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EERIK JÕGI

Development and Applications of *E. coli* Immunosensor

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Development and Applications of *E. coli* Immunosensor

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Institute of Chemistry, Faculty of Science and Technology, University of Tartu, Estonia

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LIST OF ORIGINAL PUBLICATIONS

The current thesis is based on three original research papers, which are referred to in the text by Roman numerals I–III.

I. Eerik Jõgi, Ingrid Väling & Toonika Rinken (2020) Assessment of bathing water quality with an *E. coli* immunosensor, International Journal of Environmental Analytical Chemistry, 1–12, DOI: 10.1080/03067319.2020.1786549

II. Eerik Jõgi, Ingrid Väling & Toonika Rinken (2022) The assessment of coli index with *E. coli* immunosensor in natural water, International Journal of Environmental Science and Technology, DOI: 10.1007/ s13762-022-04280-y

III. Merit Nikopensius, Eerik Jõgi & Toonika Rinken (2021) Determination of Uropathogenic *Escherichia coli* in Urine by an Immunobiosensor Based Upon Antigen-Antibody Biorecognition with Fluorescence Detection and Bead-Injection Analysis, Analytical Letters, 55:7, 1040–1051, DOI: 10.1080/00032719.2021.1982958

Author's contribution

Paper I: Performed the experimental work and calculations, and was responsible for the interpretation of results and preparation of the manuscript.

Paper II: Performed the experimental work and calculations, and was responsible for the interpretation of results and preparation of the manuscript.

Paper III: Performed part of experimental work and calculations and was responsible for the interpretation of results and preparation of the manuscript.

ABBREVIATIONS AND SYMBOLS

ATCC	American Type Culture Collection
BIA	bead injection analysis
BSA	bovine serum albumin
CCE	calibrator cell equivalents
CFU	colony-forming units
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
Fc	immunoglobulin crystallizable fragment
FITC	fluorescein isothiocyanate
IgG	Immunoglobulin G
K_d	dissociation constant
λ_{ex}	excitation wavelength
λ_{em}	emission wavelength
LAMP	loop–mediated isothermal amplification
LOD	limit of detection
LOQ	limit of quantification
LOV	lab-on-valve
NASBA	nucleic acid sequence-based amplification
OmpA	outer membrane protein A
PBS	phosphate buffered saline
PCR	polymerase chain reaction
S. aureus	*Staphylococcus aureus*
SDS	sodium dodecyl sulphate
SpA	*Staphylococcus aureus* cell wall protein A
spp.	species
UPEC	uropathogenic *Escherichia coli*
UTI	urinary tract infections
VBNC	viable but noncultivable

INTRODUCTION

The quality of water is among the major global problems which is usually associated with drinking water quality. However, problems related to the physical, chemical, and biological pollution of bathing water are increasing.

Biological pollution is commonly assessed using microbiology methods by identifying and quantifying microbial indicator organisms. There are several bacterial species which have been selected as indicator organisms. One of the main indicator species for water analysis is *Escherichia coli* – gram-negative, rod-shaped bacteria generally found in the guts of warm-blooded animals. The identification and enumeration methods for *E. coli* vary from specific isolation and cultivation techniques, microscopic analysis, and biochemical tests to modern molecular analysis (DNA/protein) or mass spectrometry. However, the most common method for *E. coli* enumeration is still microbiological analysis. The aforementioned methods all suffer from similar problems, such as the long analysis time and the need for special labs. So, there is an urgent need for rapid on-site *E. coli*-associated analyses.

Biosensor-based systems can serve as a good alternative for the identification and enumeration of *E. coli*, as biosensors can provide short analysis time, high specificity, and selectivity. Biosensors also offer good possibilities for automated analysis and on-site measurement. There are numerous studies about the development of *E. coli* specific biosensors. However, the studies on practical applications of *E. coli* specific biosensors for real-life sample analysis, e.g., for environmental analysis or clinical application, are scarce, and most studies are currently focused on novel technological aspects of these sensors.

The objective of this thesis was the designing and production of *an E. coli*-specific immunobiosensor, it's testing for potential applications in environmental and clinical laboratory analysis, and the validation of the biosensor results using microbiological cultivation and qPCR methods.

1. LITERATURE OVERVIEW

1.1. *E. coli*-a human symbiont, pathogen, and indicator organism

Escherichia coli (*E. coli*) was first isolated in 1885 by Theodor Escherich from the faeces of healthy individuals [1]. *E. coli* is a common intestine habitant of most warm-blooded animals. It is a Gram-negative, facultative anaerobic, and rod-shaped bacterium (2 μm long, diameter 0,2–1 μm) [2]. *E. coli* is the most numerous bacteria in the colon of the majority of mammals, and different *E. coli* strains originating from humans and animals can serve as an indicator of faecal pollution in the environment. [3].

Hundreds of different strains have been identified for *E. coli* [4], making *E. coli* the most thoroughly studied prokaryotic model organism [5]. *E. coli* is described as one of the most diverse bacterial species – only 20% of the typical *E. coli* genomic genes are shared among all strains [6]. Systematically *E. coli* belongs to the family of *Enterobacteriaceae*, which is part of the domain *Bacteria* and order *Enterobacterales*. There are over 30 genera and 120 species of *Enterobacteriaceae* [7]. In *the Enterobacteriacea*e family, there is a specific group of bacteria, defined as coliform bacteria. Coliforms are rod-shaped Gram-negative non-spore-forming and motile/non-motile bacteria.

E. coli is accepted as the most relevant faecal indicator as its species are considered the only non-reproducing faecal coliforms in the environment [8]. However, in tropical and subtropical environments, *E. coli* can survive over a month [9]. Besides *E. coli*, different microbiological indexes, like the number of total coliforms, faecal coliforms, or enterococci, have been used for the evaluation of water quality [10].

Some *E. coli* strains have a reputation as hazardous human pathogens. The clinical syndromes generated by pathogenic *E. coli* strains can be generally divided into three groups: enteric/diarrhoeal diseases, urinary tract infections (UTIs), and sepsis/meningitis [11]. The most important and well-known pathogenic *E. coli* strain is O157:H7 [12]. Uropathogenic *E. coli* (UPEC) infects the urinary tract, and it is the most common pathogen there (60–95% of urinary tract infections) [13,14].

As a gram-negative bacterium, *E. coli* has a thin peptidoglycan cell wall, which is surrounded by an outer membrane composed of up to 80% of lipopolysaccharides (LPS). On the *E. coli* outer membrane, there are also species-specific β-barrel proteins or OMPs (outer membrane proteins: OmpA, OmpX) [15]. These proteins, along with LPSs, serve as the main targets of *E. coli*-specific antibodies and aptamers. These bio-recognition elements can be both strain - or species-specific.

1.2. Detection of *E. coli*–microbiological and molecular methods

The "gold standard" for the detection of *E. coli* is microbiological analysis. The classical microbiological methods require cultivation steps of sample material and the differentiation and enumeration of colonies formed. The microbiological differentiation of *E. coli* in media depends on specific biochemical/cultivation features of species. *E. coli* strains are commonly identified by their ability to produce β-D-glucuronidase and β-D-galactosidase [16]. For the detection of these enzymes, there are different types of chromogenic mediums available, indicating the production of specific compounds. The selection of a media is dependent on the sample matrix analyzed [17]. This method for the detection of *E. coli* has a sufficient drawback, as some critical *E. coli* strains do not exhibit β-D-glucuronidase activity (for example, pathogenic *E. coli* O157:H7). However, some pathogenic *Shigella* and *Salmonella* strains have a similar β-D-glucuronidase activity [18]. So, in water samples, cytochrome c-oxidase tests [19] (*E. coli* does not contain this specific enzyme) are additionally used the confirmation the presence of *E. coli*.

In clinical lab practice, matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectroscopy is an extensively used method for bacterial pathogen identification. This method requires sample microbiological cultivation to obtain pure bacterial cultures for the correct identification of pathogens. There are also direct MALDI-TOF studies without using the cultivation step), but the identification rate of pathogens (including *E. coli*) is low [20,21].

The limit of detection (LOD) of microbiological cultivation depends on the sample size used for analysis. For water analysis, the sample size is usually 100 ml, and the LOD value of *E. coli* analysis can be as low as 1 CFU/100 ml. For urine analysis, the sample size for cultivation is 10 µl. This means that in clinical practice, the LOD value for the detection of *E. coli* in urine is around 100 CFU/ml [22].

