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DELIA JURONEN

Biosensing system for the rapid multiplex detection of mastitis-causing pathogens in milk

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Biosensing system for the rapid multiplex detection of mastitis-causing pathogens in milk

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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 in the text by Roman numerals I–III.

I. **Peedel, D.**, Rinken, T., Rapid biosensing of *Staphylococcus aureus* bacteria in milk. Analytical Methods, 6 (2014) 2642–2647.

II. **Juronen, D.**, Kuusk, A., Kivirand, K., Rinken, A., Rinken, T., Immunosensing system for rapid multiplex detection of mastitis-causing pathogens in milk. Talanta, 178 (2018) 949–954.

III. Mihklepp, K., Kivirand, K., Nikopensius, M., **Peedel, D.**, Utt, M., Rinken, T., Design and production of antibodies for the detection of *Streptococcus uberis*. Enzyme and Microbial Technology, 96 (2017) 135–142.

Author's contribution

Paper I: Performed all the experimental work and calculations, responsible for the interpretation of results and writing of the paper.

Paper II: Performed all the experimental work and calculations, responsible for the interpretation of results and writing of the paper.

Paper III: Performed the experimental work and calculations and was involved interpretation of results and writing of the paper.

ABBREVIATIONS AND SYMBOLS

A	slope of the calibration curve
ATCC	American Type Culture Collection
B	intercept of the calibration curve
BSA-PBS	Bovine serum albumin in phosphate buffer solution
CFU	colony-forming units
CFU/ml	colony-forming units per millilitre
CMT	California Mastitis Test
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
Fc	crystallizable fragment
FISH	fluorescent in situ hybridization
FITC	fluorescein isothiocyanate
I	signal intensity
IgG	Immunoglobulin G
K_d	dissociation constant
λ_{em}	emission wavelength
LAMP	loop–mediated isothermal amplification
LBP	lactoferrin binding protein
LOD	limit of detection
LOV	lab-on-valve
M. bovis	*Mycoplasma bovis*
n. a.	data not available
NASBA	nucleic acid sequence based amplification
OmpA	outer membrane protein A
PBS	phosphate buffer solution
PCR	polymerase chain reaction
PDMS	poly(dimethylsiloxane)
PMMA	poly(methyl methacrylate)
S. aureus	*Staphylococcus aureus*
SCC	somatic cell count
SM	subclinical mastitis
SpA	*Staphylococcus aureus* cell wall protein A
Spp.	Species
Str. agalactiae	*Streptococcus agalactiae*

Str. bovis	*Streptococcus bovis*
Str. dysgalactiae	*Streptococcus dysgalactiae*
Str. parauberis	*Streptococcus parauberis*
Str. uberis	*Streptococcus uberis*
SUAM	surface adhesion molecule
tmRNA	transfer-messenger RNA
v/v	volume by volume
VBNC	viable but nonculturable
vs	*versus*
w/w	weight by weight

INTRODUCTION

Mastitis, mostly caused by bacterial infection of the mammary gland, is a major health problem of dairy cows. The resulting decrease of milk production and reduction of its quality along with medication costs and probable premature culling of animals cause essential economic burden. The total mastitis caused losses in dairy industry are estimated to be 16–26 billion € annually in view of a global population of 271 million dairy cows [1]. It has been estimated, that about 1/3 of all dairy cows are suffering from mastitis at least once per year [2].

Mastitis is a dynamic disease and has different forms. Clinical mastitis is easily detected by visible abnormalities of milk and external symptoms. The diagnostics of subclinical infections is more problematic as there are no detectable signs besides the changed characteristics of milk.

For the identification of mastitis-causing pathogens, the gold standard is microbiological culturing of bacteria, which in recent years has been partially replaced by polymerase chain reaction analyses of bacterial DNA. Although reliable, these methods require hours to obtain results, not allowing effective treatment of animals and optimal milk processing. Therefore, to assure timely and correct treatment of animals, there is a great need for a method applicable for rapid automatic detection of mastitis causing pathogens in milk.

A challenging tool for on-line detection of pathogens is the application of biosensors. The benefits of biosensors are their high selectivity, low cost, simplicity and short analysis time. There have been already proposed several biosensors for detection of particular bacteria, while biosensing systems for rapid simultaneous detection of pathogens, especially the specific mastitis-causing ones in milk matrix, are scarce.

The main goal of the present work was to develop a rapid method for multiplex detection of major mastitis causing pathogens, which can be used in farms for automatic monitoring of raw milk. We used an immuno-biosensing system, based on bead injection analyses. This biosensor system allowed semi-specific capture of bacteria onto a micro-column and consecutive specific detection of the attached bacteria with differently labelled secondary antibodies. We found the optimal conditions for both single and multiplexed pathogen detection and used the developed protocols for the studies of the selectivity and sensitivity of the biosensing system in buffer and in milk matrix. A novel approach for the calibration of biosensor for single pathogens was proposed and the characteristic parameters calculated for the detection of all studied pathogens (*Staphylococcus aureus*, *Escherichia coli* and S*treptococcus uberis)* in milk. In Estonia, these three pathogens are responsible for nearly 40% of all cases of mastitis [3]. Finally, the biosensing system was used for the detection of pathogens in the milk of infected cows, whereas the unknown pathogens were quantified both with the biosensor and microbiological cultivation.

1. LITERATURE OVERVIEW

1.1. Mastitis

Mastitis is an inflammation of the mammary gland, caused by the invasion of various pathogens through the teat canal, allergy or physical trauma [4]. In dairy industry, mastitis is one of the most costly problems. The resulting economic burden includes direct losses as temporary/permanent decrease in milk production and full rejection of milk in cases of clinical mastitis or due to the presence of antibiotic residues in the milk of treated animals. Mastitis also affects the quality and composition of milk: contents of lactose and casein are decreased while contents of whey protein and total protein can be increased [5–7]. Indirect losses include increased veterinarian and medication costs, additional diagnostics/laboratory expenses, decreased animal sale prices and finally premature culling of dairy cows and their early replacement costs [5–7]. The total mastitis-caused losses in global dairy industry are estimated to be 16–26 billion € per annum based on the overall dairy cow population of 271 million [1].

Mastitis is the most frequent disease in dairy farms, it has been estimated that about 1/3 of all cows in the world are suffering from mastitis at least once per year [2]. Mastitis pathogens are usually bacteria and more than 150 different bacterial species and subspecies may be involved in the induction of bovine mastitis [6]. In addition molds, yeast, and prototheca can act as mastitis inducing agents [6]. However, in majority cases are caused by one primary pathogen from approximately ten bacterial species or groups of species, like *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), *Streptococcus uberis* (*Str. uberis*), *Streptococcus agalactiae* (*Str. agalactiae)*, *Streptococcus dysgalactiae* (*Str. dysgalactiae)* and *Mycoplasma spp* [6;8]. In Estonia, three major pathogens *S. aureus*, *E. coli* and *Str. uberis* are responsible for nearly 40% of all cases of mastitis [3]. In Israel the proportion of mastitis caused by *E. coli* is even as high as 60% [9].

Bovine mastitis can be classified as contagious or environmental, depending of pathogens primary reservoir and how they are contracted and transferred between animals [10]. Contagious pathogens are usually found on the udder or teat surface of infected cows and are the primary sources of infection between uninfected and infected udder quarters, which occurs during milking. This kind infections are usually caused by *S. aureus*, *Str. agalactiae*, *Str. dysgalactiae*, *Mycoplasma bovis* and *Corynebacterium bovis* [11].

Environmental mastitis is caused by organisms such as *E. coli*, *Str. uberis* and *Klebisella pneumoniae*, which normally do not live on skin or in udder. These pathogens enter the teat canal when a cow comes in contact with contaminated environment. These bacteria are often found in faeces, bedding materials and feed [4]. The control of environmental mastitis in farms has been less effective than of the contagious one and therefore proportion of intramammary

infections caused by environmental pathogens has been increased markedly [12].

Milk of animals suffering from clinical mastitis is characterized by visible abnormalities like flakes, clots and a watery appearance; external symptoms of clinical mastitis are usually associated with udder heat, redness, swelling and sensitivity to touch [4]. Subclinical mastitis (SM) is a non-symptomatic form of intramammary inflammation. Upon one clinical case, comes new 15–40 subclinical cases, making this the most frequent form of mastitis [13]. Compared to healthy animals, subclinically infected cows produce less milk, and the quality of the milk is reduced similar to clinical mastitis. In addition, infected cows can be a source of infection to other animals in the herd [14].

Mastitis may often to turn chronic, so it is important to identify new cases in the herd in time. Early detection and identification of pathogens allows to accelerate decisions on treatment, thereby contributing to animal health, reducing the time required to recover the production of normal milk and reducing antibiotics use [15].

Clinical mastitis is easily detected by visible signs; diagnosis of SM is more complicated and challenging. The most common methods for the detection of SM are California Mastitis Test (CMT), somatic cell count (SCC) and electrical conductivity test [16;17]. Unfortunately any of these methods does not indicate the causative agent of mastitis nor allows their quantitative detection. For example, somatic cells including lymphocytes, macrophages, polymorphonuclear and epithelial cells are part of the natural defence mechanism [18]. SCC threshold of 200,000 cells/ml is sometimes used to indicate the status of subclinical mastitis under field conditions [19], but as SCC number actually depends on genetic factors and feeding of animals has it just an indicative parameter.

