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METHODS

Electrical protein array chips for the detection of staphylococcal virulence factors

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Abstract A new approach for the detection of virulence factors of *Staphylococcus aureus* and *Staphylococcus epidermidis* using an electrical protein array chip technology is presented. The procedure is based on an enzyme-linked sandwich immunoassay, which includes recognition and binding of virulence factors by specific capture and detection antibodies. Detection of antibody-bound virulence factors is achieved by measuring the electrical current generated by redox recycling of an enzymatically released substance. The current (measured in nanoampere) corresponds to the amount of the target molecule in the analyzed sample. The electrical protein chip allows for a fast detection of *Staphylococcus* enterotoxin B (SEB) of *S. aureus* and immunodominant antigen A homologue (IsaA homologue) of *S. epidermidis* in different liquid matrices. The *S. aureus* SEB virulence factor could be detected in minimal medium, milk, and urine in a concentration of 1 ng/ml within less than 23 min. Furthermore, a simultaneous detection of SEB of *S. aureus* and IsaA homologue of *S. epidermidis* in a single assay could be demonstrated.

Keywords Electrical protein array chips · Sandwich immunoassay · *Staphylococcus aureus* · *Staphylococcus epidermidis* · Virulence factors · Diagnostic

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Introduction

Staphylococcus aureus is one of the most common causes of nosocomial infections worldwide. Significant morbidity and mortality are associated with these infections (Bryan et al. 1984; Freeman et al. 1990; Crossley and Archer 1997; Todd 1997). Similar to *S. aureus*, *Staphylococcus epidermidis* plays an important role in nosocomial infections. *S. epidermidis* is responsible for more than 75% of catheter-associated infections. Its pathogenicity is mainly due to the ability to form biofilms on indwelling medical devices (Vuong and Otto 2002). Staphylococci can cause various diseases, such as pneumonia, inflammation of the heart and skeletal muscle, encephalitis, and food poisoning (Archer 1998; Petti and Fowler 2003; Tenover and Gorwitz 2006). Staphylococcal food poisoning results from the consumption of food in which *Staphylococcus*, mainly *S. aureus*, has

grown and produced exotoxins. Among these exotoxins are enterotoxins (SEs) which may trigger vomiting and diarrhea (Balaban and Roosley 2000; Mc Cormick et al. 2001).

Staphylococcus enterotoxin B (SEB) is an important superantigen. Superantigens are characterized by the activation of large subpopulations of T lymphocytes by directly cross-linking certain T-cell receptor Vβ domains with conserved structures on major histocompatibility complex class II molecules (Muraille et al. 1997; Proft and Fraser 1998, 2007).

Many *Staphylococcus* strains carry immunodominant structures such as the immunodominant *Staphylococcus* antigen homologues of *S. epidermidis* (IsaA homologue) (Sellman et al. 2005; Ohlsen and Lorenz 2007). IsaA was first described as a staphylococcal-specific antigenic structure, which is expressed *in vivo* during an *S. aureus* induced sepsis and might thus be a potential target for the development of antibody-based therapies against *S. aureus* infections (Lorenz et al. 2000). While the functional impact of IsaA homologue is still unclear, the protein is abundantly found in supernatants of both *S. aureus* and *S. epidermidis* cultures and is considered a virulence factor.

To date, the detection of *Staphylococcus* strains and their exotoxins is achieved by various testing methods based on different technologies. For example, identification of *Staphylococcus* species and strains is carried out by polymerase chain reaction (PCR)-based genotypification assays (Mc Lauchlin et al. 2000; Stuhlmeier and Stuhlmeier 2003; Martin et al. 2003; Palomares et al. 2003; Hwang et al. 2007; Sabet et al. 2007). Previous studies demonstrated a combined detection of staphylococci by a PCR-enzyme-linked immunosorbent assay (PCR-ELISA) (Wellinghausen et al. 2004) or using latex agglutination tests (Summers et al. 1998; Louie et al. 2001; Soloaga et al. 2004). Mass spectrometry has been suggested for the identification of specific virulence factors (Sellman et al. 2005; Alladyce et al. 2006). For diagnostic purposes, mainly, ELISA formats are applied (Park et al. 1996; Giletto and Fyffe 1998; Khreich et al. 2008). In addition, due to their higher sensitivity, fluorescence-based immunoassays are frequently used (Khan et al. 2003; Rucker et al. 2005; Medina 2006). A relatively new technique is the DELFIA® time-resolved fluorometry assay system (Peruski et al. 2002), which is highly sensitive.

