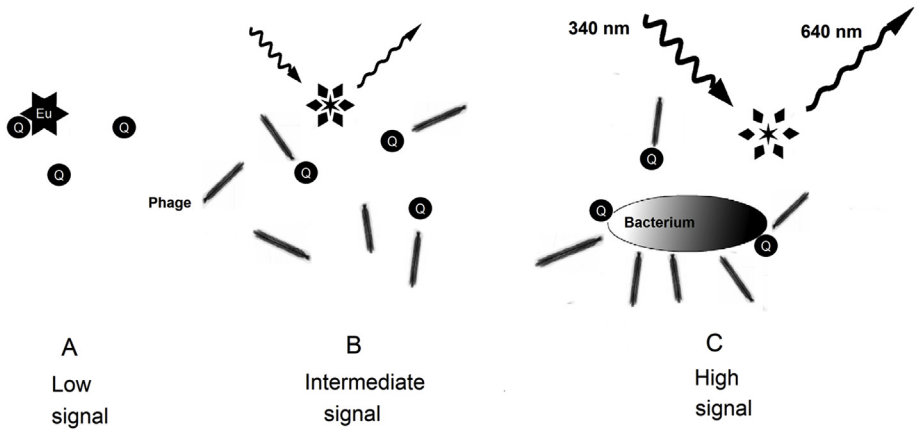

Fig. 1. The principle of biosensing screening system with phages. Q illustrates a potential explanation for quenching copper metal – label interaction. (A) Without phages the metal freely quenches europium signal, that is detected via time-resolved fluorescence (TRF). (B) Higher signal is gained when metal binding phages are moving randomly in the solution and capturing copper. (C) In case of target bacteria, phages attach to it and further interact with copper. Phages organized in the vicinity of bacteria cause high TRF-signal.

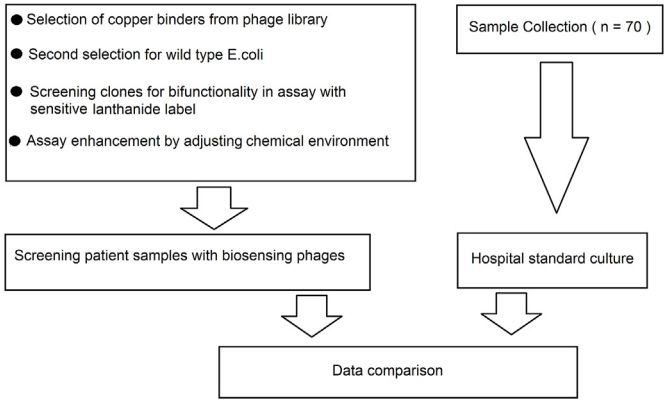

Fig. 2. Outline of the creation of the biosensing phage system. The developed method was then compared to validated culture method.

possible enhancement of the biosensing capability (Supplementary Table S1). Finally a panel of 70 urine samples were analyzed with the developed system. Ultimately the accomplished data was compared with the standard urine culture data which is a golden standard method in the laboratory diagnosis of UTI.

In order to confirm that affinity selected phage binds to copper we isolated the clones and these were tested in series of different copper chloride concentrations. A comparison was made between wild-type phage and copper/*E. coli* B selected phage in the presence of used lanthanide label Eu^{3+}:TTA:TOPO (Fig. 3). The protocol was first tested with different copper chloride concentrations. The relative fluorescence signal was higher with copper selective phages than wild type phages. This indicates that under these conditions, copper selective phages seem to protect lanthanide label from quenching. Wild type M13 phage