The influence of mastitis-causing pathogens and SCC on milk quality must be minimal, because milk should be safe for human consumption. The cut-off value for the acceptance of a pathogen in human food depends on its ability to grow in refrigerated conditions, at which psychotrophic bacteria, such as *Listeria monocytogenes*, are able to proliferate [20]. The total bacterial count in raw milk used in processing should be lower than 10^5 CFU/ml [21]. According to Regulation No. 71 of the Estonian Ministry of Agriculture, the specific limit for *S. aureus* in raw milk for direct marketing and raw milk products should be under 500 CFU/ml and 2000 CFU/ml, respectively [22]. The European Commission has set limits to the titters of *Enterobacteriaceae* in pasteurized milk (<10 CFU/ml), and *E. coli* for milk that has undergone a lower heat treatment than pasteurisation (<100 CFU/ml). The presence of *E. coli* in primary milk production is considered to be an indicator of faecal contamination and low hygienic conditions [23].

1.2. Short characterization of the major mastitis pathogens studied

1.2.1. *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive round shaped facultative anaerobe (≈ 1 μm), which form grape-like microscopic clusters [24;25]. *S. aureus* is nonmotile, non-spore forming, non-moving, catalase and coagulase positive bacteria [24;26]. *S. aureus* is part of the natural micro-flora of humans, commonly found on skin and hair as well as in noses and throats of mammalians [27].

Protein A is a 42 kDa protein originally found on the surface of *S. aureus* cell wall [28]. Practically all (~99%) *S. aureus* pathogenic strains possess surface protein A (SpA) and therefore is this protein a typical marker for the identification of pathogenic *S. aureus* strains [29–31]. Only very few other bacterial species (some strains of *Staphylococcus epidermidis*) can produce SpA and only in minute amounts [30;31]. It has been estimated that at least 1,200,000 protein molecules are represent the machinery of life in a single *S. aureus* cell in average [32]. Although the distribution of SpA changes during the cell life-cycle, it is proposed to cover comprises 7% of the cell wall [33]. SpA can serve as a characteristic target for the identification of pathogenic *S. aureus* cells in mastitis milk, as it is absent in nonpathogenic staphylococci (*Staphylococcus carnosus*, some strains of *Staphylococcus xylosus* and *Staphylococcus equorum*) [34;35]. Each individual SpA molecule reacts with mammalian immunoglobulins (IgGs), or in fact with the Fc fragment of IgGs, therefore can this protein used as a marker for quantitative and qualitative immunological techniques [36;37]. The affinity of SpA to the Fc region of human IgG is very high; the value of affinity constant is 10^8 l/mol [38].

S. aureus is one of the most widely spread contagious pathogens in dairy cattle, causing both subclinical and clinical forms of mastitis. In Estonia, it caused 11.7% of all registered cases of subclinical and 16.6–22.8% of clinical mastitis registered from 2007 to 2009 [39].

1.2.2. *Escherichia coli*

Escherichia coli is characterized as Gram-negative, chemoorganotrophic, oxidase negative commensal bacteria that was first described by Theodor Escherich in 1885 [40]. *E. coli* is a rod-shaped bacterium with 0.5 μm in diameter and 2 μm in length [41;42]. Most *E. coli* strains are harmless and colonize gastrointestinal tract of humans and animals as a normal flora [43]. *E. coli* is regarded as opportunistic and environmental pathogen [44].

In *E. coli*, 150 different proteins have been reported to be present in the cell envelope [45]. There is also the outer membrane protein A (OmpA), which aids the attachment and detection of the bacteria and are present at 10^5 copies per

cell [46]. This highly expressed protein is an ideal antigen for sensitive immunobiosensing, as each cell can potentially bind numerous detecting molecules.

E. coli is one of the major causative pathogens of clinical mastitis [47]. In 2007–2009 *E. coli* was causing 15.9% of clinical and 2.5% of subclinical mastitis in Estonia [39]. The minimal number of viable *E. coli* bacteria that can induce the disease, known as the infectious dose, fluctuates between 10^4 and 10^8 cells, depending on the strain and the individual affected [48]. Clinical *E. coli* mastitis in cows is generally recognized as acute and its results are often fatal [49]. The presence of *E. coli* is usually connected with faecal contamination, a phenomenon that is exploited by public health microbiologists as an indicator of faecal pollution of water sources, drinking water and food [27].

1.2.3. *Streptococcus uberis*

Streptococcus uberis is a Gram–positive, catalase negative bacteria belonging to the *Streptococcaceae* family. The bacterium is aerotolerant and anaerobial and its cells have coccoid form with diameter 0.5 to 1 μm, occurring in pairs or chains [50]. This pathogen has been localized in farm environment including straw bedding, soil, faeces and water, but also on animals lips, skin and in wounds, where it incurs via licking [50;51].

Str. uberis is one of the major environmental pathogens causing bovine mastitis [50]. Depending on the severity of inflammation, this pathogen can cause subclinical, clinical and chronic forms of udder infection [52]. *Str. uberis* has been identified as cause of clinical mastitis in 18.4% cases, while for subclinical mastitis its involvement was 11.8% of cases in Estonia between 2007–2009 [39].

Str. uberis cells express special membrane-bound proteins, known as surface adhesion molecule (SUAM) or lactoferrin binding protein (LBP) [53]. This protein is an important factor in the pathogenesis of *Str. uberis* caused mastitis as it is connected with in the adherence to and internalization of micro-organisms into bovine mammary epithelial cells [53]. The SUAM is exposed on the cell surface and antibodies against this protein significantly reduce bacterial adherence to bovine mammary epithelial cells [54]. It has been found that approximately 7800 binding sites of the protein molecules are expressed on the membrane surface of each bacterial cell with a dissociation constant (K_d) 1.0×10^{-7} M towards bovine lactoferrin [55].

1.3. Methods for the detection of mastitis causing pathogens

Mastitis has usually detected by indication of inflammation, estimation of somatic cell counts, or measurement of enzymes associated with tissue damage (e.g. N-acetyl-beta-D-glucosaminidase and lactate dehydrogenase) in milk; and identification of the causative microorganisms, which usually involves culturing methods [17].

The gold standard for identification of bacteria in milk is conventional microbiological bacterial culturing, where the presence of particular bacteria is indicated by their growth on an appropriate growth medium after incubation [56]. Microorganisms are identified according to their phenotypic characteristics as colony morphology, growth and serotyping [57]. The limit of detection (LOD) of microbiological method is 100 CFU/ml (there is 1 CFU per plate after cultivation of 0.01 ml sample) [58]. The cultivation method is quite time consuming and takes usually more than 48 h. Some bacteria, like *Str. uberis* and *Str. parauberis* cannot be clearly distingushed by biochemical assays [59]. In addition, false results can be obtained when mastitis is induced by bacteria capable of intracellular survival [15].

The microbiological culturing is suitable to detect only viable bacterial cells. Bacteria in the viable but nonculturable (VBNC) state fail to grow on the normal culturing media where they would normally grow [60]. However, these VBNC bacteria are still alive and capable of causing inflammation. Optimal culturing conditions for each microorganisms are different depending on the bacterial nature [61].

The novel methods are connected with direct detection of mastitis-causing bacteria using different molecular technologies, enzyme-linked immunosorbent assay (ELISA), nucleic acid amplification, flow cytometry, spectrometry and biosensing techniques. An emerging alternative for pathogen identification is polymerase chain reaction (PCR) test. PCR detects DNA sequences that are unique to specific bacteria. PCR is highly selective and is able to detect subdominant species. For the detection of six major mastitis causative pathogens a PCR-based assay has been proposed. The available test, directly usable for milk samples without any culturing steps, is specific for *E. coli*, *S. aureus*, *Str. uberis*, *Str. parauberis*, *Str. agalactiae* and *Str. dysgalactiae*. The LOD of the assay is 5×10^3 CFU/ml and it can be performed within 4.5 hours [62]. Lee *et al.* have developed a multiplex PCR biochip which can detect seven most common species of mastitis-causing pathogens like *Str. bovis, Str. uberis, Str. agalactiae*, *Str. dysgalactiae*, *Corynebacterium bovis, S. aureus* and *M. bovis* in milk within 6 h with the LOD value of 10^3 CFU/ml [15]. A new Thermo Scientific™ PathoProof™ Complete-16 Kit identifies even 16 main mastitis causing bacteria or bacterial groups (including *S. aureus* and all major coagulase-negative staphylococci) [63]. Despite the fact that PCR is a reliable and considerably fast method, the application of PCR methods requires

preliminary isolation of genetic material, being not suitable for in field analysis [64]. In addition, the determination process can be inhibited by a wide range of inhibitors present in mastitis milk such as proteinases, calcium ions, lactoferrin (leukocytes) and hemes (in blood containing milk) [6]. It is not possible to discriminate between live, nonculturable and dead cells with PCR methods.

