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Immunodetection of *Streptococcus uberis* pathogen in raw milk

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ABSTRACT

Streptococcus uberis is a major mastitis-causing environmental pathogen, which rapid immunodetection has not been possible due to the absence of specific anti-*Str. uberis* antibodies. Recently, a specific antibody against the *Str. uberis* adhesion molecule (SUAM) has been designed. In the present study, the specificity and affinity of this antibody towards SUAM antigenic region SAPVYLGVSTE and *Str. uberis* cells are characterized, using experimental and *in silico* bioinformatic methods. The selectivity studies and bioinformatic analyses revealed high specificity of the antibody towards *Str. uberis*. The K_d value of SAPVYLGVSTE/anti-*Str. uberis* antibody complex was 27 ± 6 nM, indicating the applicability of this antibody for the detection of *Str. uberis*. The anti-*Str. uberis* antibody was used as a specific biorecognition element of a biosensor for the detection of *Str. uberis* bacteria in phosphate buffer and in milk and these analyses took less than 20 min. The *Str. uberis* biosensor was also tested in the milk of cows suffering from mastitis and the obtained results were in good agreement with the conventional identification of this pathogen by microbiological plating.

1. Introduction

Mastitis or the mammary gland infection is the most common and the most costly disease in dairy cattle. It causes significant economic burden worldwide due to the reduction of milk amount and quality, but also premature exclusion of affected animals from herds [1]. Bovine mastitis has been associated with over 100 different pathogens [2,3] that adapt well to the bovine host resulting in clinical signs or subclinical infections [4]. According to the mode of pathogen transmission, mastitis is classified as contagious or environmental. Nowadays the implementation of mastitis control plans has reduced the incidence of bovine mastitis caused by a contagious route of bacterial transmission, but has had little effect on the occurrence of mastitis due to bacteria which infect the gland from an environmental reservoir [5]. Recent studies show that environmental pathogens are becoming more prevalent and in 2016, over 40% of the analyzed cases in Europe were prevailed by *Streptococcus uberis* [6]. This presents a high risk of *Str. uberis* to become a serious threat to farms all over Europe.

Str. uberis is a gram-positive environmental pathogen [7], commonly found in manure and bedding [8]. The *Str. uberis* cells have a coccoid form (Ø 0.5–1 μm) and they are occurring in pairs or in chains as cell division occurs along a single axis [9]. *Str. uberis* bacteria possess a special membrane bound protein, known as surface adhesion molecule (SUAM) which plays a central role in adherence of *Str. uberis* into bovine mammary epithelial cells *via* bovine lactoferrin (bLF) [10,11]. The affinity of SUAM towards bLF is characterized by the value of K_d, which is 1.0×10^{-7} M [12]. SUAM is well exposed on the cell surface and there are about 7800 SUAM molecules on the membrane of each bacterial cell [12]. Being vital for the internalization of *Str. uberis* bacteria in udder and due to the big number of molecules on cell membrane, SUAM molecules establish an excellent target to be used for the immunodetection of *Str. uberis* pathogen.

At present, microbiological cultivation techniques and DNA-based molecular diagnostics are the most common tools for the detection of bacteria. The major drawback of conventional microbiological culturing is the time necessary to obtain results as the required incubation of milk samples on blood-esculin agar at 37 °C is 48 h [13]. Another limitation of microbiological methods is the need to use specific broths for the cultivation of particular strains. The usual sample size for cultivation is 10 μl, yielding an estimated limit of detection 10^2 CFU/ml.