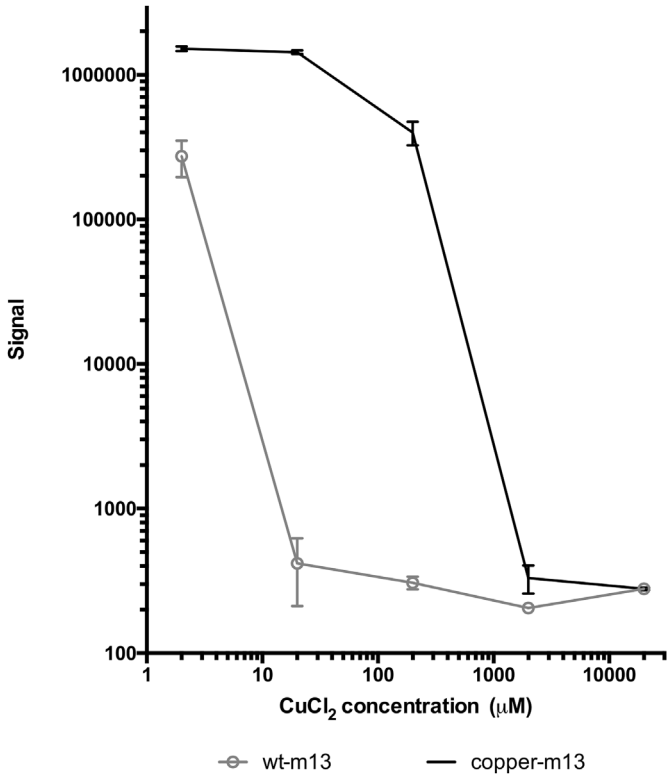

Fig. 3. Evaluation of copper binding by phage assay. Both wt-m13 and copper-m13 concentration was 10^{12} pfu/mL. Among phages the assay reaction also comprised lanthanide label and varying amounts of copper chloride (0–20 000 μM). Results were obtained after 10 min incubation.

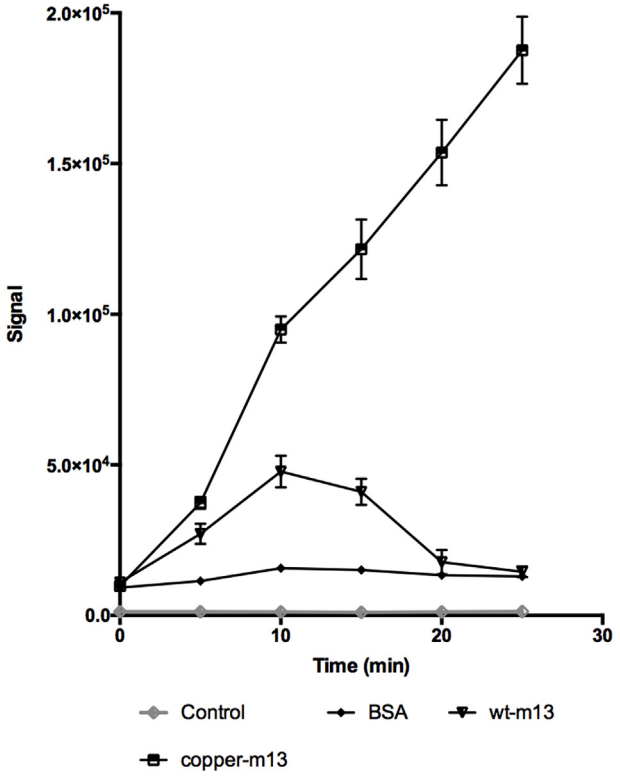

Fig. 4. Kinetic comparison of copper/*E. coli* B specific m13, wt-m13 and 0.5% BSA.

was used throughout the experiments as a control because it was purified with exactly the same procedure as copper/*E. coli* B specific phage. This reduces the possibility that the assay measures irrelevant parameters.

To gain further insight into the activity of copper binding phage a kinetic comparison was made (Fig. 4.). The aim was to observe how much different assay compositions protect from the quenching effect of 1 μM copper chloride solution. After 10 min copper specific phage continued to protect label from quenching while in the cases of BSA, wt-m13, and control the relative signal started to decline. BSA is known to bind nonspecific binding sites and metals [33]. Control phage and its random sequence was not assumed to interfere with copper and results indicate the same. Figs. 3 and 4 show clearly that S/N continues to increase after 10 min. Ten minutes incubation time was chosen because patient samples have a relatively wide range of pH differences and unknown interfering factors. Longer incubation time causes some samples to have almost completely quenched luminescence signal.

As a step toward biosensing system confirmed copper binding phages were subsequently biopanned against *E. coli* B. The main idea was to find clones interacting with both quenching copper and *E. coli*. Fig. 5 Shows an example how copper/*E. coli* B binding phage gives a different signal when compared with wt-m13 in same concentrations (> 10 000 cfu/mL) of *E. coli* B and 1 μM copper chloride concentration.

Each M13 phage has 5 copies of minor coat protein at the one end of the phage. These are binding sites for copper. In each mL there are 10^{12} phage particles and number of copper ions per mL is 10^{15}. Considering these parameters, the protection from quenching is not completely due to copper-specific binding sites in minor coat proteins. To some extent the phage particle itself could provide protection for the used lanthanide label.