Nucleic acid sequence based amplification (NASBA) has been used for pathogen analysis in food. NASBA is based on the enzymatic activity of reverse transcriptase that amplifies RNA templates into complementary DNA. O'Grady et al. introduced NASBA for the amplification of transfer-messenger RNA (tmRNA) for the detection of *S. aureus* in raw milk with detection limit of 1 CFU/ml within 3–4 h, there was no need for any culture enrichment of the samples [65].

A promising method for rapid pathogen detection in mastitis milk is loop–mediated isothermal amplification (LAMP), which is an alternative method of nucleic acid amplification in PCR. LAMP method is faster than traditional PCR and it does not require expensive specific equipment. Several LAMP assays have been described for detecting single mastitis causing pathogens such as *S. aureus* with LOD $1x10^2$ CFU/ml in mastitis milk [66], *Str. uberis* with LOD in raw milk 2.4×10^4 CFU/ml [67] and *M. bovis* with LOD in pasteurized milk 50 CFU/ml [68]. The detection time for each pathogen was approximately 1 hour. In addition Kawai et al. described a LAMP test for detecting 12 main mastitis causing pathogen families and/or genera in milk within few hours [61]. Unfortunately, this particular method does not provide any quantitative results.

Gey et al. have introduced a technique called fluorescent in situ hybridization (FISH), which utilizes oligonucleotides (labelled with some fluorophore) that are specific to a string target DNA/RNA. FISH has been applied for the detection of seven mastitis pathogens (*S. aureus, Str. agalactiae, Str. uberis, Enterecoccus faecalis, Enterecoccus faecium, E. coli* and *Trueperella (Arcanobacterium) pyogenes*) directly from milk samples. The method is capable to detect pathogens in real mastitis milk samples with LOD of 10^6 CFU/ml. However, the detection time is relatively long as sample pre-treatment is required (savinase treatment 45 min and hybridization 105 min) [69].

Another commonly used method for pathogen quantification is enzyme-linked immunosorbent assay (ELISA), which is a biochemical technique combining an immunoassay with an enzymatic assay. ELISA is a widely used immunological approach that is less complicated and less expensive. It takes typically 3–4 h, even 6 h, while it includes several consequent incubation steps [70;71]. ELISA is not suitable for on-line detection.

A promising technology for monitoring the safety of milk is flow cytometry. This method can provide accurate multiple bacterial counts with LOD below 10^4 cells/ml in ultra-heat-treated milk within 1 h [72]. Although flow cytometry is a fast method, it needs additional steps to distinguish between cell types. Its high cost and poor signal/noise ratio in complex matrices have prevented its uptake by the food industry [73]. A condensed summary of currently available technologies for the detection of pathogens in milk is given in Table 1.

Table 1. Methods for detection of major mastitis causing pathogens in milk.

Method for pathogen detection	Pathogens	Linear range, CFU/ml	LOD, CFU/ml	Time, h	Ref.
ELISA	*E. coli* O157:H7	n. a.	10^2	< 6	[71]
Flow cytometry	Total bacteria count in milk	n. a.	$< 10^4$	1	[72]
ELISA based PCR	*E. coli*	1–10^4	10^2	5	[74]
PCR	*Str. bovis, Str. uberis, Str. agalactiae*, *Str. dysgalactiae*, *C. bovis, S. aureus*, *M. bovis;* Simulataneously	10^3–10^7	10^3–10^5	6	[15]
NASBA	*S. aureus*	n. a.	1–10	3–4	[65]
Conventional microbiological culturing	*E. coli, S. aureus, Str. uberis;* Separately	n. a.	100	24–48	[58]
LAMP	*S. aureus*	10^2–10^6	10^2	< 1	[66]
FISH	*S. aureus, Str. uberis, Str. agalactiae, Enterococcus faecalis, Enterococcus faecium, E. coli, Trueperella pyogenes;* Simultaneously	10^6–10^8	10^6	≥ 3	[69]

ELISA – enzyme-linked immunosorbent assay; n. a. – data not available; PCR – polymerase chain reaction; NASBA – nucleic acid sequence based amplification; LAMP – loop–mediated isothermal amplification; FISH – fluorescent in situ hybridization

1.4. Biosensors for the pathogen detection

A fast developing tool for pathogen detection is biosensor technology, since it is portable, rapid and especially suitable for conducting on- and in-line multiplex measurements, operating in fully automatic or manual mode [75]. Biosensors are devices that convert the signal of a specific biorecognition reaction into a measurable, e.g. electrical or optical signal.

Biosensors are suitable for direct rapid analysis of complex samples requiring minimal or no sample treatment. Despite the great potential of biosensors, their use is still mostly limited to laboratories and only a limited number of biosensors for foodborne pathogens, like X-MARK™ (nanoRETE Inc.) and Aegis 1000 (BioDetection Instruments) are commercially available [73].

Different bioreceptors as antigen/antibody, enzymes, nucleic acid/DNA and cells have been used for the detection of bacteria in milk [76]. Currently available biosensing systems are typically limited for the detection of single pathogens, mostly *S. aureus* and *E. coli*. A selection of available biosensors for single pathogen detection in milk matrix is given in Table 2.

Table 2. Biosensors for single pathogen detection in milk.

Method for pathogen detection	**Pathogen**	**Bio-receptor**	**Linear range, CFU/ml**	**LOD, CFU/ml**	**Time**	**Ref.**
Immunomagnetic separation and colorimetric detection	*S. aureus*	Antibody	1.5×10^5–1.5×10^6	$1.5x10^5$	40 min	[77]
Amperometric magnetoimmunosensor	*S. aureus*	Antibody	4–$5x10^4$	1	2 h	[78]
Amperometric biosensor	*E. coli*	Enzyme	10^2 –10^8	10^2	1 h	[79]
Optical biosensor	*E. coli* O157:H7	Antibody	10–10^6	10	2 h	[80]
Potentiometric biosensor	*E. coli*	Aptamer-based	6–10^4	6	Few minutes	[81]
Piezoelectric quartz crystal microbalance	*E. coli* O157:H7	Antibody	10–10^6	53	4 h	[82]
Semi-quantitative magnetic detection	*Str. uberis*	Antibody	n. a.	10^2	5 h	[83]

Despite the increasing scientific interest and numerous biosensors, which have been proposed for the detection of single bacteria, biosensing systems for multiplex detection of pathogens, especially in milk matrix, are scarce (Table 3). At present, there are no biosensors, which could be used for automated simultaneous detection of pathogens in milk in time frame of minutes.

Table 3. Biosensors for multiplex pathogen detection.

Biosensors for multiple pathogen detection	**Pathogens**	**Bio-receptor**	**Mat-rix**	**Linear range, CFU/ml**	**LOD, CFU/ml**	**Time**	**Ref.**
Chemiluminecent antibody array	*E. coli, Salmonella spp.*	Antibody	Milk	n. a.	8×10^4 5×10^7	75 min	[84]
Microfluidic impedance immunosensor	*E. coli O157:H7, S. aureus*	Antibody	Milk	10^2–10^5	10^2	2 h	[85]
Immunomagnetic separation	*E. coli,* *Salmonella enteritidis*	Antibody	PBS	$5x10^2$–$5x10^5$ $4x10^2$–$4x10^5$	5×10^2 4×10^2	2 h	[86]
Flowbased kinetic exclusion fluorescence immunoassay	*S. aureus,* *Pseudomo-nas aeruginosa*	Antibody	BSA-PBS	$5.2x10^4$–$1.0x10^9$ $4.1x10^6$–$1.6x10^{10}$	5.2×10^4 4.1×10^6	<15 min	[87]
Glucose oxidase – peroxidase composite amperometric biosensor	*E. coli, S. aureus, Salmonella chole-raesuis*	Enzyme	PBS	6.5–650	6.5	7 h	[88]

2. AIMS OF THE STUDY

The main goal of the present work was to develop a rapid method for multiplex detection of major mastitis causing pathogens (*S. aureus*, *E. coli* and *Str. uberis*), which can be used in farms for automatic monitoring of raw milk. This main goal was divided to the following objectives:

- Design of a biosensing system for multiplex detection of bacteria.
- Design and separation of anti-*Str. uberis* antibody for application in biosensing system.
- Founding of optimal conditions and implementation of a protocol for the detection of major mastitis-causing pathogens.
- Studying the system's sensitivity in buffer and in milk matrix.
- Study of the system's selectivity in the presence of different pathogens.
- Calibration of the biosensor for the multiplex detection of all studied pathogens in milk matrix.
- Validation of the biosensor results and testing the system's applicability in real mastitis milk, whereas the unknown pathogens were identified and quantified both with the biosensor and standard microbiological cultivation method.

3. EXPERIMENTAL

3.1. Design of a biosensor for multiplex detection of bacteria

For the multiplex detection of mastitis-causing bacteria in milk an immunobiosensing system, based on specific antibody/antigen interaction, was proposed. The key aspects of the biosensor design were its selectivity, sensitivity, working range and applicability in complex biological sample matrix like milk.

First, pathogens were concentrated and captured using semispecific single-use renewable micro-columns, consisting of differently bioactivated Sephadex beads. The unbound sample components were removed by washing the column with buffer. The captured bacteria were selectively detected using specific antibodies conjugated with different fluorescence markers. The choice of fluorescence markers was based on the consideration that the absorption and emission peaks of different markers were not overlapping [I–III]. The illustrative scheme of the detection of the three most common mastitis-causing pathogens is shown in Figure 1.