There are two major limitations considering the microbiological analysis. First, the cultivation of targeted bacterial species (e.g., *E. coli*) assumes that most of these bacterial cells are cultivable and represent the majority of species in the sample. In addition to cultivable bacteria, samples also contain viable but noncultivable (VBNC) (persistors), which are not detectable via microbiological cultivation [23]. Other methods, like DNA/RNA detection methods or biosensors, allow to detect also non-cultivable cells. The second limitation of the microbiological analysis is the long cultivation time, as most microbiological methods require at least 18–24 h [10].

Several *E. coli* identification methods are associated with the detection of *E. coli*-specific nucleic acids (DNA/RNA) sequences. Because the amount of (*E. coli*) specific DNA (or RNA) in samples is low, most nucleic acid detection methods use the amplification step. These molecular methods can be divided into polymerase chain reaction methods (PCR) and isothermal methods,

depending on amplification types. The most popular method for *E. coli* identification and quantification is quantitative PCR (qPCR). The very high specificity of the qPCR method is secured by the primer and probe interaction with a unique target sequence. The method's sensitivity is based on iterative thermostable DNA polymerase amplification steps where the applicable PCR target sequence is doubled in every step. This gives the possibility to detect even a single target molecule of nucleic acid. qPCR requires the extraction of the targeted DNA/RNA, so the complete analysis takes 5 [24] to 12 hours [25]. The LOD value of qPCR method for the detection of *E. coli* is found to be between ~ 2 CFU/100ml [24] to 10^3 CFU/100ml [25], depending on the sample preconcentration level, but also the specific procedures used [26].

Isothermal amplification is more simple in instrumentation terms compared to qPCR, as it does not require temperature changes during nucleic acid amplification. One of the isothermal amplification methods, nucleic acid sequence-based amplification (NASBA), allows detecting *E. coli* at the level of 10 copies if we measure *E. coli clp*B gene mRNA [27] or 1.7 CFU/ml targeting 16S ribosomal RNA [28]. The main drawback of isothermal amplification is the high price of analysis due to complex RNA isolation (especially from environmental samples) and detection.

There is also an isothermal amplification method for the detection of *E. coli* DNA, loop-mediated isothermal amplification (LAMP) [29,30]. The sensitivity of the LAMP method for the detection of *E. coli* s 10 copies *of* genomic DNA per reaction, but it is not a quantitative method such as qPCR [31].

The main problem of molecular methods is the sample preparation, i.e., DNA or RNA extraction. Samples, especially the environmental ones, can contain compounds that can act as PCR inhibitors, not allowing obtaining correct results [32]. In addition, DNA/RNA amplification techniques are sensitive to possible contamination problems with non-sample nucleic acid. This aspect requires special care during sample preparation and analysis.

1.3. Biosensors for *E. coli*

Biosensors allow the identification of both cultivable and non-cultivable *E. coli* cells and significantly shorten the analysis time. There are ~ 2 500 publications about *E. coli* biosensors (Web of Science search, "*E. coli* + biosensor" 18.05.2022) because *E. coli* is used as a model organism also in biosensor studies. Most proposed biosensor platforms for the detection of bacteria are tested with *E. coli* standard strains or using *E. coli* cells as a sensing element of the biosensor [33,34]. The number of publications focusing on the detection and enumeration of *E. coli* cells is over two hundred. The majority of these studies deal with the *E. coli* biosensor development and its initial testing, and data about testing of *E. coli* biosensors in real-life samples are scarce.

E. coli-specific biosensors, like any biosensors, generate a measurable signal after a specific biorecognition of the targeted analyte [35]. One of the first *E.*

coli-specific biosensors, which was based on piezoelectric crystal, covered with Enterobacteria-specific antibody, having a relatively high LOD value of 10^6 cells/ml, was published already 30 years ago in 1992 [36]. The first attempts to develop a pathogenic *E. coli* specific biosensor were based on uropathogenic *E. coli* P-fimbria interaction with immobilized multivalent galabiose sugar and were published 2 years later [37].

The working principle of the proposed biosensors varies in a wide range – from measuring the *E. coli* specific enzyme reaction metabolites [38–42] to detecting the specific outer membrane antigenic regions [43–47]. There are also remarkable differences in the sensitivity (8 CFU/ml [44] to 6.54×10^5 CFU/ml [48] and analysis time (20 minutes [44] to 7 hours [47]) of the proposed sensors.

As mentioned above, there is little information available about testing *E. coli* biosensors in complex environmental samples. The *E. coli* content in drinking water, where the microbiological background is low, has been studied by Hesari *et al.* [38] and Wang *et al.* [47]. There are laboratory studies for the application of *E. coli* biosensors in the presence of other bacterial species like *B. subtilis* [49], *Klebsiella sp., Salmonella sp, Enterobacter sp*, *Bacillus sp* [38], and *S. enterica, S. aureus, and P. aeruginosa* [50]. In the milk matrix, the *E. coli* has been detected with a bead-injection-analysis (BIA)-based *E. coli* biosensor [44].

An essential problem of biosensors is regeneration. This aspect is often overlooked, although it is of utmost importance for biosensor applicability. The biosensor regeneration is described only for ZnO/GaAs bulk acoustic wave biosensor [49]. Single-use biosensors are based on the application of soluble reagents, like the detection of *E. coli* produced β-D-glucuronidase activity [38]; renewable microcolumns [44]; and bio-specific chips integrated with surface acoustic wave platform [48]. The selection of applying single-or multiple-use biosensors depends on the aim of analyses-reusable biosensors is commonly more complicated and expensive (e.g., using Au chips) [43], while single-use biosensors comprise more simple/inexpensive materials [48]. The main problem with biosensor regeneration is the partial non-reversibility of the bio-recognition reaction and the need for frequent recalibration. Commonly biosensors can be reused 5 [49] to 25 times without recalibration [51].

1.4. The application of *E. coli* biosensors

E. coli biosensors are used for the evaluation of microbiological safety and the major fields of their application are environmental, clinical, and veterinary analyses, but also food analyses.

1.4.1. Detection of *E. coli* in water samples

The microbiological background of environmental samples is diverse, so specific polyclonal or monoclonal antibodies should be used for the specific bio-recognition of *E. coli* with biosensors. For the detection of the immunobio-

recognition reaction, various signal transduction platforms allowing to achieve a LOD value from 10 to 220 cells/ml in water in less than 1 hour have been used in *E. coli* biosensors [51–56] (Table 1).

In environmental analysis, it is optional to concentrate samples before analysis, as the amount of the targeted analytes in the sample can be low. However, the enrichment procedure is not commonly considered in biosensors [51,53,56]. Only in some *E. coli* biosensors the time-consuming stages of pre-filtration [58] or material pre-cultivation [54] are used. In addition, an enhanced flow-through measuring cell has been used for the sample concentration [59]. A condensed overview of the *E. coli* biosensors proposed for water analysis is given in Table 1.

Table 1. *E. coli* immunobiosensors for water analysis

Detection principle	**Bioselective element**	**LOD (cells/ml)***	**Analysis time**	**Reference**
Electrochemical impedance sensor with screen-printed gold electrode	Polyclonal *E. coli* antibody	30	1 hour	[59]
Label-free capacitive immunosensor	Polyclonal *E. coli* antibody	220	1 hour	[52]
Visible paper chip immunoassay	Polyclonal *E. coli* antibody	10	No data	[53]
Paper microfluidics, immuno-agglutination	Polyclonal *E. coli* antibody	10	90 seconds (+20 hours sample pre-cultivation)	[54]
Surface plasmon resonance	Polyclonal *E. coli* antibody	90	30 min	[51]
Bifunctional linker-based immunosensing	Polyclonal *E. coli* antibody	10	30 min	[55]
Optical immunosensor using dual labeled Ag@SiO_2 core-shell nanoparticles	Monoclonal *E. coli* antibody	5	> 1 hour	[56]

*the biosensor studies usually do not differentiate between cells/ml and CFU/ml, so these units are combined in the present table

1.4.2. Clinical analysis of *E. coli* in urine

Several different biosensors have been proposed to detect uropathogenic *E. coli* (UPEC) in urine in recent years. UPEC is a subgroup of extra-intestinal pathogenic *E. coli*, and it is the major causative agent of urinary tract infections (UTI). The UPEC biosensors are based on the bio-recognition of UPEC-specific antigens but also species-specific antigenic regions (e.g., OmpA), which are

common for all *E. coli* strains (Table 2). As non-pathogenic *E. coli* strains besides UPEC are never present in urine, UPEC-specific bio-recognition is not necessarily required [46]. The LOD value of UPEC biosensors ranges from 10 to 10^5 cells/ml, and as a rule, this value is in an inverse relationship with the time required for analysis.