Methods based on polymerase chain reaction (PCR) are being used in increasing trend for identification of infectious pathogens and mastitis diagnostics [14,15]. The first specific PCR assay for *Str. uberis*, worked out by Gillespie et al. in 2005 included an overnight enrichment step and allowed correct identification of up to 100% of *Str. uberis* in studied samples [16]. Currently, different commercial PCR assays for

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multiplex detection of mastitis-causing bacteria are available. For example, the latest PathoProof™ kits allow simultaneous detection of 16 major mastitis-causing bacteria [17]. PCR can provide bacteriological diagnosis in nearly half cases where conventional culturing results are negative [18]. Although PCR methods allow identification of specific bacterial strains, they still require several hours to obtain results and have some technical drawbacks as these methods are mostly based on the comparative study of 16S rRNA gene sequence, which sometimes fails to discriminate between related species and intragenomic heterogeneity [19].

Therefore, excessive interest still exists to find alternative methods for specific detection of infectious microorganisms in the timeframe of several minutes and various assays have been proposed for the detection of *Str. uberis* bacteria. Milner et al. suggested a method to detect pathogens causing subclinical infections by using electrical conductivity [20]. Unfortunately, this assay did not work for infections caused by *Str. uberis*. Thereafter Gardner et al. designed an assay comprising a pair of shear horizontal (SH) surface acoustic wave (SAW) devices which could detect *Str. uberis* at the level of ~ 10^2 CFU/ml [21]. A fluorescent *in situ* hybridization (FISH) method using oligonucleotides specific to *Str. uberis* was proposed by Gey et al. [22]. However, the FISH method was rather laborious and had relatively high detection limits - with enzymatic treatment, the assay was able to detect bacteria at $\geq 10^6$ CFU/ml. A loop-mediated isothermal amplification (LAMP) method for the detection of *Str. uberis* in raw milk was developed and evaluated by Cornelisson [23]. With this assay, it was possible to identify seven different *Str. uberis* strains among other mastitis-causing pathogens in milk with an analytical sensitivity of 2.4×10^4 CFU/ml within 60 min (detection time decreased along with the increase of the concentration of bacteria).

Recently our workgroup designed a specific antibody against the *Str. uberis* adhesion protein molecule (SUAM) with the aim to use it for selective biorecognition of *Str. uberis* [24], as there are no commercially available antibodies for this pathogen. In the current study, the specificity and affinity of this anti - *Str. uberis* antibody towards SUAM antigenic region SAPVYLGVSTE and intact *Str. uberis* cells are characterized using *in silico* and experimental studies. In addition, the applicability of this monospecific antibody as an immunosensor's biorecognition element for the detection of *Str. uberis* in the milk of mastitis-infected cows in less than 20 min is demonstrated. The obtained results are compared and validated with the gold standard of identification of bacteria in milk - microbiological culturing [25].

2. Materials and methods

2.1. Bioinformatic analysis

The bioinformatics search in order to estimate the uniqueness of the selected epitopes of SUAM and the according selectivity of the anti - *Str. uberis* antibody towards *Str. uberis* was conducted in comparison with 24 most common mastitis causing pathogens in Estonia [26]. Corresponding bioinformatical analysis was performed using the BLAST tool (Basic Local Alignment Search Tool) [27]. The alignment was performed against non-redundant protein sequences database with the default BLASTP parameters [28] at NCBI (The National Center for Biotechnology Information).

2.2. Purification of anti-Str. uberis antibody

Anti-*Str. uberis* antibodies were purified from the antisera of immunized rabbits with ÄKTA purifier UPC 10 (GE Healthcare) following the earlier published 2-step affinity chromatography protocol [24]. The immunization of rabbits was ordered from LabAs OÜ, Estonia and the schedule used two rabbits. For immunization, the 1st booster was made 3 weeks and the 2nd booster 6 weeks after the primary injection. Injection amounts were 0.5 mg antigen each, and oligopeptide SAPVYLGVSTE (with purity 99%) conjugated with carrier protein KLH was used as an antigen.

The amount of purified antibody was determined by absorbance at 280 nm using extinction coefficient 1.37 in a 1 cm light path cuvette for 1.0 mg/ml IgG solution. For the characterization and further use in *Str. uberis* biosensor, part of the purified monospecific antibodies was directly conjugated with a fluorescent DyLight® 550 marker (LightningLink®Rapid DyLight®550 antibody labelling kit, Innova Biosciences Ltd., UK) following the company's standard protocol.