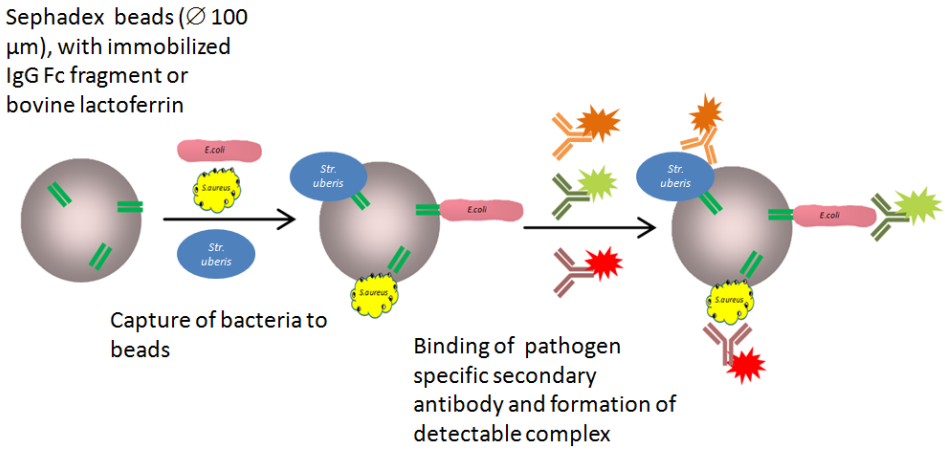


Figure 1. Scheme of multiplex biosensing of mastitis-causing pathogens using bead injection analysis platform. The sample is injected into the system, where pathogens are captured to the bio-functionalized beads. The captured pathogens are detected with specific antibodies conjugated with different fluorescence markers.

3.2. Biosensor set-up

Measurements were carried out using FIALab 3500 system (FIALab Instruments). This unit, comprising a high-resolution micro-syringe pump and a multi-position valve (Lab-on-valve, LOV), use computer control software to

carry out measurements in flows and allows to optimize all steps of assay protocol. The FIALab unit was connected with a light source (DH-2000, Ocean Optics) and spectrophotometer (USB 2000+ equipped with advanced electronics and extended 200 µm wide slit, Ocean Optics) to perform fluorescence measurements. For light transmission we used fibres with core diameter of 400 µm. The excitation light was filtered with an adjustable bandpass linear variable filter (Ocean optics LVF-HL), optimized for 300–750(±5) nm wavelength ranges. The LOV was additionally equipped with a moving plug to assure the capture of the beads into appropriate channel's geometry and shielded with a black light-tight cover to minimize the interference of incidental light. The biosensor set-up and the assay scheme on LOV are shown in Figure 2.

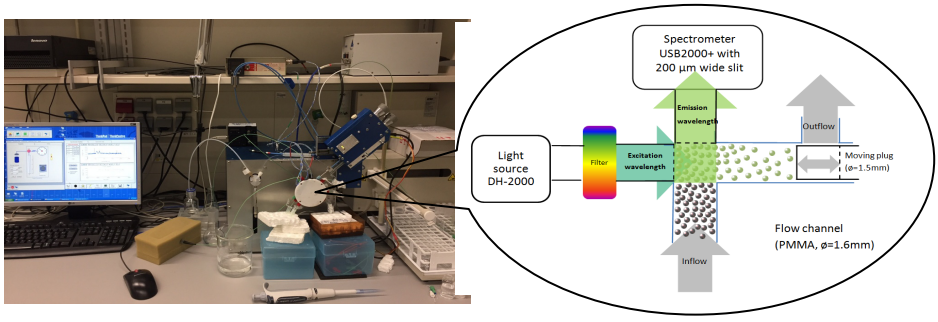


Figure 2. The biosensor set-up. Flow and light directions in measurement valve are shown with arrows: grey arrows show the direction of flow and beads movement; coloured arrows show the direction of excitation and emission light.

3.3. Measurements

3.3.1. Preparation of bioactivated beads and microcolumns for pathogens assay

For the binding of the studied pathogens from buffer or milk samples, human IgG Fc fragment and/or bovine lactoferrin were immobilized onto beads to form capturing microcolumns.

Human IgG was separated from the blood serum of healthy volunteers (donated by the Department of General and Molecular Pathology, University of Tartu) using affinity chromatography on protein G column, cleaved with papain and the Fc fragment purified on protein G column [I].

For the immobilization of pathogen-capturing proteins, Sephadex G50 Medium gel was first swollen in MilliQ water (1: 20 w/w) and activated with epichlorohydrin [I]. After that, the activated beads were incubated with IgG Fc fragment or bovine lactoferrin for 24 h and washed carefully to remove the unbound material. The only difference in the procedure of immobilizing human IgG Fc fragment and bovine lactoferrin were the pH values of binding buffers,

which were 9.5 and 13.0, respectively. Different pH values were selected according to different isoelectric point values of these proteins.

The free binding sites, potentially available for non-specific adsorption, were blocked with ethanolamine and after thorough washing the prepared biospecific gel was stored in Na-phosphate buffer (PBS, 0.01 M, pH 7.2, 0.15 M NaCl) at 4 ºC for further use. The presence of immobilized proteins on beads was controlled by staining the beads with Coomassie Brilliant Blue G-250 after bioactivation [I]. There was no significant change in binding activity of the gel for at least 2 months [I]. Biospecific beads were used for the production of microcolumns either as single agents or were mixed at different ratios depending on the aim of analyses.

3.3.2. Specific detecting antibodies

For the detection of *S. aureus* and *E. coli* commercial goat polyclonal anti-Protein A antibody (10 mg/ml; NB120–7243) conjugated with Texas Red (maximal emission at λ_{em}=615 nm) and rabbit polyclonal anti-*E. coli* antibody (4.0 mg/ml; NB 100–64448) conjugated with fluorescein isothiocynate (FITC, λ_{em}=525 nm), respectively (all from Novus Biologicals) were used [I, II]. As there are no commercial anti-*Str. uberis* antibodies available, these particular antibodies were designed using bioinformatic analysis and solid phase peptide synthesis to mimic the adhesion protein SUAM of *Str. uberis.* The antibodies were purified from the blood antisera of immunized rabbits (obtained from LabAs AS) with affinity chromatography, using synthetic peptides as affinity ligands [III]. The purified anti-*Str. uberis* antibody was conjugated with Lightning-Link® Rapid Dylight® 550 fluorescence marker (Innova Biosciences Ltd, λ_{em}=576 nm) [III].

3.3.3. Optimization of assay parameters

Optimal experimental conditions along with volumes of sample and analytical agents were found for the assay. First, to produce semiselective microcolumns we modified the volume of bio-activated beads from 10 to 40 µl (dry weight 1 mg to 4 mg). To secure the homogeneity of beads suspension, the beaker was constantly shaken during the inflow of beads. Transport of beads to the cell was secured by adding 30 µl PBS at a flow rate of 2 µl/sec. After forming of the pathogen-capturing column, a sample was introduced at a flow rate 1–5 µl/sec. We commonly used a sample volume of 150 µl to achieve a detection limit around 10 pathogen cells per ml. For the attachment of bacteria onto column, the sample incubation time was dependent on the bacteria and was up to 540 sec. The sample matrix and unbound bacteria were removed from the column with 700 µl PBS at the flow rate 10 µl/sec. Finally, 30 µl of secondary detecting antibody conjugated with a fluorescence marker was injected at flow

rate 1 µl/sec, incubated for 60–120 sec and the unbound antibodies were removed with 150 µl PBS at a flow rate 2 µl/sec. All measurements were carried out at room temperature [I, II].

3.3.4. Data acquisition and analysis

The presence of pathogens in a sample was detected by the fluorescence intensity of the complex of fluorescence marker conjugated detecting antibody and captured pathogen in a definite part of the microcolumn. FIALab 5.0 (FIALab Instruments, Medina, WA) software was used to control the system, and for data collection. Depending on the aim of the analysis, the fluorescence signal was recorded at up to 3 different wavelengths. The biosensor signal, characterizing a particular sample was calculated as a difference (ΔI) of average signal intensity before adding the secondary antibody and after removing unbound secondary antibody since the signal was stabilized (Fig. 3).

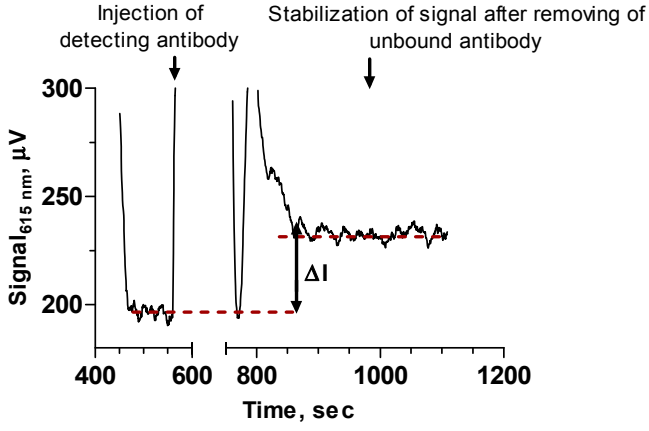


Figure 3. An example of biosensor signal intensity change during the experiment. The analysis was carried out at a *S. aureus* concentration of $4x10^8$ CFU/ml in PBS (0.01 M, pH 7.2, 0.15 M NaCl).