Table 2. Biosensors for the detection of uropathogenic *E. coli.*

Detection principle	**Bioselective element**	**LOD (cells/ml)***	**Analysis time**	**Reference**
Paper-based immunosensor with gold nanoparticles	Polyclonal *E. coli* antibody	10^5	7 min	[46]
Label-free impedimetric sensor	-	7	5 h	[60]
Metallic nanohole array through surface plasmon resonance imaging technique	Uropathogenic *E. coli* specific polyclonal antibody	100	35 min	[57]
Label-free, long-range surface plasmon waveguide biosensor	$Gram^-$ and $gram^+$ specific antibodies	10^5	-	[61]
Electrochemical immunosensor	Uropathogenic *E. coli* specific polyclonal antibody	50	3 h	[62]
Crossed surface-relief gratings nano metallic label-free immunosensor	Uropathogenic *E. coli* specific antibody	10^5	35 min	[63]
Surface-enhanced-Raman-scattering-based biosensor with covalently linked antibodies	Antifimbrial polyclonal antibody	10	3 h	[45]
Electrochemical nitrite sensor	-	10^5	-	[40]
Amperometric, 16S rRNA DNA probes-based biosensor	16S rRNA DNA probes	10^3	1 h	[64]

*the biosensor studies usually do not differentiate between cells/ml and CFU/ml, so these units are combined in the present table

In UTI diagnostics, the appropriate UPEC level in urine indicating infection is considered to be $\geq 10^5$ CFU/ml [22]. However, on several occasions (e.g., re-current UTI, related diseases), the concentration of infection-causing pathogens is significantly lower ($\geq 10^3$ CFU/ml) [22]. This means that for practical

applications, UPEC specific biosensors should have LOD values significantly lower than 10^5 CFU/ml. However, to achieve such low LOD limits, it takes at least 35 min to obtain analytical results with biosensors [45,60,62,63].

The vast majority of UPEC biosensors employ polyclonal antibodies [45,57, 65], although in this case, careful pre-analytical handling of samples is required to avoid sample contamination with other *E. coli* strains of human origin [46]. There are also biosensors based on the detection of nucleic acids of the pathogens [64], which assure high specificity and multiplex detection of pathogens but require additional effort for sample preparation (extraction of nucleic acids). Gayathri *et al*. proposed a label-free impedimetric biosensor with a detection limit 7 CFU/ml, which does not contain any specific bio-selective elements, but a pre-cultivation step of *E. coli* inside the sensor is necessary [62]. Some biosensor systems are without selective elements: a label-free impedimetric UPEC sensor [60] and an electrochemical nitrite sensor for measuring nitrite generation by bacterial pathogens [40].

2. AIMS OF THE STUDY

The goal of the present study was to develop a biosensing system for the rapid detection of *E. coli* in selected natural matrixes. The research activities were divided into six interrelated objectives, each of which was focused on a specific research question:

1. Design of *E. coli* immunosensor
2. The fabrication & setup of *E. coli* immunosensor (incl. the separation of human IgG Fc fragment and the preparation of bioactive beads for the attachment of *E. coli* cells)
3. Optimization of *E. coli* measurement protocols
4. Characterization of the analytical performance of *E. coli* immunosensor regarding its sensitivity, LOD, time of analysis, and selectivity
5. Testing the applicability of the proposed immunosensor in bathing water and urine samples; characterization of matrix effects.
6. Validation of results, obtained with *E. coli* immunosensor, using the PCR and microbiology methods

3. EXPERIMENTAL

3.1. The *E. coli* immunosensor design

The proposed *E. coli* immunosensor system is an optical biosensor incorporating the BIA technology for the transport of samples and necessary reagents. An essential part of the system is the single-use renewable microcolumn, which allows sample enrichment, removal of possible contaminants, and does not need any regeneration.

3.2. The biosensor setup

The immunosensor setup consists of a bead injection analysis platform for fluidics (FIALab 3500B, FIAlab Instruments) and an optical system for the generation and detection of fluorescence signals. All components of the optical system were from Ocean Optics: a DH-2000 halogen lamp equipped with an adjustable bandpass linear variable filter LVF-HL (300–750 nm) was used as a light source for the excitation of fluorescein isothiocyanate marker (FITC, λ_{ex}=495 nm), and a USB 2000+ spectrophotometer (with advanced electronics and extended 200 μm wide slit) for the detection of the emitted fluorescent signal at 525 nm. Covered quartz fibres with a core diameter of 400 μm were used for light transmission. The flow cell was covered with a custom-made, extra light-tight shield to eliminate all incident light and equipped with a moving piston to assure the capture of the beads and formation of a microcolumn in the appropriate flow channel's geometry (Figure 1).

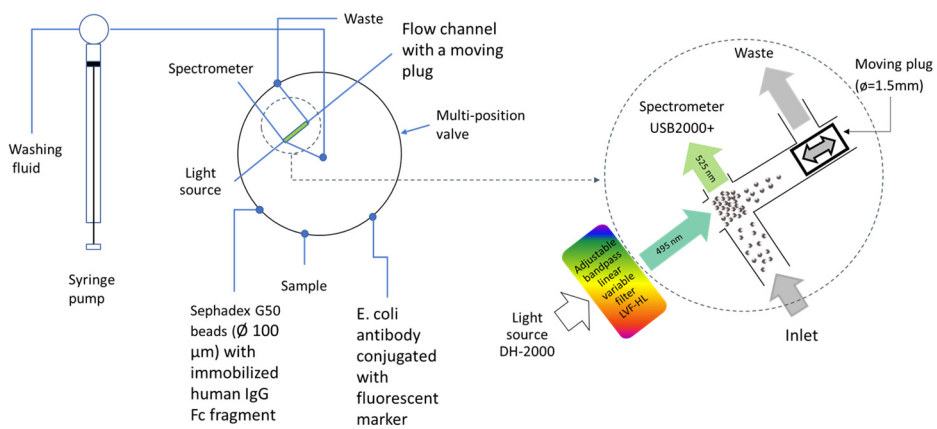

Figure 1. The immunosensor setup. The directions of fluid flow and excitation/emission light in the measurement valve are shown with grey and colored arrows, accordingly.

First, bacteria with outer membrane protein OmpA (*E. coli*, coliforms, etc.) were captured from samples onto a single-use renewable microcolumn. The column consists of Sephadex G50M beads, functionalized with the Fc fragment of human IgG [66]. The interaction between human IgG Fc fragment and the *E. coli* outer membrane protein A (OmpA), characterized by the dissociation constant K_d value of ~50–200 nM [67], allows effective preconcentration and capture of specified bacteria. The unbound sample components were removed by washing with phosphate buffer, and the captured bacteria were selectively detected with anti-*E. coli* antibody conjugated with FITC (Figure 2) [I,II,III].

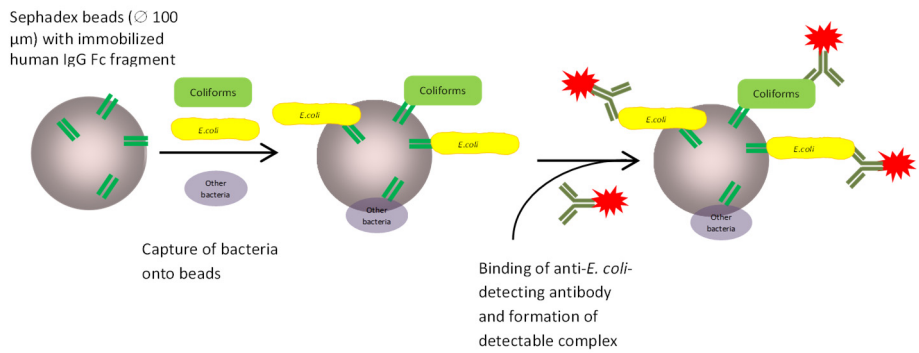

Figure 2. The principal scheme of an immunobiosensor for the detection of *E. coli*

3.2.1. Preparation of the capturing beads

The capturing beads for bacterial concentration were prepared using Sephadex G50 Medium beads, which were first swollen in MilliQ water. The swollen beads were activated with epichlorohydrin, which reacts with OH groups of Sephadex G50, and the human IgG Fc fragment is bonded over the epoxy group. The incubation time of the activated beads with human IgG Fc fragments was 24h, followed by blocking free epoxy groups with ethanolamine. The bio-activated beads were stored in PBS at 4ºC. The efficiency of the immobilization of IgG Fc fragment on beads was tested by staining the beads with Coomassie Brilliant Blue G-250 [I,II,III,66].