The amount of monospecific anti-*Str. uberis* antibody in antisera was 0.30 ± 0.12 mg/ml (depending on the batch of rabbit antisera). According to earlier studies, the obtained amount of the specific antibody in antisera was in line with the common yields of specific antibodies in antisera, where the proportion of the desired polyclonal antibody has been found to be between 0.05–0.5 mg/ml of serum [29].

2.3. Characterization of the affinity of anti-Str. uberis antibody

For the characterization of the binding affinity of anti-*Str. uberis* antibodies, we used different experimental methods. First, surface plasmon resonance (SPR) analysis using a BIAcore3000 instrument with carboxymethylated dextran polymer coated CM5 sensor chips (GE Healthcare, Sweden) was performed to study the affinity of anti-*Str. uberis* antibodies towards SUAM. The oligopeptide fragment of SUAM with the best antigenic score SAPVYLGVSTE [24], which had also been used for the immunization of rabbits, was attached to the chip surface and binding of the anti-*Str. uberis* antibodies to this oligopeptide was followed by measuring the change in SPR refractive index in arbitrary response units (RU). For the immobilization of the oligopeptide, the chip surface was activated by injecting 35 µl of EDC (N-(3-dimethylaminopropyl)-N´-ethylcarbodiimide hydrochloride)/NHS (N-hydroxysuccinimide) mixture (0.2 M/0.05 M) at a flow rate of 5 µl/min. Then 60 µl of the oligopeptide (1 mg/ml in 500 mM carbonate buffer, pH 8.3) was injected at flow rate 2 µl/min. Excess non-bound peptide was washed away with 10 mM phosphate saline buffer with 0.15 M NaCl (PBS, pH 7.2). Free reactive ester groups were blocked by injection of 30 µl of 1 M ethanolamine (pH 8.5) at a flow rate 5 µl/min. The surface was washed with 10 mM PBS (pH 7.2). Measurements of binding kinetics were performed by injection of 100 µl of antibody solution in 10 mM PBS buffer (pH 7.2) at flow rate 5 µl/min. After each measurement, the bound antibodies were removed from the surface and the chip regenerated by injecting 30 µl of 6 M guanidine chloride at flow rate 5 µl/min. Kinetic analysis was performed using the BIAevaluation software.

2.4. Cultivation of Str. uberis bacteria

Str. uberis (ATCC strain BAA-854/0140J) was cultivated on sheep blood agar plates in aerobic conditions at 37 °C for 48 h and re-cultivated in the same conditions for another 48 h. Bacterial cells were collected, then suspended in 10 mM PBS (pH 7.2), washed twice with 10 mM PBS (pH 7.2) and centrifuged (JOUAN CR3i, 20 min, 4000 rpm). The optical density of the pathogen suspension was measured by DEN-1B densitometer (McFarland Densitometer 1B Biosan) with standard accuracy ± 3% of McFarland unit. The *Str. uberis* suspension was diluted to 10^9 CFU/ml and stored with 0.1% of sodium azide (MERCK, Germany) at 4 °C for maximum 2 months.

2.5. Immunosensor for the detection of Str. uberis bacteria

For the detection of *Str. uberis*, we used a similar immonosensing system as applied earlier for the detection of other pathogens like *Staphylococcus aureus* and *Escherichia coli* [30,31]. The measurements were carried out in a modified FIAlab 3500B system (FIAlab Instruments), which flow cell was shielded with an original black light-tight cover to minimize the interference of incidental light. As a light source we used DH-2000 (Ocean Optics) and the illumination light at

Table 1
Overlapping of SAPVYLGVSTE oligopeptide in *Streptococcus* spp. predicted with BLAST tool.