However, most of these techniques are laborious and expensive for routine use in laboratory diagnostics. Recently, it has been demonstrated that electrical chips can be used as an alternative technique for the detection and quantitative analysis of biomolecules, such as DNA, rRNAs, and mRNAs (Albers et al. 2003; Gabig-Ciminska et al. 2004; Jürgen et al. 2005; Pioch et al. 2008; Elsholz et al. 2006, 2009). In this study, an enzyme-linked immunoassay was successfully combined with the biochip technology for a fast and sensitive diagnostics of staphylococcal virulence factors. The approach was tested with the enterotoxin SEB of *S. aureus* and the IsaA homologue of *S. epidermidis* as model virulence factors on *S. aureus* and *S. epidermidis*. A fast and automated detection of these selected staphylococcal exoproteins by electrical protein array chips was investigated.

Materials and methods

Strains and sample preparation

The *S. epidermidis* strain RP62a (ATCC 35984) (Gill et al. 2005) is available from the ATCC biological resource center (www.atcc.org). The *S. aureus* strain NCTC 8325 (Novick 1967) was obtained from the National Collection of Type Cultures (NCTC) (www.hpacultures.org.uk/collections/nctc.jsp). The strain *S. aureus* COL (Shafer and Iandolo 1979) is available from The Staphylococcal Genetic Stock Center of the John J. Iandolo Lab at the University of Oklahoma Health Sciences Center in Oklahoma City, OK (www.ouphysicians.com/body.cfm?id=4285).

The strains *S. aureus* NCTC8325, *S. aureus* COL, and *S. epidermidis* RP62a were grown in tryptone soy broth medium (Difco, Lawrence, Kansas, USA). Samples were taken from exponentially growing cells and from cells of the stationary phase. The samples were centrifuged at 13,000 rpm for 10 min and 4°C. The supernatant was stored at −20°C until further processing.

Antibodies, enzymes, and substrates

In order to ensure high specificity, all assays were carried out using target-specific polyclonal capture antibodies and appropriate monoclonal antibodies for the detection of bound targets. The monoclonal antibody facilitates the binding of an enzyme-conjugate, which finally induces the signal generation. For the capturing of the *S. aureus* SEB or the *S. epidermidis* IsaA homologue, purified polyclonal rabbit antibodies were used (Sigma Aldrich, Steinheim, Germany; BioGenes GmbH, Berlin, Germany). The monoclonal mouse antibodies anti-SEB 148 and anti-IsaA homologue 14017 (Helmholtz-Zentrum für Infektionsforschung GmbH, Braunschweig, Germany) were used for the detection of the bound target proteins SEB and IsaA homologue. All antibodies applied in this study were first tested by Western blot analyses to check for successful specific antigen–antibody binding (data not shown). The polyclonal antibodies were used as capture molecules with a final concentration of 0.5 mg/ml [diluted in phosphate-buffered saline (PBS), pH 7.4], and the monoclonal antibodies were used in a concentration of 2 µg/ml [diluted in Tris-buffered saline (TBS)–bovine serum albumin

(BSA)–Tween buffer: 1% BSA, 0.01% Tween 20 in TBS buffer, pH 8].

For the detection of purified SEB toxin in various matrices, the monoclonal antibody against SEB toxin was conjugated with biotin using the EZ-Link® Sulfo-NHS-LC-Biotinylation Kit (Thermo Fisher Scientific, Rockford, USA) according to the manufacturer's instructions. Unbound biotin was removed by dialysis against PBS (pH 7.4). The monoclonal biotinylated detection antibody was used with a final concentration of 5.5 µg/ml (diluted in PBS–BSA–Tween buffer: 0.5% BSA, 0.02% Tween 20 in PBS buffer, pH 7.4).

As positive control for the streptavidin-β-galactosidase-based assays, a biotinylated antibody (Sigma Aldrich, Steinheim, Germany; 1 µg/ml solved in PBS buffer, pH 7.4), which does not cross-interact with any of the target sequences was chosen. This antibody directly binds the enzyme streptavidin-β-galactosidase. For the assays using alkaline phosphatase as a reporter enzyme, the positive control was Set15, a monoclonal antibody (Helmholtz-Zentrum für Infektionsforschung GmbH, Braunschweig, Germany; 0.1 mg/ml solved in PBS buffer, pH 7.4), which directly binds the anti-mouse alkaline phosphatase conjugate (Sigma Aldrich, Steinheim, Germany). The negative control was, in all cases, BSA (0.1% in PBS buffer, pH 7.4).