3.2.2. Detection of *E. coli*

We modified and optimized the protocol for the detection of *E. coli* in natural water. As a starting point, a protocol for detecting *E. coli* in milk was used [44], although comparing the detection of *E. coli* in the samples of natural water and raw milk, there are significant differences (e.g., sample matrix, viability, and concentration of bacteria).

Micro-columns were formed by injecting 20 µl of suspended bio-activated beads into the partially closed flow cell (1 µl/s). Transport of the beads to the cell was secured by adding 30 µl PBS (0.01 M, pH 7.2, 0.15 M NaCl) (2 µl/s). Partial closing of the flow channel assured the capture of beads into an appropriate geometry and free flow of solution. Then 150 µl of the sample was added (1 µl/s), and the flow was stopped for 180 s to allow the attachment of bacteria onto the column. All unbound material was removed from the column with 150 µl (500 µl urine samples) PBS (2 µl/s). Next, 20 µl FITC – conjugated *E. coli* antibodies (125 µg/ml) were injected at a flow rate 1 µl/s and incubated for 120 s. The unbound antibodies were removed with 150 µl PBS (2 µl/s), and the fluorescence intensity was measured at $\lambda = 525$ nm. After each measurement, the flow channel was opened, washed thoroughly with 2000 µl PBS (100 µl/s), and the system was ready for the next analysis [I,II,III].

The biosensor signal $\Delta I_{\lambda=525nm}$ was determined as an average of 3–5 measurements as the difference of signal intensity before addition and after removing the unbound detecting antibody (Figure 3). The signal was considered to be stable if its change in 100 seconds was less than 1%, and its value, which was used for biosensor calibration, was calculated as an average of 100 experimental points [I,II,III].

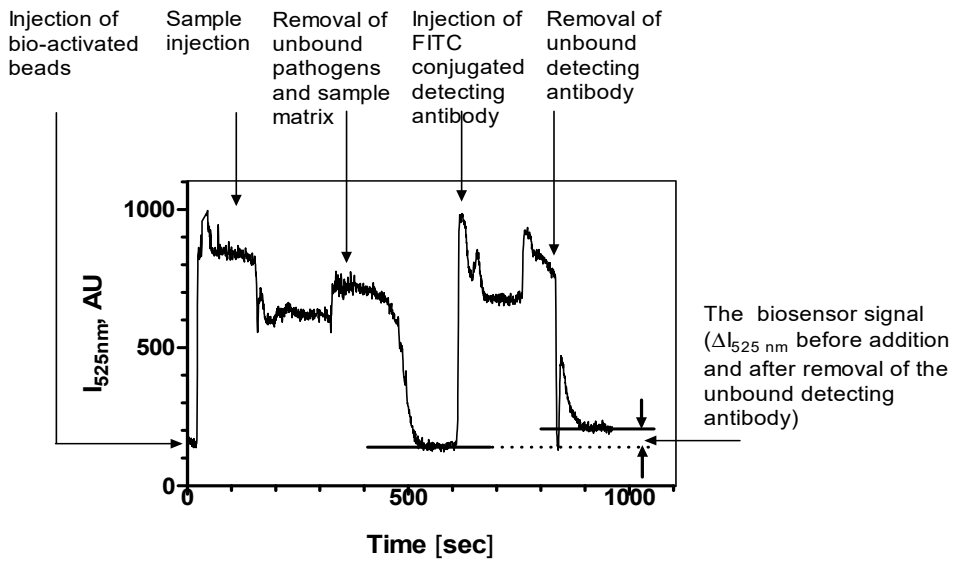

Figure 3. Example of the signal time course during a measuring cycle. The concentration of *E. coli* in urine is 10^8 cells/ml.

The average detection time, including the final wash of the flow channels, was 17–20 min (depending on analysis type-water or urine analysis) [I,III].

3.3. Cultivation of *E. coli*

E. coli (ATCC 25922) was cultivated on PCA (plate counting agar, Oxoid) plates for 24 h at 37 °C. Aliquots of bacteria were collected with a sterile spatula, dissolved in PBS, and stored at -20 ºC. The number of bacterial cells was determined by the optical density of the bacterial suspension at λ=600 nm [I,II,III].

3.4. Fragmentation of *E. coli* cells

The fragmentation of *E. coli* cells was carried out with an ultrasound sonicator (Bandelin HD 2020 Sonopuls, horn ⦰ 3 mm) with cycle intensity 7/10 and 90% power of 70 W for 2–5 min. For the chemical treatment of *E. coli* cells, we used SDS (final concentration 0.1%, Amresco). We also combined the chemical treatment with enzymatic fragmentation: *E. coli* cells were first treated with lysozyme (1mg/ml, BioChemica) for 30 minutes at 37ºC, and after that, SDS was added to a final concentration 0.1% [II].

3.5. Collection of samples

3.5.1. Bathing water samples

Water samples were collected from 4 different locations of a popular swimming place, Anne Canal in Tartu, in the summer of 2018. The samples were collected into 1 L sterile glass bottles 1.5–3.0 m from the shoreline at depth 0.5–0.7 m and stored on ice until microbiological analysis on the same day. For biosensor measurements and DNA extraction, sample aliquots were frozen and stored at -20ºC [I].

3.5.2. Urine samples and analyses

UPEC positive urine samples from anonymous UTI patients of the Tartu University Hospital (leftovers from clinical analysis) were collected after obtaining approval from the local ethics committee (UT protocol 340T-10). These samples underwent standard analytical procedures for the identification of uroinfection pathogens: overnight cultivation on CLED (cystine lactose electrolyte deficient) agar (Liofilchem) at 37ºC, combined with MALDI-TOF for the identification of bacteria. The undiluted urine samples' immunosensor measurements were carried out on the next day with samples stored at 4 ºC [III].

3.6. Isolation and characterization of the strains of coliform bacteria

The coliform isolates originated from the water samples of Anne Canal in 2019, and strains selection was based on the isolation of pink colonies from coliform chromogenic agar (Biolife Italiana Srl), all of which were checked for purity. For coliforms, identification was first amplified 16S rDNA by PCR with primers 27f and 1525r [68]. The resulting PCR fragments were sequenced in the Institute of Genomics (University of Tartu, Estonia), and the 16S rDNA sequences of isolated species were identified with Ribosomal Database Project (http://rdp.cme.msu.edu) tools [II].

4. RESULTS AND DISCUSSION

4.1. The analytical performance of *E. coli* biosensor

The analytical performance of the *E. coli* biosensor was characterized in terms of sensitivity, LOD, analysis time, and selectivity.

The immunosensor signal versus *E. coli* concentration in PBS was linear on a semilogarithmic scale, demonstrating the immunosensor's wide working range from 10 to10^7 cells/ml (Figure 4) (I, II). The calculated background signal of the system (y-intercept) was 5.70±0.46 AU. This value was similar to the background signal 5.61±0.41 AU measured experimentally in *E. coli*-free PBS solutions. The slope of the calibration plot, indicating the sensitivity of the *E. coli* biosensor, was 4.05±0.11 AU/log (CFU/ml) [I,II].