An average signal value for each pathogen concentration in the range 10^1–10^9 CFU/ml was calculated on the basis of 3–5 identical measurements. For data analysis and calculation of optimal parameters for biosensor calibration, we used GraphPad Prism® 5 software (GraphPad Software, San Diego, CA, USA).

3.5. Culturing of bacteria

E. coli (ATCC 25922) and *S. aureus* (ATCC 6538) were cultivated on blood agar plates in aerobic conditions for 24 h at 37 ºC. Bacterial cells were collected with sterile tampon, dissolved in Na-phosphate buffer solution (0.01 M, pH 6.5, 0.15 M NaCl) and stored at 4 ºC [I, II].

Str. uberis (ATCC BAA-854/0140J) was cultivated on sheep blood agar plates in aerobic conditions at 37 ºC for 48 h. After harvesting, the bacteria were recultivated in similar conditions for another 48 h. *Str. uberis* cells were collected, suspended in Na-phosphate buffer solution (0.01 M, pH 7.4, 0.15 M NaCl), washed twice with the same solution, centrifuged (JOUAN CR3i, 20 min, 4000 rpm) and stored at 4 ºC [III].

The bacterial concentrations were determined by optical density of the bacterial suspension at a wavelength λ=600 nm.

3.6. Milk samples

Milk was collected from healthy cows from the experimental farm of the Estonian University of Life Sciences, Tartu. Raw milk was centrifuged for 5 min (2450 x g) to remove fat. Milk samples were diluted 1:1 (v/v) with PBS (0.01 M, pH 7.2, 0.15 M NaCl) and used as a negative control. For a positive control, PBS used for dilution contained a fixed amount of bacteria and it was added to milk immediately before measurements.

Milk samples of cows suffering from acute clinical mastitis were collected from different farms in Estonia. The mastitis-causing pathogens in milk samples were at first identified with microbiological tests. Samples, where a major contagious pathogen was identified, were used for further analyses with the biosensor. To avoid the growth of bacteria in the collected samples, the samples were instantly freezed at –20 ºC and melted at 4 ºC before analyzing. The processing of the mastitis milk samples were similar to that described above for milk samples of healthy cows, except the added PBS contained no bacteria.

4. RESULTS AND DISCUSSION

4.1. Optimal protocol for pathogen detection

4.1.1. Detection of single pathogens

The following operational parameters of the biosensing system were optimized [I]:

1) Amount of bioactivated beads to form reproducible pathogen-sensitive micro-columns.
2) Input/output flow rates for gel, samples, washing buffer and detecting secondary antibodies.
3) Incubation times for antibody/antigen interactions.

A sufficient volume of the bioactivated gel suspension to form a micro-column was 20 µl. This volume contained ~2 mg dry beads and formed a stable micro-column. The reproducibility of column packing with such an amount of beads was very good, as no signal fluctuations exceeding background noise were detected in the series of 5 identical measurements [I]. The number of binding sites on the beads, with IgG Fc fragment or bovine lactoferrin could quantitatively bind pathogens with concentration up to 2×10^9 CFU/ml and 4×10^9 CFU/ml, respectively. It was sufficient to capture interacting bacteria in quantitative mode even at the highest studied concentrations which were exceeding in case of 150 µl sample the total bacterial number in mastitis milk at least 10 times in the case of all common mastitis-causing bacteria [69].

An optimal sample volume for the measurements was 150 µl [I]. This volume, assuming that at least one pathogen cell is statistically present in a sample, theoretically allows to achieve a LOD value of 6 CFU/ml [II]. In case lower LOD values are required, it is possible to use bigger sample volumes, although this may require longer time for analysis.

The sample input flow rates were varied in the range of 1–5 µl/sec. At flow rate 5 µl/sec, the maximal signal was achieved in 300 sec. The optimal input flow rate was 1 µl/sec, at which the maximal signal was achieved in 120 sec. To ensure the maximal value of the measured signal, an incubation time of 180 seconds, corresponding to 10 half-times of the association at a flow rate of 1 µl/sec was used for the attachment of pathogens onto the column [I].

High selectivity of the biosensor was achieved by dual step biosensing – the initial attachment of pathogens to the column (or pre-concentration) and the following specific antigen/antibody interaction with the bound pathogen. The incubation time of the detecting secondary antibody was varied from 0 to 180 sec. It was found that the optimal incubation time for forming detectable antibody-antigen complex was 60 sec for *S. aureus* and *Str. uberis* and 120 sec for *E. coli*, since the fluorescence signals did not increase during longer incubation times [I].

The background signal of the micro-column, caused by Sephadex G50 Medium beads was 3.2±1.3 μV (~5% of maximum signal) determined at different wavelengths between 500 and 700 nm [I].

To regenerate the biosensing system, the moving plug was opened and the flow channel was washed 3 times with 1 ml of PBS (0.01 M, pH 7.2, 0.15 M NaCl) (200 μl/sec), 3 times with 1 ml of 70% ethanol (200 μl/sec, ethanol was hold in a system for 3 min after last washing) and 2 times with 1 ml of PBS (200 μl/sec). After analyses of milk samples, 0.01 % Tween 20 was added to PBS used for washing.

4.1.2. Multiplex pathogen detection

For the simultaneous attachment of different pathogens onto microcolumn, a mix of beads functionalized with human IgG Fc fragment or bovine lactoferrin was used. Both *S. aureus* and *E. coli* bind to human IgG Fc fragment showing comparable affinity (with K_d values of 10^{-8} M) [89;90], whereas *Str. uberis* and *S. aureus* bind to bovine lactoferrin (with K_d values of 10^{-7} M and 10^{-8} M, respectively) [55;91]. Our experiments and the absence of data in literature indicated that there was no binding of *Str. uberis* to human IgG Fc or *E. coli* to lactoferrin (our unpublished data).

For the multiplex detection of all studied pathogens within their presumed concentration range from $<10^3$ to 10^7 CFU/ml in mastitis milk [69], we determined the optimal ratio of differentially functionalized beads for the formation of micro-columns. First we calculated the number of binding sites potentially available on the biofunctionalized beads for the attachment of pathogens from 150 μl sample. Assuming that binding agents form a monomolecular layer on the bead surface, we found that beads within 20 μl gel suspension, activated with human IgG Fc fragment or bovine lactoferrin, could quantitatively bind pathogens up to 2×10^9 CFU/ml [II] and 4×10^9 CFU/ml, respectively. This shows that the detection of pathogen concentrations up to 10^8 CFU/ml requires less than 10% of the specifically bioactivated beads of the total amount of the beads of the micro-column. As we used a two-component mixture of differently activated beads, the biosensor optimal working range was secured by mixing equal amounts of IgG Fc fragment and bovine lactoferrin functionalized gels and not changing the total volume of gel compared with single pathogen detection.

For the multiplex detection of pathogens, we combined the optimized protocols for single pathogen detection and used the longest required incubation time, being 180 sec for the attachment of pathogens to the column and 120 sec for the attachment of the detecting antibodies.

Assessing the sensitivity of the biosensor system towards different pathogens, we should also consider the number of antigenic determinants on the bacterial cell, accessible to detecting antibodies. In principle this epitope number is the multiplication factor of the measurable fluorescence signal, generated by

a single cell. It has been found that the number of protein A molecules on *S. aureus* cell outer membrane and outer membrane protein A (OmpA) molecules on *E. coli* membrane, which both interact with human IgG Fc fragment, is 10^4 and 10^5 respectively [II]. The number of *Str. uberis* adhesion protein molecules (SUAM) on the outer membrane of the cell, which ensure the binding of this pathogen to host cells and which fragment was used for the production of anti–*Str. uberis* antibodies is estimated to be 7800 [55].

4.2. Detection of pathogens

4.2.1. Detection of single pathogens

Using optimized protocols [I, II] we studied the dependences of the biosensing system signal on single pathogen concentrations both in PBS (0.01 M, pH 7.2, 0.15 M NaCl) and in milk matrix. The pathogen concentration range in our studies was chosen according to the predicted concentration of pathogens in mastitis milk [69]. An additional criterion was to meet the limits of allowed pathogen concentrations in milk. For direct marketing of raw milk and raw milk products the upper limit for *S. aureus* is 500 CFU/ml and 2000 CFU/ml, respectively [22]. The maximum limit for *E. coli* in raw milk is not officially established, but according to good practice is 10^3 CFU/ml [92].

S. aureus could be detected in a wide range – from 30 CFU/ml to 10^9 CFU/ml [I]. The dependence of the signal on *S. aureus* concentration in semi-logarithmic scale both in PBS and in milk is shown on Figure 4.