Bacteria	Host	AA[2]	Maximum score[3]	Query cover %[4]	Identity %[5]
Streptococcus hongkongensis	marine flatfish [32]	11/11	1752	97	99
Streptococcus pseudoporcinus	female gastro urinary tract [34]	9/11	843	97	50
Streptococcus porcinus	infections in swine [35]	8/11	842	97	51
Streptococcus iniae	Amazon freshwater dolphin [33]	10/11	770	97	48
Streptococcus didelphis	skin lesions, spleen, liver and lungs of opossums [36]	5/11	732	97	46
Streptococcus parauberis[1]	olive flounder, bovine [37]	8/11	718	97	45
Streptococcus equi	horses [38]	8/11	640	97	44
Streptococcus castoreus	European beaver (*Castor fiber*) [39]	9/11	627	96	41
Streptococcus phocae	Atlantic salmon (*Salmo salar*) [33]	9/11	621	96	42
Streptococcus ictaluri	channel catfish [40]	7/11	621	96	41
Streptococcus canis FSL Z3-227	dogs and cats [41]	8/11	605	97	41
Streptococcus pyogenes[1]	human diseases: skin infections to systemic diseases [42]	8/11	605	96	40

[1] Potentially mastitis-causing.
[2] AA – overlap of amino acid residues comparing the sequence of SAPVYLGVSTE and the membrane proteins of other *Streptococcus* sp.
[3] Maximum score – score of single best-aligned sequence.
[4] Query cover – input sequence (or other type of search term) to which all of the entries in a database are to be compared.
[5] Identity – the extent to which two amino acid sequences have the same residues in the same positions in an alignment, expressed as a percentage.

absorption wavelength of DyLight® 550 marker ($\lambda = 562$ nm) was set with an adjustable bandpass linear variable filter (Ocean Optics LVF-HL). The intensity of fluorescence at 576 nm was measured perpendicularly to the excitation with Ocean Optics USB 2000+ spectrophotometer equipped with advanced electronics and extended 200 µm wide slit. Fibres with core diameter of 400 µm were used for light transmission.

Str. uberis cells from the sample solution were captured and concentrated onto single-use renewable micro-columns, using their interaction with bovine milk lactoferrin (bLF, purity ≥85%, Sigma-Aldrich) - activated Sephadex G50 Medium (GE Healthcare) beads. The beads were prepared as described earlier [30]. The binding of bLF onto beads was controlled by staining with Coomassie Brilliant Blue G-250. The micro-columns were produced by injecting 20 µl suspension of bio-activated beads (approx. 2 mg beads in dry weight) from a constantly shaken beaker into the flow cell, where partial closing of the flow channel's end secured the capture of beads into appropriate geometry and free flow of solution. 150 µl of sample was added and the flow was stopped for 180 s to ensure the attachment of bacteria onto beads. The sample matrix and unbound bacteria were removed from the column with 150 µl 10 mM PBS (pH 7.2) and 30 µl anti–*Str. uberis* detecting antibody (10 µg/ml) conjugated with DyLight® 550 was injected and incubated for 60 s. The biosensor signal of the bound *Str. uberis* was calculated as the difference of the fluorescence intensity before and after the attachment of the detecting antibody as an average signal of 3–4 identical measurements. After each measurement the flow channel was opened, beads discarded and the system carefully washed with 10 mM PBS (pH 7.2) to prepare the system for next analyses. After analyses with milk samples the system was washed with 10 mM PBS (pH 7.2) containing 0.1% Tween 20 in order to remove residues of milk fat from the system. The total time of analysis was approximately 17 min.

2.6. Milk samples and validation of biosensor results

Milk samples, collected from healthy cows (from the experimental farm of the Estonian University of Life Sciences, Tartu), were centrifuged for 5 min (2450 × *g*) to remove fat and the remaining fraction was diluted 1:1 with 10 mM PBS (pH 7.2), containing a definite amount of *Str. uberis*. Milk samples of the cows, suffering from mastitis were collected from different farms in Estonia. To avoid the growth of bacteria in the collected samples, these samples were immediately frozen at −20 °C after collection and melted at 4 °C before analyzing.