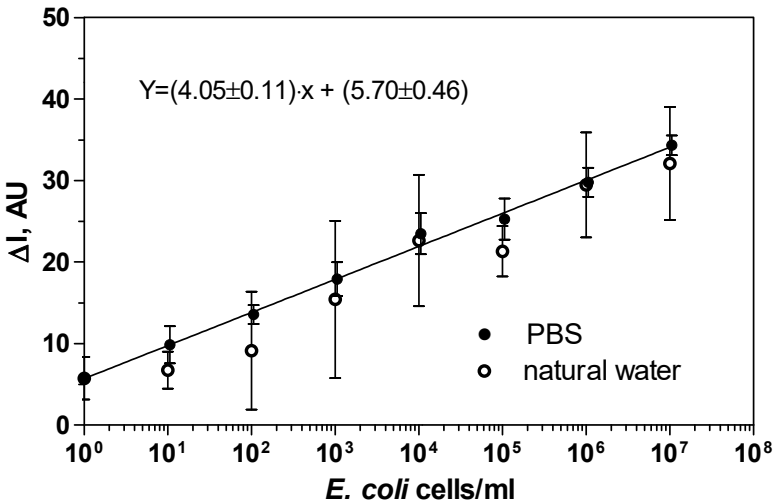

Figure 4. Dependence of the immunosensor signal $\Delta I_{\lambda=525nm}$ on the concentration of *E. coli* in PBS and natural water matrix. The biosensor signal and error bars were calculated from 3–5 independent experiments.

The limit of *E. coli* detection from water samples with the current biosensor setup, calculated as *E. coli* concentration corresponding to the signal exceeding the average background signal by the value of 3 standard deviations, was < 10 CFU/ml in PBS [I]. The limit of quantification (LOQ); *E. coli* concentration corresponding to the signal exceeding the average background signal by the value of 10 standard deviations) was < 30 CFU/ml [I]. The low LOD and LOQ values demonstrate the high sensitivity of this biosensor system, allowing the detection of a single pathogen cell in a sample. This high sensitivity is achieved due to the great number of antigenic binding sites on the outer membrane of the *E. coli* cells. As reported earlier, the OmpA molecules are present at 10^5 copies

per single *E. coli* cell [69], indicating the high signal amplification potential of immunosensor [I,II].

The detection and quantification limits of the *E. coli* biosensor were also in the same range in urine samples [III]. The achieved with the biosensor LOD and LOQ values for the detection of *E. coli* is significantly lower than the pathogenesis limit of microbiological cultivation $>10^5$ CFU/ml [22].

Applying a semilogarithmic scale for immunosensor calibration does not allow the correct determination of pathogen concentrations exceeding 10^7 cells/ml. To extend the working range of the immunosensor in urine, we also applied a double logarithmic scale for the immunosensor calibration (Figure 5). This approach has been used earlier to calibrate pathogen sensors for the analyses of pathogens in raw milk [44].

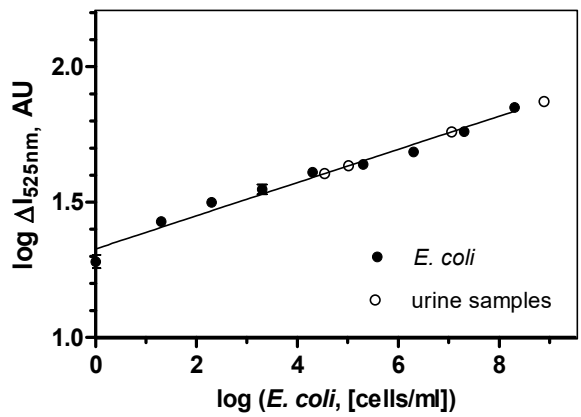

Figure 5. The immunosensor calibration plot in urine. The results of the samples of UTI patients are marked with ○.

The double logarithmic dependence was linear within the whole studied *E. coli* concentration range from 10 to 10^8 CFU/ml with the slope 0.061±0.002 (log AU/log(cells/ml)) and intercept 1.33±0.01 AU [III].

We also studied the matrix effect of the natural water (Figure 4) [I]. It was evaluated in different samples collected from different places of the Anne Canal in different years (natural water 1 *vs*. natural water 2) [II]. The comparison of the slope and background signal in PBS and natural water revealed that the background signal and the slope were not significantly different ($P < 0.05$) in PBS and natural water samples, indicating that the analyzed natural waters had no detectable matrix effect [I,II].

Comparing *E. coli* immunosensor response in PBS and urine matrix, the sensitivity was almost 2 times higher in PBS than in urine matrix, with the slope of calibration plots being (9.1±0.4) and 5.6±0.3) AU·cells/ml, respectively [III].

In nature, there is a vast diversity of different *E. coli* strains, which outer membrane composition can be different. So, we tested the selectivity and sensitivity of our immunosensor towards different *E. coli* strains isolated from nature and a type strain ATCC 25922. It was found that there was no significant difference in immunosensor signals produced by different *E. coli* isolates in the whole studied concentration range (10–10^7 CFU/ml), although some strains generated somewhat bigger experimental errors [II]. Based on the obtained results, it can be assumed that the constructed *E. coli* immunosensor is universal and can be used to detect different *E. coli* strains in samples regardless of their origin.

Our studies showed that immunosensor measurements resulted in sufficiently higher *E. coli* concentrations than cultivation data and qPCR results [I,II]. That kind of discrepancies in *E. coli* measurements can be expected, as different methods are based on the detection of different characteristics of the *E. coli* cell. Microbiological cultivation allows the detection of only cultivable bacteria. However, water samples, particularly natural water samples, may contain non-cultivable bacterial material in addition to cultivable bacteria, which is not detectable by microbiological techniques [23]. The non-cultivable material includes live VBNC cells (persistors), intact dead cells (quantifiable by qPCR), and different fragments of dead cells. The membrane fragments have a notable effect on the signal of *the E. coli* immunosensor, as the sensor is based on the bio-recognition of *E. coli* outer membrane proteins. There is also information about the prolonged existence of cell fragments in marine environments [70,71], so the effect of cell fragments can be expected in bathing water samples. The presence and amount of cell fragments are dependent on the origin of the sample analyzed. In some cases, like in urine, this is not a remarkable problem because the number of other pathogens in urine is limited [13], and the proteolytic environment is not favourable for the preservation of dead cells or cell fragments [72].

To study the impact of the fragmented *E. coli* cells at different degradation levels on the immunosensor signal, we used ultrasonic, chemical (detergent), and enzymatic treatment of *E. coli* cells. The ultrasonic treatment provides totally disrupted cells with intracellular proteins, lipopolysaccharide (LPS) structures, and nucleic acids sheared [73]. Our results showed that ultrasonication of *E. coli* cells even up to 5 min did not remarkably affect the immunosensor signal, even if the cells were totally disrupted. The obtained immunosensor response was similar to the one of intact *E. coli* cells (Figure 6) [II].

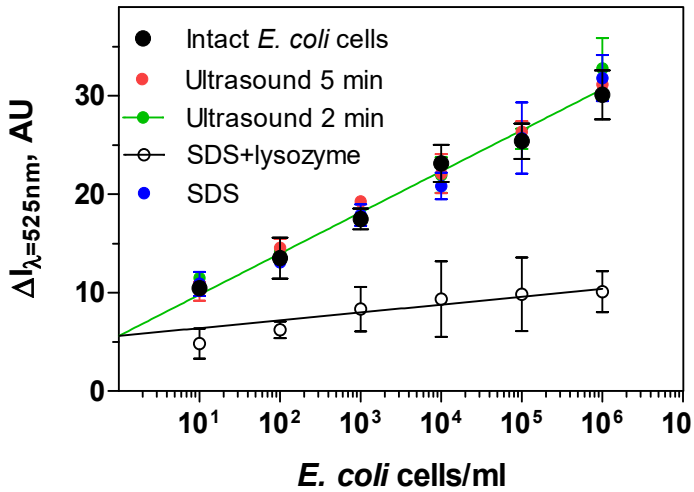

Figure 6. The immunosensor signal $\Delta I_{\lambda=525nm}$ for intact and disrupted *E. coli* cells in PBS. The immunosensor signal and error bars were calculated from 3–5 independent experiments.

The immunosensor response also did not change when the *E. coli* cells underwent chemical treatment with 0.1% SDS (Figure 6). In addition, we combined the chemical treatment with the enzymatic treatment with lysozyme, which is mostly used to decompose the peptidoglycan cell wall of Gram-positive bacteria. However, it also works effectively for some Gram-negative bacteria [74] and can disrupt the integrity of the outer and cytoplasmic membrane of *E. coli* [75]. Although the SDS treatment of *E. coli* cells did not influence the immunosensor signal, the combined treatment decreased the signal significantly. The output signal up to *E. coli* concentrations 10^5 cells/ml was not statistically different from the background signal (Figure 6) [II].