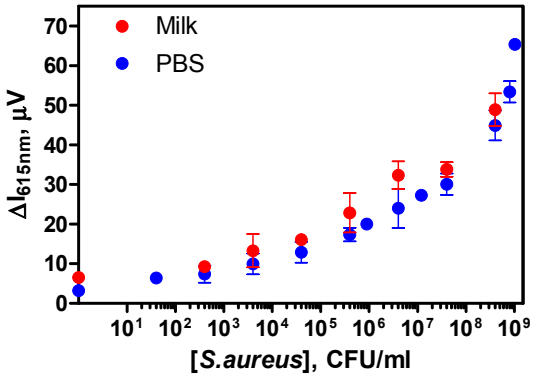


Figure 4. Dependence of the signal on *S. aureus* concentration in PBS (0.01 M, pH 7.2, 0.15 M NaCl,) and in milk (diluted 1:1 with PBS). The biosensor signal was calculated as a difference of average signal intensity ΔI before adding the secondary antibody and after washing unbound secondary antibody since the signal was stabilized at **λ**=615 nm.

In semi-logarithmic scale, we determined the linear working ranges for all studied single pathogens, although the determination of the limiting values of the working range was problematic, as the signal dependence obeyed exponential pattern. In PBS (blue circles in Fig. 4) the linear working range was from 3×10^3 to 4×10^7CFU/ml and in milk spiked with *S. aureus* (red circles in Figure 4) from 4×10^2 and 4×10^7 CFU/ml. The slopes, characterizing the linear part of the dependence, in PBS and in milk were 5.12±0.39 µV/log(CFU/ml) and 5.33±0.53 µV/log(CFU/ml), respectively [I]. The slopes of the linear range dependence in semi-logarithmic scale were similar in PBS and in milk, although the absolute value of signal in milk was about 5 µV higher than that of the signal in PBS at similar *S. aureus* concentrations. Higher signal in milk compared to PBS can be caused by matrix effect of milk and the aggregation of bacteria [II]. The detection limits in PBS and in milk were 30 and 200 CFU/ml, respectively, calculated as the *S. aureus* concentration corresponding to the signal, exceeding the average signal in pure PBS (3.2±0.2 µV) or milk (6.5±1.2 µV) by the value of 3 standard deviations [I].

For the detection of *E. coli* we used the biosensor system in pathogen concentration range from 30 to $3\text{x}10^6$ CFU/ml in a buffer solution and in milk (Fig. 5) [II].

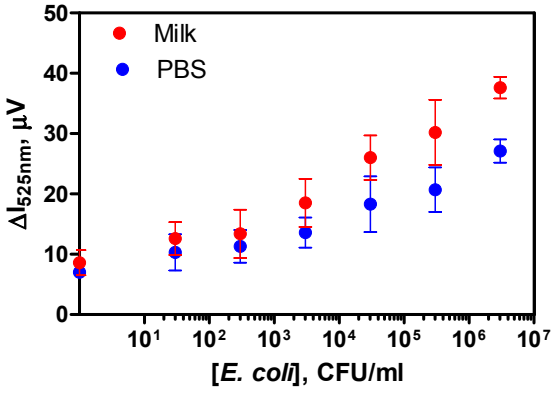


Figure 5. Dependence of the biosensing system signal on the *E. coli* concentration in PBS (0.01 M, pH 7.2, 0.15 M NaCl) and in milk (diluted 1:1 with PBS). The biosensor signal was calculated as a difference ΔI of average signal intensity before adding the secondary antibody and after washing unbound secondary antibody since the signal was stabilized at **λ**=525 nm.

The dependence of biosensor signal on *E. coli* concentration in semi-logarithmic scale was linear within the range 300–3×10^6 CFU/ml both in PBS (blue circles in Fig. 5) and in milk (red circles). The slope of linear part, which characterize the system sensitivity to *E. coli,* was 3.87±0.38 µV/log(CFU/ml) in PBS and 6.01±0.28 µV/log(CFU/ml) in milk. As already pointed out above, the

higher sensitivity in milk may be caused by the aggregation of bacteria, not occurring in buffer solution [II].

For the detection of *Str. uberis* a preliminary semi-logarithmic calibration curve was composed within a concentration range from 10–10^9 CFU/ml in PBS and in milk (Fig. 6).

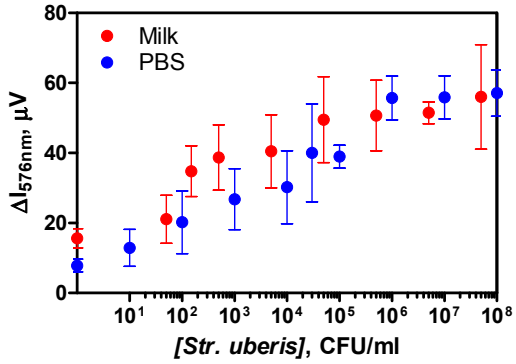


Figure 6. Dependence of the biosensing system signal on the *Str. uberis* concentration in PBS (0.01 M, pH 7.2, 0.15 M NaCl) and in milk (diluted 1:1 with PBS). The biosensor signal was calculated as a difference ΔI of average signal intensity before adding the secondary antibody and after washing unbound secondary antibody since the signal was stabilized at λ=576 nm.

The dependence of biosensor signal on *Str. uberis* concentration in semi-logarithmic scale was linear within the range from 100 to 10^6 CFU/ml both in PBS (blue circles in Fig. 6) and in milk (red circles). The slope of linear part of the biosensor signal dependence on the concentration of *Str. uberis* was 6.1±0.3 in PBS and 6.8±0.9 μV/log(CFU/ml) in milk.

The total time of analysis for each single pathogen with the biosensor was approximately 17 minutes.

4.2.2. Multiplex pathogen detection

To study the system's selectivity and possible interference of different pathogens on the biosensor signal of a particular pathogen, measurements were carried out in mixtures of three pathogens (*S. aureus, E. coli* and *Str. uberis*) at different concentration ratios.

Based on the obtained results, calibration graphs for the detection of different pathogens were constructed, wherein the results were compared with the results in samples, containing only one single pathogen. The statistical analysis revealed that the presence of several pathogens in samples had no relevant impact on calibration parameters within pathogens' concentration range 10^3–10^8 CFU/ml and the detected signals coincided within the range of errors [II]. These results confirm that the selectivity of the biosensing system in

the presence of several pathogens (even at concentrations up to 10^8 CFU/ml) is very good and it is applicable for the multiplex detection of different pathogens both in PBS and in milk. The micro-column formed with 20 µl of bead suspension, activated with human IgG Fc fragment or bovine lactoferrin, could quantitatively bind pathogens with concentration up to 2×10^9 CFU/ml [II] and 4×10^9 CFU/ml, respectively.

We also studied the possibilities to expand the linear region of the dependence of biosensor signal *vs* pathogen concentration over the whole studied pathogens' concentration range. So graphs on log (measured signal) *vs* log (pathogen concentration) scale were constructed for the determination of all three pathogens (Fig. 7).

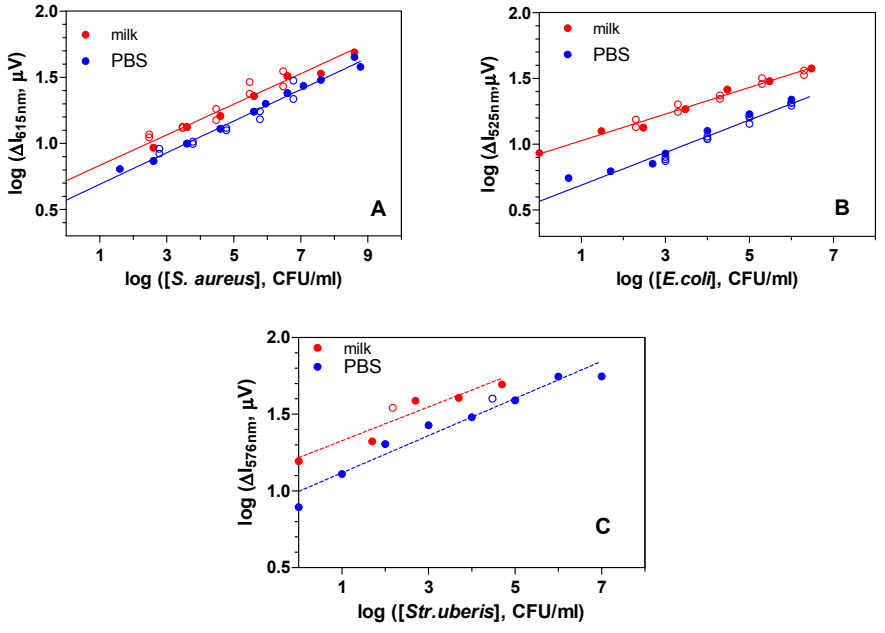


Figure 7. The calibration curves in PBS (0.01 M, pH 7.2, 0.15 M NaCl) and in milk (diluted 1:1 with PBS) in log-log coordinates. A: *S. aureus*; B: *E. coli*; C: *Str. uberis*. Data of experiments carried out with a single pathogen (filled circles) and in the presence of other pathogens at different concentration (open circles) are combined. Each point is an average biosensor signal value of 3–5 identical measurements.