The assay cross-reactivity was evaluated by comparing *E. coli* and *S. aureus*. The difference between wt-m13 and specific phage is 58% points in case of *E. coli* and 11% points in case of S. aureus. This preliminary analysis showed that phages interacted less with *S. aureus* and

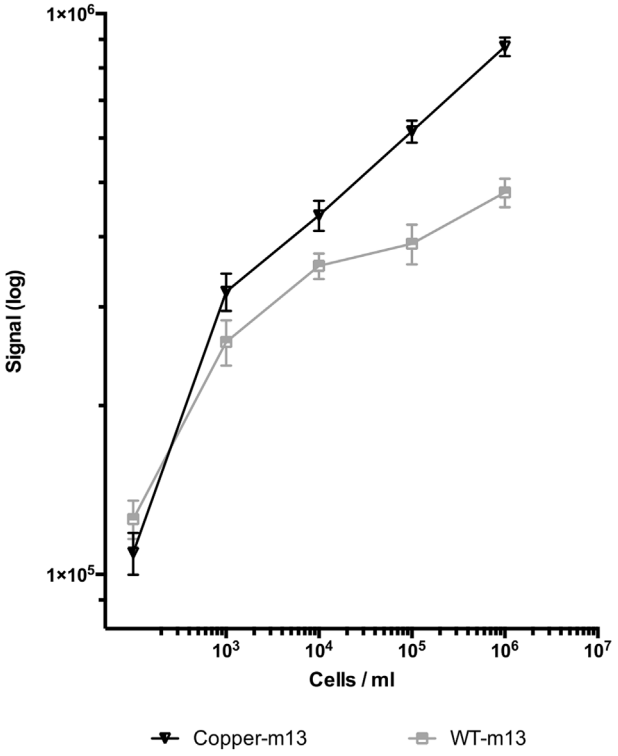

Fig. 5. Comparison of wt-m13 and copper-m13 in various (0–1000 000) *E. coli* B concentrations. Measurement made after 10 min of incubation.

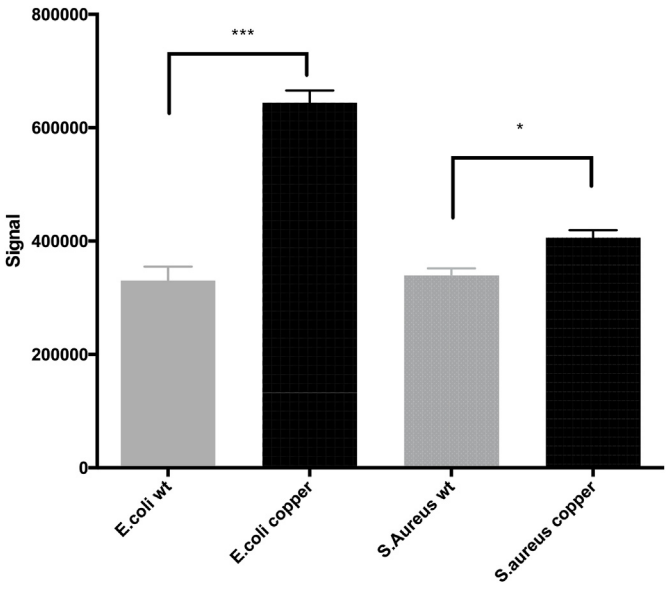

Fig. 6. Observed time-resolved signal with two different bacterial species. Copper chloride, europium-label, wt-M13 or specific phage was added to the samples. The measurement was made after 10 min of incubation in RT. Bacterial cell concentration was 10 000 cells/mL.

the assay had more specificity towards *E. coli* (Fig. 6).

We chose to analyse 70 patient samples for a proof-of-concept demonstration of our application. Fig. 7 Presents screening of the samples in three chemical environments. Each sample was divided in three 100 µl replicates for wild type reference phage and specific phage. For the reason that three chemistries or assay chemical environments was utilized, the obtained data was analyzed with K-Nearest Neighbor (KNN) classification method. Since the response differed between these chemistries (increase/decrease/magnitude) we assumed each of the