The obtained results showed that the integrity of target cells (cell membrane/wall) was not important for raising the biosensor signal, and the immunosensor signal was dependent on all noncultivable, dead, and (partially) fragmented *E. coli* cells [II]. So, for the application of *E. coli* immunosensor for the assessment of environmental water, the potential presence of dead and fragmented cells in samples should be considered. This issue is crucial for samples taken from water bodies with no continuous flow or from lakes where there and much sediments, which are a potential source of the dead cells and their fragments.

The other potential reason for apparently higher *E. coli* concentration obtained with the immunosensor is caused by the cross-reactivity of the polyclonal *E. coli* antibody. However, this antibody should not exhibit any cross-reaction with other *Enterobacteriacea*e and coliform [76]. We tested the immunosensor against different coliform strains isolated from the water of

Anne Canal. One of these coliform isolates, strain T12 (*Raoultella sp.*), generated a substantial signal (11.4 AU, incl. background) which was only about 3 times lower than the *E. coli* signal at the same concentration of bacteria. The average signals generated by other coliform strains were 1–3 AU above the background signal (Table 3). As the number of coliform bacteria is always high in bathing waters [I], one should consider the impact of coliforms on the immunosensor output signal [II].

Table 3. *E. coli* immunosensor signal (three independent measurements) in different coliform isolates.

Isolate No	Coliform family/species	Average immunosensor signal at 10^6 CFU/ml	Family/species average signal (AU)
1.	*Aeromonas sp.*	N/A	6.9±2.3
2.	*Aeromonas sp.*	10.54±3.24	
3.	*Aeromonas sp.*	N/A	
4.	*Aeromonas sp.*	5.24±0.76	
5.	*Aeromonas sp.*	8.3±1.25	
6.	*Aeromonas sp.*	7.53±2.74	
7.	*Aeromonas veronii*	7.24±1.51	7.24±1.51
8.	*Enterobacter sp.*	6.59±1.50	6.59±1.50
9.	*Klebsiella sp.*	7.09±1.69	7.09±1.69
10.	*Raoultella sp.*	5.28±1.81	6.5±4.4
11.	*Raoultella sp.*	N/A	
12.	*Raoultella sp.*	11.39±1.97	

The immunosensor output in the mixtures of coliform isolates at different concentrations of live bacteria (10^3–10^7 cells/ml) is shown in Table 4. The chemical and combined treatment for the degradation of coliform bacteria had the same effect on the immunosensor signal as the similar treatment of *E. coli* cells [II].

Table 4. The immunosensor signal in untreated and treated mixtures of coliform isolates

Coliforms CFU/ml + treatment	Average immunosensor signal (AU)
10^3/PBS	N/A
10^4/PBS	5.55±0.73
10^5/PBS	6.71±0.86
10^6/PBS	7.71±1.09
10^7/PBS	10.1±0.49
10^6/0.1 % SDS	8.16±1.35
10^6/5 min ultrasound	7.82±1.35
10^6/SDS+lysozyme	N/A

Other tested bacteria like *Staphylococcus* and *Streptococcus* species did not generate any detectable signal with the *E. coli* immunosensor up to concentration 10^6 CFU/ml [44].

4.2. Application of *E. coli* immunosensor for the analysis of bathing water

The *E. coli* immunosensor was applied to evaluate the microbiological quality of Anne Canal, which is one of the most popular beaches in Tartu. The samples of bathing water were collected during the summer of 2018. To validate the biosensor results, the number of *E. coli* was assessed with three different methods-microbiological cultivation (membrane filtration method), qPCR, and *E. coli* immunosensor. In addition, the number of coliform bacteria was also determined using microbiological cultivation [I].

In ideal conditions-in fresh exponential phase cultures, where all cells are integral, viable and the genomic DNA is intact, the results of all three methods are similar [I].

Depending on the detection method, different factors influence the outcome of bacterial enumeration in natural water. For example, the variability and different viability (e.g., persistors) of cells affect the results of microbiological cultivation, and the presence of inhibitive compounds affects the efficiency of qPCR. [77,78]. The biosensor signal is dependent on the number of cell membrane proteins targeted by the detecting antibody and varies due to environmental stress, cell viability, homogeneity of population, etc. [79,80]. Each method for *E. coli* measurement has its shortcomings and advances. Microbiology is the standard method, but it is time-consuming and needs a specific lab. Molecular methods provide very high specificity, but there is also a need for preparatory steps and specific equipment. As microbiological cultivation still serves as the standard for *E. coli* enumeration, results obtained with all other methods should be compared with the cultivation results. The warm and dry summer of 2018 in Tartu caused a rise in the total number of bacteria.

Both *E. coli* cultivation and immunosensor measurements and coliform cultivation numbers increased in Anne Canal, peaking by the 3rd decade of August with *E. coli* cultivation maximum > 500 CFU/100ml. The number of coliform bacteria achieved a number of 10^5 CFU/100 ml, and the immunosensor indicated >5000 cells/100ml (Figure 7) [I].

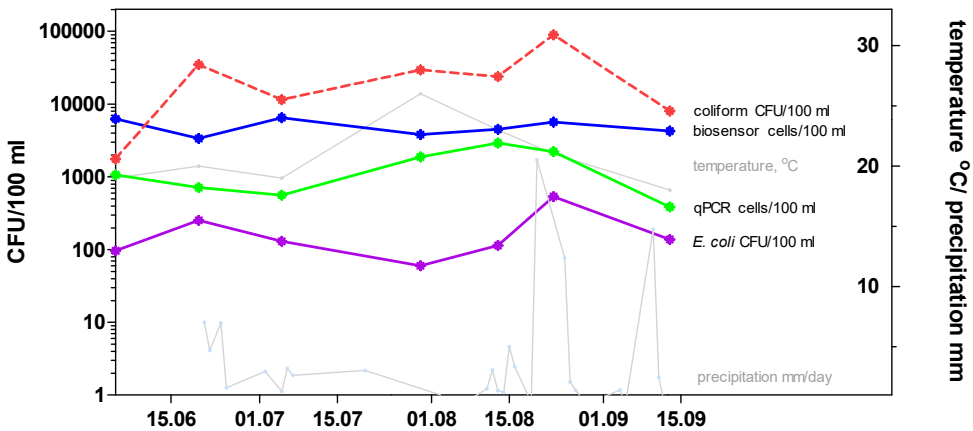

Figure 7. The number of *E. coli* in Anne Canal in Tartu (Estonia) in the summer of 2018 was assessed with microbiological cultivation, qPCR, and *E. coli* immunosensor (marked in violet, green and blue, respectively). The total number of coliform bacteria is marked in red, and the meteorological data is grey.

The maximum result of *E. coli* quantitation by the qPCR analysis 3000 cells/ 100ml was detected two weeks earlier. The overall dynamics of the immunosensor and microbiological data were similar during most of the sampling period, except for the 1st half of June (lower temperatures) when the immunosensor results decreased in parallel with qPCR data, contrary to the cultivation data. With temperatures falling in September, the number of cultivable *E. coli* dropped, but the immunosensor results were stable. The qPCR and cultivation results had opposite trends also in July. The immunosensor results were significantly higher than the enumeration of *E. coli* with cultivation and qPCR but lower than the total number of coliform bacteria. This indicates that immunosensor targets *E. coli* cells, cell fragments dynamics are different in water bodies compared to cultivation (viable *E. coli* cells) or qPCR (genomic DNA from *E. coli* cells) [I].

The median value of *E. coli* concentrations obtained with the immunosensor was approximately 4 times bigger than the outcome of qPCR, indicating that the biosensor results are likely to contain signals from other bacteria than *E. coli;* or in addition to intact live and dead cells, the sensor also detects proteins on membrane fragments of broken cells. Based on the comparison of the median values of *E. coli* enumeration with different methods, the threshold for *E. coli*

concentration determined with an immunosensor is indicatively 40 times higher than the maximum number of *E. coli* determined by cultivation [I]. For the evaluation of biosensor results and characterization of its selectivity, we also determined the total number of coliform bacteria in water samples, although currently, no maximum allowed coliform numbers in recreational water had been set. The median value of the total coliform number (16 625 CFU/100 ml) was extraordinarily high and exceeded (over two magnitudes) the results of *E. coli* cultivation. It is also interesting to mention that the currently abandoned threshold of this number for bathing water was 10 000 CFU/100 ml [81]. Comparing the number of total coliform bacteria and biosensor results, it turned out that these were in significant correlation (Pearson's r=0.495; p=0.0370), indicating that the anti-*E. coli* antibody used for *E. coli* biorecognition showed some unexpected affinity towards other coliforms in samples [I].