The dependence of the biosensor signal on a particular pathogen concentration on Fig. 7 is characterized by the following equation [II]:

$$\log(\Delta I) = A\log[pathogen] + B, \quad (1)$$

where parameter A denotes the slope and B the intercept of the calibration curve. From Eq. 1, the concentration of a single pathogen can be calculated as follows [II]:

$$[pathogen] = 10^{\left[\frac{\log(\Delta I)-B}{A}\right]}. \quad (2)$$

According to Eq. 2, we calculated the value of parameters A and B for all studied pathogens for our biosensor set-up and used these for the determination of *S. aureus*, *E. coli* and S*tr. uberis* in milk samples. The obtained values of these parameters for *S. aureus*, *E. coli* and *Str. uberis* from the combined experimental data in milk are shown in Table 4.

Table 4. Calibration parameters for the detection of single pathogens in milk with our biosensor setup.

Pathogen	**A value**	**B value**
Staphylococcus aureus	0.116±0.006	0.718±0.031
Escherichia coli	0.101±0.003	0.925±0.015
Streptococcus uberis	0.0928±0.0169	1.25±0.06

As it can be seen from Table 4, the slopes of the calibration curves for different pathogens using different fluorophores were quite similar meaning that the biosensor sensitivity (parameter A) towards different pathogens in milk was alike. This is a promising starting point for the development of pathogens biosensors for practical applications.

The value of background signal (parameter B) for different pathogens was different, being the smallest for *S. aureus* and the largest for *Str. uberis*. This may be caused by light scattering on different wavelengths in milk caused by colloidal particles in milk, as well as the different dimensions of the bacterial cells determined.

Due to technical problems, the total time for multiplex pathogen detection was 35 minutes, but the analysis time can be reduced to the same time frame as for single pathogen detection by using combined light for the simultaneous excitation of fluorescent markers at different wavelengths or a different set of fluorescent markers, which have similar excitation, but different emission wavelengths.

4.3. Milk analysis and validation of biosensor results

To validate the results obtained with the biosensor system and test the system's applicability in real mastitis milk samples, a series of analyses were carried out, where unknown mastitis causing pathogens in infected cows' milk samples were detected and quantified both with the biosensor and microbiological cultivation.

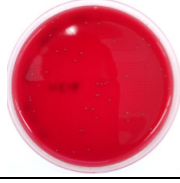
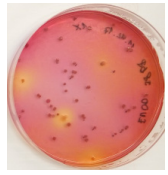
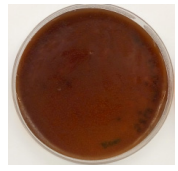
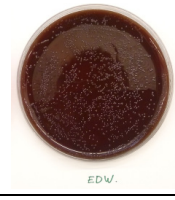
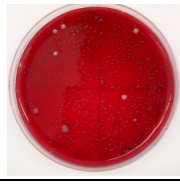
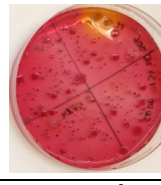
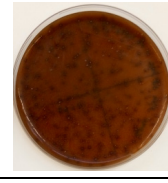
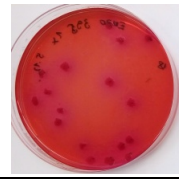
For the detection of *S. aureus* in mastitis milk, blood agar, where *S. aureus* typically forms a light to golden yellow pigment colonies which are surrounded by zones of clear beta-hemolysis, was used [93]. Coliforms and enteric pathogens were detected using their ability to ferment lactose on MacConkey agar, where lactose-fermenting bacteria, like *E. coli* form red to pink colonies while non-lactose fermenting bacteria appear as colourless or transparent colonies [94]. Edward's aesculin crystal violet blood agar was used as a selective medium for detecting streptococci. Aesculin-fermenting organisms like *Str. uberis* are forming black colonies; other streptococci are forming colourless colonies [95]. The results of microbiological analyses along with biosensor results for selected mastitis milk samples are shown in Table 5.

As it can be seen from Table 5, the identification of mastitis causing pathogens with different methods gives well–matched results. Differences between results with biosensor and microbiological cultivation in case of *E. coli* are caused by that, this medium is not totally specific towards *E. coli* and all lactose-fermenting bacteria form colonies. Therefore additional biochemical tests are needed for the confirmation of *E. coli.*

At the same time, the quantitative results obtained with different methods are varying. The correlation is better for samples, which contain blood and are curd-like than for samples, which externally look like normal milk. For these samples, the difference in the number of bacteria is several orders of magnitude. Such difference is caused by the fact that using different methods, the bacterial abundance is determined differently: biosensor detects reproductive as well as viable but nonreproductive and dead bacteria; while with microbiological cultivation it is possible to detect only reproductive bacteria. The other reason for different quantitative results can be the collection of samples, as in this study the farmers collected the samples theirselves and some milk samples were probably collected from cows whose treatment with antibiotics had been already started. So there may be many dead bacteria in milk, which also could cause overestimation of the number of bacteria in a sample with biosensor. For the implementation of cut-off values for pathogen concentrations in mastitis milk and the development of biosensor based diagnostic tests for the identification of animals suffering from different forms of infection, further studies should be performed to study the correlation of results obtained with different methods.

One mastitis causing pathogen was found in only two of the studied 17 samples, while in other samples there were two or more different pathogens. According to current practice, the simultaneous presence of two or more bacterial species in milk is considered to be a sample contamination during collection. On the other hand, there are references in the literature [69] that mastitis can be caused by several pathogens at the same time. Thus, the biosensor results can give additional information about the presence and concentration of several pathogens and help to modify the schemes of animal treatment, which are currently focused only on the main mastitis-causing pathogen.

Table 5. Concentrations of *S. aureus*, *E. coli* and *Str. uberis* in mastitis milk samples, determined with biosensing system and microbiological cultivation (pictures of representative samples).

	***Staphylococcus aureus*, CFU/ml**		***Escherichia coli*, CFU/ml**		***Streptococcus uberis*, CFU/ml**		**Description of the milk sample**
	Bio-sensor*	**Hemolytic colonies on blood agar****	**Bio-sensor***	**Pink colonies on MacConkey agar**/*****	**Bio-sensor***	**Black colonies on Edwards agar****	
1	3.8×10^6	5.04×10^5 	10^3	4.9×10^2 	10^3	80 	Medium fat consistence, like a normal milk
2	–	–	–	1.4×10^3 	5.4×10^5	7.6×10^3 	Bloody, curdlike, low fat consistence
3	8.6×10^6	5.12×10^5 	10^2	3.36×10^3 	10^5	2.76×10^3 	High fat consistence, flaky
4	90	0	–	1.8×10^2 	3.9×10^5	0	High fat consistence, like a normal milk
5	–	0	$4,7\times10^2$	$2,4\times10^3$ 	–	0	Very low fat consistence, lumpy

*– An average concentration of 3–5 measurements
**– Determined from the results of three different sample dilutions (nondiluted; 10×; 100×)
***– Total quantity of lactose-fermenting bacteria
– – Under the limit of detection

CONCLUSIONS

As a result of these doctoral theses, a novel method and an immuno-biosensing system have been developed and applied for the rapid multiplex detection of major mastitis causing pathogens in milk.

The key features of the biosensor development were its selectivity, sensitivity, working range and applicability for rapid automated analyses in complex biological sample matrix like milk.

To carry out the analyses, pathogens were first concentrated by semispecific capturing onto single-use renewable micro-columns and then specific antigen/antibody interaction was used for the detection of individual pathogens. Specific antibodies conjugated with different fluorescence markers of non-overlapping emission maximums are required to identify different pathogens at the same time. We used commercial fluorophore labelled antibodies for the detection of *S. aureus* and *E. coli*. As no anti-*Str. uberis* antibodies are commercially available, these particular antibodies were designed using bioinformatic analysis to mimic the antigenic regions of adhesion protein SUAM, exhibited on *Str. uberis* membrane. The antibody production was ordered by immunizing rabbits with the conjugates of the selected antigenic peptides, which were synthesized in solid phase. The monospecific anti-*Str. uberis* antibodies were purified from the blood antisera using affinity chromatography, and conjugated with Dylight 550 marker for their application in *Str. uberis* immunosensor. In our biosensor, *S. aureus* was detected at wavelength λ=615 nm, *E. coli* at λ=525 nm and *Str. uberis* at λ=576 nm.

Optimal experimental conditions for single as well as for multiplex pathogen detection were found by varying sample volume, incubation time, etc. Using these optimized protocols, the dependences of the signal of the biosensing system on three major mastitis-causing pathogens (*S. aureus, E. coli* and *Str. uberis*) concentrations were studied both in buffer and in milk matrix. The biosensor signal was a bit higher in milk than in buffer in case of all studied pathogens.

The system's selectivity and possible interference of different pathogens on biosensor signal was also studied. The obtained results confirmed that there was no cross-reactivity in the presence of other bacteria at their concentrations up to 10^8 CFU/ml, confirming the applicability of the biosensor for the multiplex detection of different pathogens.