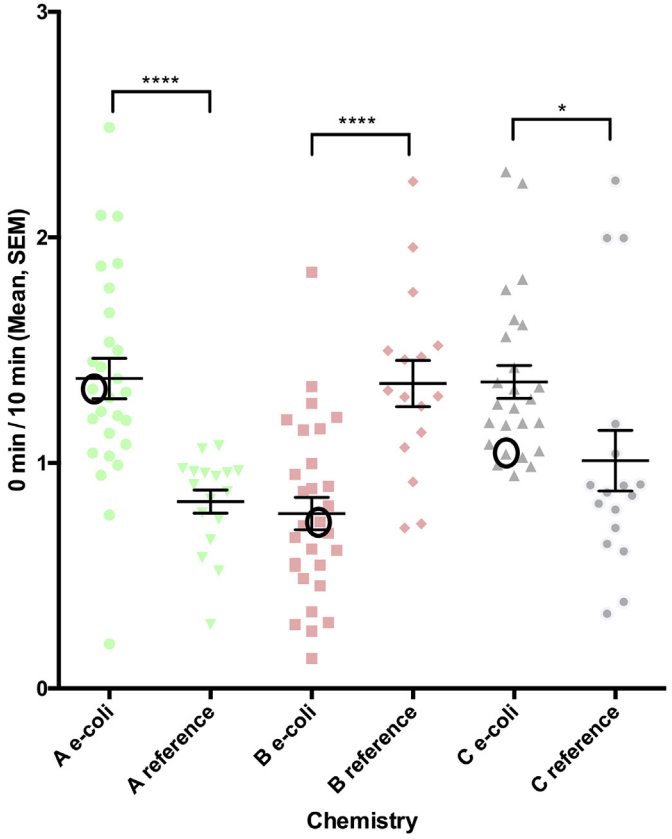

Fig. 7. Screening of patient samples with the developed method. Used chemistries: (A) diethyl malonate, (B) 2,3-Dichloro-5,6-dicyano-*p*-benzoquinone, (C) triisopropylsilane. Urine test results 10 min, normalized with 0 min sample (ratio of specific and nonspecific phage). The circled points are of a single sample that would have been misclassified by one chemistry (C) but was correctly classified by KNN and the two other chemistries.

three to reveal varying properties of the sample-label interaction rather than to be simple repeats of the same measurement. This assumption was also supported by the fact that using the three different chemistry results instead of the repeats improved the classification precision. The KNN method was taught by using artificial random noise contaminated averages for each of the categories. The algorithm classified *E. coli* from sample data with the output of 90% sensitivity and specificity. This is a competitive result when compared with other rapid screening methods for urine [8–14]. Comparing the categories in Fig. 7, it is evident, that although the differences between reference and *E. coli* patient samples was in all cases significant, using a simple cut-off value would have not reached the same 90% mark as we reached with fingerprinting and the K-Nearest neighbor algorithm.

Our simplified explanation for reaction (Fig. 1) indicates that phages provide protection for the used label. Nevertheless, the three used chemistries modulated signal differently as seen in Fig. 7. This could be explained by added chemical capturing copper, protecting the label or interfering assay chemical environment in a way that increases the performance of the screening assay. Probably the resulting TRF-signal is a sum of multiple non-specific interactions in a liquid sample.

Out of analyzed panel of samples, five had turbidity or reddish colour. This is likely due to red blood cells leaking from urinary tract system and precipitation of salts. All these samples were correctly classified by KNN algorithm. Healthy individuals' urine pH is between 5.5 and 6.5. Lower pH causes e.g. precipitation of calcium phosphate [34]. In general, disease state changes urine composition as it often is in the case of UTI infection. For instance UTI is associated to increased nitrite concentration [7]. Furthermore urine consists of many acids like

oxalic acid (range: 1–30 mg/L) and citric acid (range: 90–930 mg/L) that has potency to influence negatively the used lanthanide label and so forth the assay [35].

4. Conclusions

E.coli is by far the most prevalent causative agent in urinary tract infection and therefore was chosen as a model bacteria to demonstrate this proof-of-concept study. We demonstrated that our alternative method has feasibility for screening UTI samples with good performance characteristics. The assay sensitivity and specificity reached the same level as current flow cytometric and dipstick methods. The limit of detection was in the range of clinical level (< 10 000 cfu/mL). In addition, results were obtained in 10 min from the time reaction was started. Rapid and reliable detection of the most important pathogen(s) would be very helpful to positively predict UTI, and could help guide the treatment. Urine samples were not treated before screening them. Nonetheless, this didn't cause interference to the method and clear cross-reactivity to other bacterial species was not seen. One of the main problems is the variation of pH among samples. This requires studies of different buffering options. Secondly, sample blood and its iron content has potency to interfere the screening assay. In this work the binding site and interacting part of phage was minor coat protein pIII. M13 has only 5 of pIII binding sites for copper. Instead, pVIII has 2700 copies per M13 phage. It would likely capture more quencher but other important characteristics might change. The use of pVIII would allow not only to have more surface to interact with the target but also to reduce the amount of phage particles needed in the assay. In the future, the bifunctional nature of the phage peptides should be examined.

Furthermore, phage affinity towards intestinal commensal *E. coli* or uropathogenic *E. coli* should be studied. Phage peptide interactions could be elucidated by sequencing the DNA of the binding region. The aim is to interact both the target and the quencher. Improper balance of binding is likely to influence the screening of the target. Finally, the target range of the biosensing screening assay should be investigated. Even smaller compound recognition may be achievable by adjusting peptide, choosing right quencher and chemical environments.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ab.2019.01.011.

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