The results obtained with the biosensing system were validated using microbiological cultivation of the analyzed samples on modified Edwards agar combined with sheep blood. For better quantification of bacteria, samples with volume of 100 µl were used for cultivation and if necessary, dilutions were made. The cultures were incubated at 37 °C for at least 24 h, after which the colonies formed were counted.

3. Results and discussion

3.1. In silico prediction of the anti-Str. uberis antibody specificity

To analyze the uniqueness of the SUAM's peptide sequence SAPVYLGVSTE, used for the production of rabbit anti-*Str. uberis* antibodies, the peptide sequence was analyzed with protein BLAST program. The BLAST alignments did not show any identity with membrane proteins from *E. coli*, *S. aureus*, *Str. agalactia*, *Str. dysgalactia* or other 20 most common mastitis-causing pathogens [26], indicating high selectivity of the produced antibodies towards *Str. uberis* among major mastitis-causing pathogens in bovine and the high application potential of these antibodies for the detection of *Str. uberis*.

An overlapping region in primary protein sequence (11/11) was found in membrane proteins expressed on other *Streptococcus* species - *Str. hongkongensis*, which is found in marine flatfish [32]; and some partial similarity with membrane proteins of *Str. iniae* (10/11) and *Str. phocae* (9/11), found in Amazon freshwater dolphins and Atlantic salmon, respectively [33]. The only potentially mastitis - causing *Streptococcus* species, which had some overlapping (8/11) with SAPVYLGVSTE oligopeptide, was *Str. parauberis*. However, this pathogen is currently very rare and in recent years there have been no cases of mastitis caused by this pathogen in Estonia [26]. The results of BLAST analyses of oligopeptide SAPVYLGVSTE similarity with membrane proteins of other *Streptococcus* spp. are shown in Table 1.

3.2. Affinity of the anti-Str. uberis antibodies

The affinity of the monospecific anti-*Str. uberis* antibodies towards the oligopeptide fragment of SUAM (SAPVYLGVSTE), which was also used for the immunization of rabbits, was characterized with SPR immunoassay. In qualitative terms the specificity of this antibody towards this antigen has been demonstrated earlier with an ELISA assay [24]. However, with ELISA it was not possible to determine the value of binding constant, essential for the construction and application of the *S. ubris* immunosensing platform.

In the SPR experiments the oligopeptide was covalently immobilized onto a sensor chip, and formation of the complex was registered by injecting the antibody onto this sensor chip. The peptide was immobilized instead of the antibody for two reasons. First, the

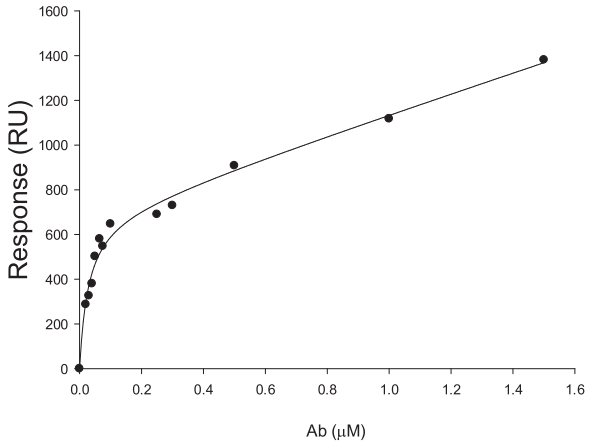

Fig. 1. Equilibrium binding curve for association of anti-*Str. uberis* antibody with immobilized oligopeptide SAPVYLGVSTE. The oligopeptide was immobilized onto carboxymethylated dextran polymer coated CM5 sensor chips. Antibody at different concentrations (from 19 nM to 1.5 μM) was injected over the chip with the peptide. SPR response values (RU) were registered at equilibrium. The presented curve was obtained by fitting SPR response data to the two binding site model. Measurements were performed at 25 °C using 10 mM PBS (pH 7.2) as a running buffer. After each measurement, 6 M guanidine chloride was used for the regeneration of sensor chip surface.

immobilized peptide did not lose its binding properties after regeneration the chip with 6 M guanidine chloride. Second, the binding of the antibody to the immobilized peptide would generate remarkably higher SPR response signal than the binding of the peptide to immobilized antibody, as the molecular weight of an IgG type antibody is more than 100 times greater than that of this peptide (150 *vs* 1.3 kDa).