The linear region of the biosensing system's working range was considerably extended by using logarithmic scale for the construction of calibration curves. Our biosensor calibration parameters for this model were calculated for all studied pathogens and used for the determination of these pathogens in milk samples. For the validation of the results obtained with our immuno-biosensing system and testing the system's applicability in real mastitis milk, a series of analyses were carried out, where unknown mastitis causing pathogens in the milk of infected cows were detected and quantified both with the biosensor and

microbiological cultivation. The identification of mastitis causing pathogens with different methods gave well–matched results, although, further studies are required to understand the correlation between quantitative results, which were obtained with different methods. Establishment of such correlations allows to define the cut-off values of pathogen concentrations in milk for the detection of potential infections and form a solid base to carry out automated monitoring of the health of cows and identification of animals suffering from subclinic or clinic mastitis in early stage of infection. Early identification of infected animals allows timely start of their accurate treatment, improving the welfare of animals. The biosensor results can give valuable information about the presence and concentrations of several pathogens and help to modify animal treatment schemes, which are currently focused on the major single mastitis-causing pathogen. The application of pathogen biosensors also allows to reduce production costs and improve the quality of raw milk and dairy products. Based on the results of the present study, the proposed biosensing system has a great potential to serve as a tool for in-line automatic monitoring of milk and animal health in dairy farms.

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SUMMARY IN ESTONIAN

Biosensorsüsteem mastiiti põhjustavate bakterite kiireks ja samaaegseks määramiseks piimas

Käesoleva doktoritöö eesmärgiks oli välja töötada biosensorsüsteem peamiste mastiiti tekitavate patogeenide (*Staphylococcus aureus*, *Escherichia coli* ja *Streptococcus uberis)* kiireks samaaegseks määramiseks piimas. Metoodika põhineb spetsiifilise antigeen/antikeha bioäratundmissüsteemi kasutamisel kombineerituna poolspetsiifilise graanulsisestusanalüüsiga kolme erineva patogeeni määramiseks [I–III].

Mastiit on udarapõletik, mis enamasti tekib patogeensete mikroorganismide sattumisel läbi nisajuha udaraveerandisse, olles peamine lüpsilehmade nakkushaigus. Mastiidi poolt põhjustatud kahju kogu maailma 271 miljoni piimalehma kohta on hinnanguliselt 16–26 miljardit eurot aastas. Suur majanduslik kahju on põhjustatud haigestunud loomade piimatoodangu ja selle kvaliteedi vähenemisest, suurenenud kulutustest diagnostikale ja veterinaarravimitele, ravimijääkidega piima utiliseerimise vajadusest ning enneaegsest loomade väljapraakimisest. Hinnanguliselt kannatab 1/3 kogu maailma lüpsilehmadest vähemalt kord aastas mastiidi all.

Kliinilise avaldumise järgi jaotatakse udarapõletikke kliinilisteks ja subkliinilisteks. Kliinilise mastiidi avastamine toimub väliste haigustunnuste alusel; samuti on täheldatavad muutused piimas, mis on tükiline, kohupiimataoline ning võib sisaldada verd. Subkliinilise mastiidi korral loomal nähtavad haigustunnused puuduvad, kuid piimas on suurenenud põletikuindikaatorite sisaldus. Kõige hõlpsamini määratavaks põletikuindikaatoriks käesoleval ajal on somaatiliste rakkude arv (SRA), mis aga ei anna infot haigustekitaja kohta.

Traditsiooniliste meetoditena mastiiti tekitavate bakterite identifitseerimiseks on tänapäeval kasutusel mikrobioloogilised analüüsid, mis võtavad aega 1–2 päeva ja laboratoorsetes tingimustes tehtavad patogeenide geenianalüüsid, mille tegemiseks kulub peale proovide laborisse jõudmist ligikaudu 6 tundi. Kuna ravi edukuse tagamiseks on selle täpsuse kõrval väga oluline ka selle võimalikult operatiivne alustamine, siis on vajalik välja töötada sellised analüüsimeetodid, mis võimaldavad patogeenide identifitseerimist oluliselt kiiremini kui praegu ning mida on võimalik kasutada farmides kohapeal. Tänasel päeval sellised meetodid puuduvad.

Üheks võimalikuks alternatiiviks traditsioonilistele kasutusel olevatele meetoditele on biosensorite kasutamine. Biosensorite eeliseks on lihtsus, lühike analüüsi aeg ja odavus ning nende sobivus kohapealseks analüüsiks. Selle metoodika puhul jääb ära aeganõudev proovide eeltöötlus.

Doktoritöö käigus töötati välja biosensorsüsteem ja mõõtemetoodika kolme peamise mastiiti tekitava patogeeni – *Staphylococcus aureus*'e *Escherichia coli* ja *Streptococcus uberis'*e määramiseks nii eraldi kui ühtlasi ka kõikide nimetatud patogeenide koos määramiseks. Biosensorsüsteemi konstrueerimisel uuriti selle tundlikkust, tööpiirkonda, selektiivsust ja sobivust rakendamiseks keeru-

listes maatriksites nagu piim. Erinevate bakterite üheaegse määramisesüsteem põhineb mitmel etapil: esmalt kogutakse patogeenid poolspetsiifiliste graanulitega, seejärel kasutatakse igale mikroorganismile selektiivset antikeha, kusjuures erinevad antikehad on seotud erineva luminestsentsmarkeriga, mille neeldumis- ja kiirgusspektrid ei kattu, mis seega võimaldab baktereid samaaegselt määrata [I–III].

S. aureuse ja *E. coli* vastased antikehad koos vastavate fluorestentsmarkeritega on kommertsiaalselt saadaval, kuid ei ole müügil antikehasid *S. uberise* vastu. Seetõttu *S. uberise* vastased antikehad toodeti, eraldati ja puhastati immuniseeritud küülikute vereseerumist, märgistati need luminestsentsmarkeriga ning kasutati saadud antikehasid *S. uberise* määramiseks biosensorsüsteemis [III].

Töö tulemusena leiti optimaalsed eksperimentaalsed tingimused nagu proovi suurus, voolu kiirus, inkubatsiooni aeg kõigi bakterite jaoks. Mitme bakteri samaaegsel määramisest lähtuti "halvimast" tulemusest [I, II]. Kasutades neid optimeeritud protokolle leiti biosensorsüsteemi signaali sõltuvused erinevate bakterite kontsentratsioonidest nii puhvrilahuses kui ka piimas. Antud lähenemine võimaldas antud bakterid identifitseerida ja kvalifitseerida vahemikus 10–10^8 CFU/ml. Piimas oli foon kõrgem, kuid see ei takistanud määramist. Oluline on, et kõiki baktereid on võimalik kvantifitseerida kuni 10^8 CFU/ml ka teiste bakterite juuresolekul.

Uuritud biosensorsüsteemide lineaarne tööpiirkond kõikide patogeenide puhul oli sobilik biosensori kasutamiseks reaalsetes piimaproovides [I, II].

Biosensori selektiivsus kindla patogeeni määramiseks teiste bakterite olemasolul (kuni kontsentratsioonini 10^8 CFU/ml) oli väga hea ning seda on võimalik kasutada mitme bakteri samaaegseks määramiseks [II]. Kõigi uuritud bakterite jaoks leiti biosensori kalibreerimisparameetrid, mis on aluseks biosensorite edaspidisele kasutamisele mastiiti põhjustavate bakterite määramiseks piimas.

Väljatöötatud biosensorsüsteemi testimiseks ja saadavate tulemuste usaldatavuse hindamiseks analüüsiti mastiiti haigestunud lehmade piimaproove kolme uuritud patogeeni sisalduse suhtes nii biosensoriga kui ka mikrobioloogiliste külvide abil. Tulemuste võrdlemisel selgus, et bakterite identifitseerimisel oli tulemuste kokkulangevus väga hea. Tulemuste erinevusi oli patogeenide kontsentratsiooni määramisel, mis tuleneb sellest, et erinevalt mikrobioloogilisest analüüsist määratakse biosensoriga lisaks kasvuvõimelistele patogeenidele ka nn „uinuvaid" ja surnud baktereid. Samuti oli erinevus kahe meetodi vahel *E. coli* määramisel, mis tulenes sellest, et konkreetne külv ei ole selektiivne *E. coli* suhtes. Erinevatel meetoditel saadud kvantitatiivsete tulemuste korrelatsioonide leidmine on edasiste uuringute oluline eesmärk, et defineerida haigestumist näitavad patogeenide kontsentratsioonid ning töötada välja alused loomade tervise automaatseks monitoorimiseks. Kuna käesoleval ajal identifitseeritakse piimaproovides tavaliselt ainult üks peamine mastiiti tekitav patogeen, siis biosensoriga saadavad tulemused annaksid edaspidi võimaluse loomade raviskeemide modifitseerimiseks ning täpsustamiseks. Väljapakutud

biosensorsüsteemil põhinevat mõõtesüsteemi on võimalik kasutada loomade tervise automatiseeritud kontrolliks farmis kohapeal ja seeläbi kiiresti identifitseerida juba varajases staadiumis potentsiaalne haigus. Varajane haiguse avastamine aitab alustada koheselt kiiret ja sobivat ravi, parandades seeläbi looma heaolu ja piima kvaliteeti ning vähendada tootmiskulusid ja majanduslikku kahju aga vähendab ka ravimijääkide sattumist loodusesse.

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PUBLICATIONS

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Mihklepp, K., Kivirand, K., Nikopensius, M., **Peedel, D**., Utt, M., Rinken, T. Design and Production of Antibodies for the Detection of Streptococcus uberis. Enzyme and Microbial Technology, 2017, 96 (1), 135–142.

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