We analyzed the immunosensor output signal assuming that the polyclonal *E. coli* antibody potentially interacts with the outer membrane proteins of *E. coli* and coliforms' cells (both intact and fragmented), generating a biosensor signal. The signals of these bacteria are additive, and the total biosensor signal can be expressed as follows:

$$S_{total} = S_{Background} + S_{E.\ coli} + S_{noncultivable\ E.\ coli} + S_{(E.\ coli\ fragments\ +coliforms)} \quad (1),$$

where $S_{Background}$ marks the immunosensor background signal in natural water, $S_{E.\ coli}$ stands for the signal of cultivable *E. coli* (which is the basis of determination of coli-index); $S_{noncultivable\ E.\ coli}$ is the signal of non-cultivable *E. coli*, and the remaining term in *Eq. 1* is the combined signal raised by the fragments of *E. coli* cells and all different coliform cells [II]. The assumption of signal additivity is based on the fact that the total number of human IgG Fc fragments on the column forming beads is sufficient to determine quantitatively > 10^9 cells/ml [82], and the measurable biosensor response is generated by different substrates [66,83]. Based on this assumption, we analyzed 16 samples of bathing water collected from different spots of Anne Canal in Tartu, Estonia (Table 5). The average background signal of the system was 5.7±0.46 AU, and as expected, this value was similar for different samples. The background was subtracted from the measured immunosensor signal for signal analysis to calculate the "pure" signal generated by bacterial material. The *E. coli* concentration in the studied samples was relatively low, <10 CFU/ml, also resulting in low corresponding immunosensor signals. In only 6 cases, the *E. coli* caused signal was significantly different (±3 SD values) from the background signal [II]. In order to increase the signal generated by cultivable *E. coli*, the sample volume can be increased. Our preliminary studies with increased 5 to 10 times standard volume (0.75 and 1.5 ml respectively) resulted in signals corresponding to the increased number of bacteria in the increased sample volumes [I].

Table 5. The impact of different components on *E. coli* immunosensor signal in different bathing water samples collected from the beach of Anne Canal in Tartu, Estonia

Sample	Date	Measured immunosensor signal (AU)		Background signal (AU)		*E. coli* Microbiol. cultivation (CFU/ml)	Signal caused by cultivable *E. coli*	*E. coli* number by qPCR, CFU/ml		*E. coli* qPCR results immunosensor signal (AU)-background	Non-cultivable *E. coli* cells, qPCR (AU) – cultivable. *coli* (AU)	Coliform microbiol conc. (CFU/ml)	Coliform cultivation signal (AU)
		avg	std	avg	std	*avg*	*avg*	avg	std	avg	avg	avg	avg
1	20.06.2018	15.48	4.98	5.7	0.46	2.52	1.63	7.09	0.72	3.44	1.82	465	N/A
2	20.06.2018	16.81	8.15	5.7	0.46	2.36	1.51	15.15	0.90	4.78	3.27	117.5	N/A
3	20.06.2018	17.97	4.94	5.7	0.46	2.72	1.76	7.20	0.12	3.47	1.71	117.5	N/A
4	05.07.2018	16.63	1.05	5.7	0.46	1.3	0.46	5.59	0.79	3.03	2.57	115	N/A
5	05.07.2018	11.67	2.10	5.7	0.46	1.61	0.84	3.89	0.12	2.39	1.55	160	N/A
6	05.07.2018	9.67	1.35	5.7	0.46	1.24	0.38	8.25	1.46	3.71	3.33	92.5	N/A
7	30.07.2018	16.36	7.84	5.7	0.46	0.6	0.00	18.82	0.75	5.16	5.16	295	N/A
8	30.07.2018	16.17	5.13	5.7	0.46	1.85	1.08	5.34	1.53	2.95	1.87	262.5	N/A
9	30.07.2018	18.45	9.57	5.7	0.46	0.84	0.00	7.62	0.94	3.57	3.57	265	N/A
10	13.08.2018	16.09	3.60	5.7	0.46	1.14	0.23	29.04	1.46	5.92	5.69	217.5	N/A
11	13.08.2018	20.20	6.69	5.7	0.46	1.4	0.59	11.44	1.00	4.29	3.70	235	N/A
12	13.08.2018	11.65	2.90	5.7	0.46	1.18	0.29	5.91	0.30	3.12	2.83	197.5	N/A
13	13.08.2018	26.56	1.61	5.7	0.46	3.25	2.07	6.40	1.22	5.26	3.19	495	N/A
14	23.08.2018	14.85	4.91	5.7	0.46	7.648	3.58	22.24	2.83	5.46	1.88	890	N/A
15	23.08.2018	19.81	5.18	5.7	0.46	2.27	1.44	12.29	0.73	4.41	2.97	405	N/A
16	23.08.2018	14.83	3.46	5.7	0.46	0.4	0.00	10.74	1.45	4.18	4.18	240	N/A

On average, the signal of cultivable *E. coli* was only ~ 10 % of the "pure" immunosensor signal. The determination of the signal caused by intact but non-cultivable *E. coli* was based on the results of qPCR analyses. This signal was more significant and formed ~ 30% of the specific signal as an average. The remaining 60% of the immunosensor signal was most probably caused by the *E. coli* fragments and all forms of coliforms (culturable, VBNC, and cell fragments) [II].

The immunosensor signal depends on *E. coli* concentration in a semilogarithmic pattern (Figure 4). The signal value corresponding to *E. coli* concentration equal to the maximum allowed coli-index (10^3 CFU/100 ml), is 4.05 AU. Assuming that the ratio of different bacterial materials does not change during the swimming season, the theoretical immunosensor signal corresponding to the allowed coli index is 27.2 AU (including background). However, the viability of the microflora depends on many unpredictable factors, and it may be necessary to use different numerical values of immunosensor signals for the estimation of the coli index in different water bodies [II].

4.3. Application of *E. coli* immunosensor for the analysis of UPEC in urine

The applicability of *E. coli* immunosensor was also tested in the urine samples of UTI patients collected from the Tartu University Hospital [III]. The obtained immunosensor results of the urine samples were validated with MALDI-TOF and qPCR methods (Table 6).

The urine analysis is a challenging task for immunosensors because of the high biological variability of samples [84]. The survival and cultivability of UPEC strains can be influenced by the patient's health parameters or the infection stage, which can also influence the reliability of the results of microbiological cultivation. Some experiments indicate that in the case of symptomatic patients, 20–30 % *E. coli* negative urine cultures give a positive result in the qPCR analysis [14]. This kind of false-negative diagnosis could be avoided by immunosensor analysis with comparable sensitivity to molecular methods.

Table 6. Assessment of uropathogenic *E. coli* concentration in urine samples with matrix-assisted laser desorption ionization-time of flight mass spectrometry (after 24h microbiological cultivation), quantitative polymerase chain reaction analysis, and an *E. coli* immunosensor.

Sample No	*E. coli* [cells/ml]			Additional information
	Matrix-assisted laser desorption ionization-time of flight mass spectrometry *	Quantitative polymerase chain reaction analysis**	*E. coli* Immuno-sensor***	
1	10^4	2.41×10^4	1.0×10^5	Urine strip analysis positive
2	$>10^5$	3.21×10^7	1.1×10^7	–
3	$>10^5$	1.12×10^8	7.6×10^8	Includes sediments
4	10^4	1.94×10^7	3.4×10^4	Includes sediments

* matrix-assisted laser desorption ionization-time of flight mass spectrometry was used for the identification of *E. coli* colonies in cultivated urine samples.
** quantitative polymerase chain reaction analyses were made from urine samples stored at -18°C and thawed at 4 °C before analysis.
*** *E. coli* immunosensor measurements (average value of 3 measurements) were made from urine samples kept at 4°C for 24 hours

The cultivation/MALDI-TOF analysis is semi-quantitative and indicates if the UPEC levels are below or above 10^5 CFU/ml. The other two assessment methods gave more detailed results, and according to the results shown in Table 6, the UPEC levels were above 10^7 CFU/ml in several cases. In general, results obtained with different methods were in good correlation, indicating that the matrix of UPEC patients' inflammatory urine did not affect the measurements with immunosensor (Figure 5). Only in the case of sample No 4, the qPCR results were considerably higher than the cultivation and immunosensor results (Table 6). This disparity can be explained by the potential specificity of the patient's urine tract (urine matrix), where proteolytic activity can be high [72], leading to the possible accumulation of the genetic material of bacteria in urine and elevated qPCR results [III].