The equilibrium binding curve, presented as a plot of SPR response at equilibrium as a function of antibody concentration (Fig. 1), revealed two oligopeptide/antibody interactions with clearly different affinities (K_d values were in nano- or in micromolar range). Analysis of the experimental data with the model of two parallel interactions ($A + P_1 \rightleftarrows AP_1$, $A + P_2 \rightleftarrows AP_2$, where A denotes antibody and P oligopeptide) resulted in a K_d value of 27 ± 6 nM for the high affinity interaction. Since the low affinity region of the curve was almost linear, it was not possible to obtain a reliable K_d value for this interaction.

A selection of SPR sensorgrams demonstrating both the association and dissociation phases of the antibody/oligopeptide interaction at different antibody concentrations is presented in Fig. 2.

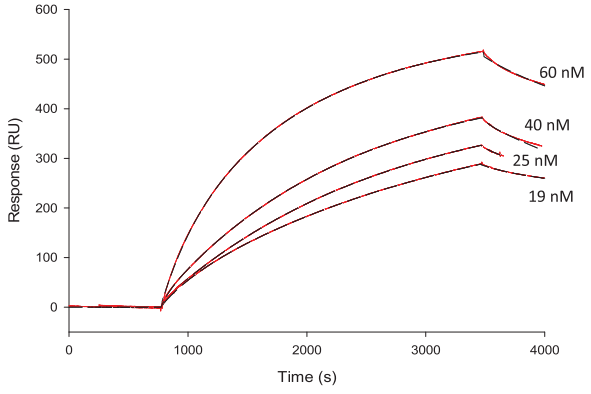

Fig. 2. Kinetics of binding the antibody to immobilized oligopeptide SAPVYLGVSTE as studied by SPR. Measurements were performed under the same conditions as described in the legend of Fig. 1. The experimental sensorgrams were fitted to the two-step binding model: A + P ⇄ AP ⇄ AP'. Red lines denote the experimental and black dashed lines the theoretical curves (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

The obtained kinetic curves were analysed with the two-step binding model A + P ⇄ AP ⇄ AP', considering formation and the following transformation of antigen/antibody complex. As can be seen in Fig. 2, there was an excellent fit of the model with the experimental data. Other models failed to describe the curves satisfactorily. Using the two-step binding model, we calculated the rate constants of antigen/antibody association for the first step of the interaction (k_{ass} = (6–8)x10^3 $M^{-1}s^{-1}$) and dissociation (k_{diss} = 1.5–3.1 × 10^{-4} s^{-1}). The relatively low value of the association constant indicates the probable conformational change of antibody in the second step of binding [43]. The value of the dissociation constant, calculated from kinetic data was 26 ± 12 nM. The nanomolar K_d value reveals high selectivity of the produced antibody and the applicability of this antibody for biorecognition of SUAM, indicating in turn the presence of *Str. uberis* bacteria.

3.3. Immunobiosensing of Str. uberis using anti–Str. uberis antibodies

The nanomolar K_d value, characterizing the affinity of the produced antibody combined with the anticipated number of SUAM molecules on the outer surface of *Str. uberis* cell membrane (n ≈ 7800) [12], determining the sensitivity of biorecognition, forms a solid basis for the application of anti–*Str. uberis* antibodies for specific and sensitive biorecognition of *Str. uberis* pathogens. In principle, the number of specific binding sites, accessible to interaction with detecting antibodies is the multiplication factor of the measurable fluorescence signal.

The dependence of the output signal of the immunobiosensing system on *Str. uberis* concentration was evaluated both in buffer and in raw milk matrix within the *Str. uberis* concentration range from 1 to10^9 CFU/ml (Fig. 3).

Comparing the calibration plots in different matrixes (buffer *vs* milk matrix); the analysis revealed that the calibration slopes were not significantly different indicating that the matrix effect has no effect on the sensitivity of the system. The value of the pooled slope was 5.6 ± 0.3 mV/log[*Str. uberis*]. The signal was linear between 10^3 and 10^7 CFU/ml in both matrixes, demonstrating the applicability of the system in different matrixes. This relatively wide range of linearity allows the application of this system for the detection of *Str. uberis* pathogens in milk of infected cows, as the level of *Str. uberis* bacteria in the milk of mastitis cows has been found to be in the same range: from 10^5 to 10^7 CFU/ml [22].

The background signal of the system was approximately 2 times higher in milk matrix than in buffer, being 17.0 ± 2.0 mV and

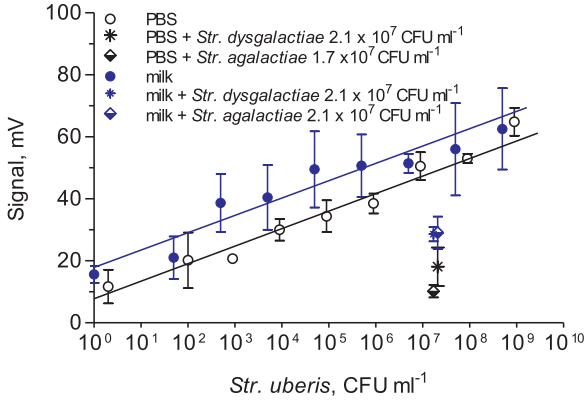

Fig. 3. Dependence of the immunosensing system signal on *Str. uberis* concentration in 10 mM PBS (0.15 M NaCl, pH 7.2) and raw milk (diluted 1:1 with 10 mM PBS, pH 7.2). The biosensor signal was calculated as a difference of average signal intensity at λ = 576 nm before adding and after removing the unbound labelled antibody from the micro-column. The signal was also determined for *Str. agalactiae* and *Str. dysgalactiae*.

Table 2
Analysis of mastitis milk samples with immunobiosensor and microbiological cultivation.

Sample number	*Str. uberis* concentration, CFU/ml		Presence of other pathogens
	Biosensor	Culturing	
1.	2.8×10^5	1.3×10^2	+
2.	1.6×10^3	5.0×10^2	+
3.	1.6×10^3	1.0×10^2	+
4.	< LOD	0	+
5.	5.3×10^7	6.0×10^2	+
6.	< LOD	0	+
7.	5.4×10^5	7.6×10^3	Only *Str. uberis*
8.	< LOD	0	Only *E. coli*
9.	1.5×10^6	1.7×10^2	+
10.	1.0×10^4	7.0×10^2	+
11.	1.0×10^3	80	+
12.	1.0×10^5	2.8×10^3	+

8.6 ± 1.9 mV, respectively. The higher values of background signals and standard errors in milk in comparison with buffer (Fig. 3) are probably due to the colloidal nature of milk matrix and potential aggregation of bacteria [30]. The limits of detection (LOD) and quantification (LOQ) of the system were calculated as the *Str. uberis* concentration corresponding to the system signal, exceeding the average signal in pathogen-free samples by the value of three or ten standard deviations, respectively. The LOD value was 15 CFU/ml and the LOQ value 3×10^3 CFU/ml. Both LOD and LOQ values were similar in buffer and in milk matrix. It is interesting to denote that the LOD values (assuming that there should be at least 1 pathogen cell in the analyzed sample volume) were in the same range of the theoretically calculated LOD value, which is 6 CFU/ml in case of 150 µl samples [30], and accordingly 13 CFU/ml for 75 µl samples.