CONCLUSIONS

A novel immunosensor system integrating polyclonal *E. coli* antibodies for bio-recognition and bead-injection-analysis system has been developed and applied for the rapid detection of *E. coli* from bathing water and urine samples.

The main performance characteristics like sensitivity, the limit of detection, selectivity, and working range of the proposed *E. coli* immunosensor were studied and characterised. For the application of the *E. coli* biosensor, we also studied the matrix effects of natural samples.

The sensitivity of the *E. coli* biosensor was 4.05±0.11 AU/log (CFU/ml). The limit of detection of the *E. coli* biosensor was below 10 cells/ml, indicating the very high sensitivity of the biosensor due to the large number of antibody binding sites used for bio-recognition on the outer membrane of *E. coli* cells. To achieve even higher sensitivity, it is possible to increase the sample volume, as the BIA platform allows a simple concentration of samples. We also found that the *E. coli* immunosensor detects live cells, intact dead cells, and fragmented cells.

The selectivity and the possible impact of other bacteria on the *E. coli* immunosensor system signal were studied. It was found that the *E. coli* immunosensor measurements were influenced by related to *E. coli* bacterial species – coliforms.

The analysis of the biosensor signal in bathing water samples revealed that the proportion of cultivable *E. coli* cells in the immunosensor entire signal was only about 10%. The signal of non-cultivable *E. coli* cells (measured by qPCR) formed 30% of the immunosensor signal. The majority of the measured signal, 60%, was most likely generated by different forms of coliform bacteria and *E. coli* cell fragments. *E. coli* immunosensor signals generated by different forms of coliform bacteria were about three times lower than the signal of *E. coli* cells at similar concentrations.

Biosensing of *E. coli* in urine analysis was not affected by other bacterial species present in urine. As there is a minor probability of the presence of dead *E. coli* cells and cell fragments in urine, the *E. coli* biosensor results were in the same range as the results obtained with qPCR and cultivation/MALDI-TOF methods.

Using renewable, single-use *E. coli* immunosensor is a good alternative to time-consuming microbiological and molecular methods for analyzing complex natural samples. This can significantly shorten the time required for the determination and quantitation of *E. coli*, and could be used for automated analyses, as quick identification of *E. coli* allows to take timely measures to minimize potential health risks.

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SUMMARY IN ESTONIAN

E. coli immunosensori arendus ja rakendamine

Doktoritöö eesmärgiks oli välja töötada immunobiosensorsüsteem *E. coli* tuvastamiseks ning testida selle biosensori rakendamise võimalusi looduslike veeproovide ja kliiniliste uriiniproovide analüüsil. *Esherichia coli* (*E. coli*) on gram-negatiivne bakter, mis esineb soojavereliste organismide seedekulglas, ning seetõttu on selle bakteri arvukus vees oluline indikaator vee (kaasaarvatud suplusvee) mikrobioloogilise kvaliteedi määramiseks. Kuigi enamus *E. coli* tüvedest pole inimesele ohtlikud, leidub nende seas ka üksikuid patogeenseid tüvesid – näiteks inimese urotrakti nakatavaid tüvesi.

Tavaliselt hinnatakse *E. coli* arvukust mikrobioloogilistel meetoditel, kultiveerides proove spetsiaalsetel söötmetel, kuid see on aeganõudev (vähemalt 24 tundi). Erinevad molekulaarsed meetodid (kvantitatiivne PCR) on küll kiiremad (2–6 tundi), kuid nõuavad proovide eeltöötlust (DNA/RNA eraldamine), keerukat aparatuuri ja spetsiaalseid laboritingimusi. Lisaks on molekulaarsed analüüsimeetodid tundlikud võimaliku saastuse ja proovi maatriksist tuleneva inhibitsiooni suhtes.

Üheks võimaluseks on *E. coli* määramisel kasutada biosensoreid. Töös kasutatud biosensori (immunosensori) bioloogilise äratundmiskomponendina kasutati fluorestsentsmärgisega (FITC) konjugeeritud polüklonaalset anti-*E. coli* antikeha. Analüüsi kõrge tundlikkus saavutati tänu proovis leiduva *E. coli* sidumisele ühekordse kasutusega mikrokolonnile ning seondunud bakterite spetsiifilisele detekteerimisele. *E. coli* biosensori tundlikkus oli 4,05±0,11 AU/log (CFU/ml), ning immunosensori detekteerimispiir oli alla 10 raku/ml. Immunosensori platvorm võimaldab tundlikkust suurendada, suurendades proovi mahtu kuni 10 korda.

Erinevatest allikatest pärinevates proovides saadud analüüsitulemusi võrreldi alternatiivsete *E. coli* määramismeetodite, mikrobioloogilise külvi ja kvantitatiivse PCR abil saadud tulemustega. Nimetatud metoodikad võimaldavad küll kõik hinnata *E. coli* arvukust, kuid mõõdavad erinevaid rakku iseloomustavaid suurusi. Mikrobioloogiliste külvide meetod võtab arvesse elusaid kultiveeritavid rakke; kvantitatiivne PCR (qPCR) hindab *E. coli* genoomse DNA kogust (elusad + mitte-kultiveeritavad ja surnud rakud), ning biosensor mõõdab *E. coli* mebraanivalkude kontsentratsiooni proovis. Mõõtes näiteks ühte ja sama veeproovi kirjeldatud meetoditega selgus, et oodatult kõige madalama tulemuse andis mikrobioloogiline meetod (40 korda madalam, kui biosensor), ning ka qPCR meetod andis keskmiselt 4 korda madalama tulemuse kui biosensor. Töö selgitati välja põhjused, mis selliseid erinevusi põhjustasid. Esiteks, biosensoris põhjustasid mõõdetava signaali ka rakkude mehhaanilisel ja keemilisel töötlemisel saadud rakumembraanide fragmendid. Teise olulise tulemusena selgus, et biosensoris kasutatava antikeha äratundmisreaktsioon oli komplekses mikrobioloogilises keskkonnas eeldatust vähem selektiivne. Lisaks *E. coli´*le on looduslikes keskkondades palju sarnaseid kolivormseid bakteriliike, millest

mõnedel on potentsiaalselt afiinsus immunosensoris kasutatud *E. coli* antikeha suhtes. Kuna selliste bakterite üldhulk looduslikes vetes võib olla kõrge, siis tuleb immunosensori mõõtetulemuste interpreteerimisel arvestada ka nende poolt genereeritava signaaliga. Arvestades erinevate rakufragmentide ning kolivormsete rakkude poolt põhjustatud signaali osakaalu, siis elusate kultiveeritavate *E. coli* rakkude poolt tingitud signaali osakaal on immunosensori kogusignaalist 10%. Enamuse mõõdetud signaalist (60%) moodustasid erinevad kolivormsed bakterid (elusad, surnud, nende membraanifragmendid). 30% immunosensori signaalist moodustasid mitte-kultiveeritavad ja surnud *E. coli* rakud.

E. coli immunosensorit kasutati ka uropatogeense *E. coli* tuvastamiseks ja kvantiteerimiseks kliinilistes uriiniproovides, kus biosensoriga saadud analüüsitulemused langesid kokku mikrobioloogiliste ja molekulaarsete (qPCR) meetoditega saadud tulemustega. Väljatöötatud biosensorsüsteem võimaldas määrata *E. coli* sisalduse vee- või uriiniproovides vahemikus 7–10^7 rakku milliliitris 20 minuti jooksul, mis loob eelduse *E. coli* automaatseks kohapealseks määramiseks, vältides vajadust proovide transpordiks laborisse ning analüüsile eelnevaks töötluseks.

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PUBLICATIONS

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