The selectivity of the *Str. uberis* immunobiosensing system was assessed with other major mastitis-causing Streptococcus species - *Str. dysgalactiae* and *Str. agalactiae* [26]. These bacteria did not generate signals significantly different from the background even at concentrations 10^7 CFU/ml both in PBS and milk matrix (Fig. 3), confirming the results of *in silico* analysis and demonstrating the high specificity of the antibody and its usability in biosensors. In multiplex assays for the detection of major mastitis-causing pathogens, the potential nonspecific binding of anti-*Str. uberis* antibodies to *S. aureus* and *E. coli via* the Fc region of IgG type antibodies to membrane protein A of these bacteria can be eliminated by blocking the Fc region of the detecting antibodies by using oriented labelling, as pointed out earlier [24].

For the demonstration of the applicability of the *Str. uberis* immunosensing system in real mastitis milk, milk samples from 12 cows suffering from mastitis were analysed. In all these samples, the causative bacteria were identified and quantified using microbiological plating. Most of the analyzed samples (10/12) contained several mastitis-causing pathogens and only 2 out of 12 samples were monocultures (1 sample contained only *E. coli* and 1 sample *Str. uberis*). The presence of *Str. uberis* was identified in 9 samples, both with biosensing system and microbiological cultivation and there were no false positive or false negative results, indicating high correlation of the biosensor and culturing results (Table 2).

The concentrations of *Str. uberis*, determined with the biosensor, were considerably higher than the results obtained with microbiological cultivation. This is caused by the different principles of analyses, as culturing reveals only live culturable bacteria, while the biosensor results are based on the detection of SUAM concentration on cell membrane (or membrane fragments) and include as well the number of live and dead, but also viable nonculturable bacteria, which in favourable conditions are capable of causing infections [44]. Therefore, the application of biosensors, similarly to qPCR assays, can provide potential tools for improved mastitis diagnostics.

4. Conclusions

The monospecific anti-*Str. uberis* antibody was characterized in terms of affinity and specificity using *in silico* and experimental methods. The bioinformatic analyses revealed no overlapping regions of the primary protein sequence of SUAM antigenic oligopeptide SAPVYLGVSTE, used for the production of anti-*Str. uberis* antibodies, and the protein sequences of membrane-bound proteins of other mastitis-causing pathogens. An overlapping region was established only with membrane protein of *Str. hongkongensis*, which is found in marine flatfish. The K_d value of antigen/antibody complex, characterizing the affinity of anti-*Str. uberis* antibody towards the SUAM oligopeptide SAPVYLGVSTE, was 27 nM. The anti-*Str. uberis* antibody was used for the detection of *Str. uberis* bacteria with an immuno-biosensing system both in PBS and milk matrix with LOD value 15 CFU/ml, and the applicability of the system for pathogen analyses in milk was demonstrated assessing the milk of 12 cows, suffering from mastitis. *Str. uberis* in the milk of infected animals was found in 9 milk samples and the biosensor and microbiological plating results were in a good correlation. The concentration of *Str. uberis* pathogens in milk, determined with the biosensor, were higher than indicated by microbiological cultivation, as the latter reveals only live culturable bacteria, while the biosensor results include also the number of dead and viable, but nonculturable bacteria. Quantitative detection of pathogens in milk with biosensors allows to study correlations between pathogen levels and mastitis symptoms in individual animals for the development of algorithms for advanced and more effective treatment schemes of animals in the future.

Author agreement

The authors of the manuscript declare that they have made the following contributions :

1 Kaisa Mihklepp - bioinformatic studies, preparation of the manuscript
2 Kairi Kivirand - experimental studies (SPR, TIRF), preparation of the manuscript
3 Delia Juronen - experimental studies (milk analyses with biosensor)
4 Aivar Lõokene - experimental studies (SPR), preparation of the manuscript
5 Toonika Rinken - methodology, conceptualization, preparation of the manuscript

All authors have approved the final article.

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