For the multiplex assays, an anti-mouse alkaline phosphatase, which was diluted 1:1,000 in TBS–BSA–Tween buffer (1% BSA, 0.01% Tween 20 in TBS buffer, pH 8), was used as a reporter enzyme with the corresponding substrate *p*-aminophenyl-phosphate (Universal Sensors, Ireland; 0.5 mg/ml in TBS buffer, pH 8.0). For the analysis of the detection limit of SEB in various liquid matrices a streptavidin-β-galactosidase (Roche Diagnostics, Mannheim, Germany) was applied as the reporter enzyme (1:400 diluted in 0.5% BSA, 0.02% Tween 20, 1 mM $MgCl_2$ in PBS buffer, pH 7.4) with the corresponding substrate *p*-aminophenyl-β-D-galactopyranoside (Sigma Aldrich, Steinheim, Germany; 1 mg/ml in 0.02% Tween 20, 1 mM $MgCl_2$ in PBS buffer, pH 7.4).

Spotting of the protein array chips

The capture antibodies for *S. aureus* SEB and *S. epidermidis* IsaA homologue as well as the positive and negative control were spotted on defined gold electrode positions on the array chips using the GeSiM piezoelectric nanodispenser NP 2.0 (Grosserkmannsdorf, Germany). Twenty-four nanoliter of the capture antibody solutions were dispensed at the appropriate gold electrode position on the array chip with a final concentration of 0.5 mg/ml in PBS buffer, pH 7.4.

After the spotting procedure, the chips were incubated for 2 h at room temperature or overnight at 4°C in a humidity chamber to allow immobilization of the antibodies to the gold surface of the electrodes. Subsequently, the protein chips were washed three times with 500 µl PBS and blocked with 1% milk (Sigma Aldrich, Steinheim, Germany; in PBS buffer pH 7.4) for 30 min in a humidity chamber, followed by three washing steps with 500 µl PBS.

For the protein chips, a special trehalose treatment is necessary. The trehalose film protects the immobilized capture antibodies and enables a longer storage time of the protein chips. For this purpose, the electrode positions were incubated with 5% trehalose solution (Sigma Aldrich, Steinheim, Germany; diluted in PBS buffer pH 7.4 and filtrated) for 1–2 h in a humidity chamber. After trehalose incubation, the chips were dried at room temperature (RT) and stored at RT (few days) or at 4°C for approximately 3 months (AJ eBiochip GmbH, Itzehoe, Germany).

Principles of the measurements with the eMicroLISA

The electrochemical detection was carried out using the eMicroLISA device of AJ eBiochip GmbH (Itzehoe, Germany). The electrical biochips are manufactured by an industrial silicon semi-conductor technology (Paeschke et al. 1995). The chips measure 9×10 mm and hold 16 working electrode positions of 500 µm in diameter. Each electrode position consists of 2×204 interdigital gold array electrode fingers with a gap of 400 nm between anode and cathode fingers and a width of 800 nm. Nineteen contact pads with eight anodes, eight cathodes, one counter electrode, and one optional reference electrode are integrated on the chip. For electrochemical reasons, the additional electrodes are made of gold like the contact pads and working electrode positions (Albers et al. 2003; Elsholz et al. 2006, 2009).

The detection of proteins with the electrical biochip sensor eMicroLISA is based on an ELISA (Fig. 1). The signal generation was achieved by reporter enzymes, coupled either to an anti-mouse antibody (Fig. 1a) or conjugated with streptavidin (Fig. 1b), which perform the hydrolysis of the redox-inactive substrate to the electrochemically active product. The readout occurs as a measurement of the electrical current, which is generated by redox recycling (Niwa et al. 1990; Paeschke et al. 1995) of the enzymatically released substance. After completion of the measurement, the electric readout was obtained in a 4-sec period in "stop flow mode." The recorded data set was transferred to the "Origin" software (OriginLab Corporation, Northhampton, USA) for further evaluation in nanoampere per minute.

The measurements were performed by applying a potential of +350 mV to the anodic fingers and −150 mV to the cathodic fingers of the interdigitated array electrodes. The spotted chip was positioned into the chip adapter, which facilitates the fluidic and electronic connections to the eMicroLISA. Reagents and the sample were placed into the separate reservoirs of the chip reader. The automatic

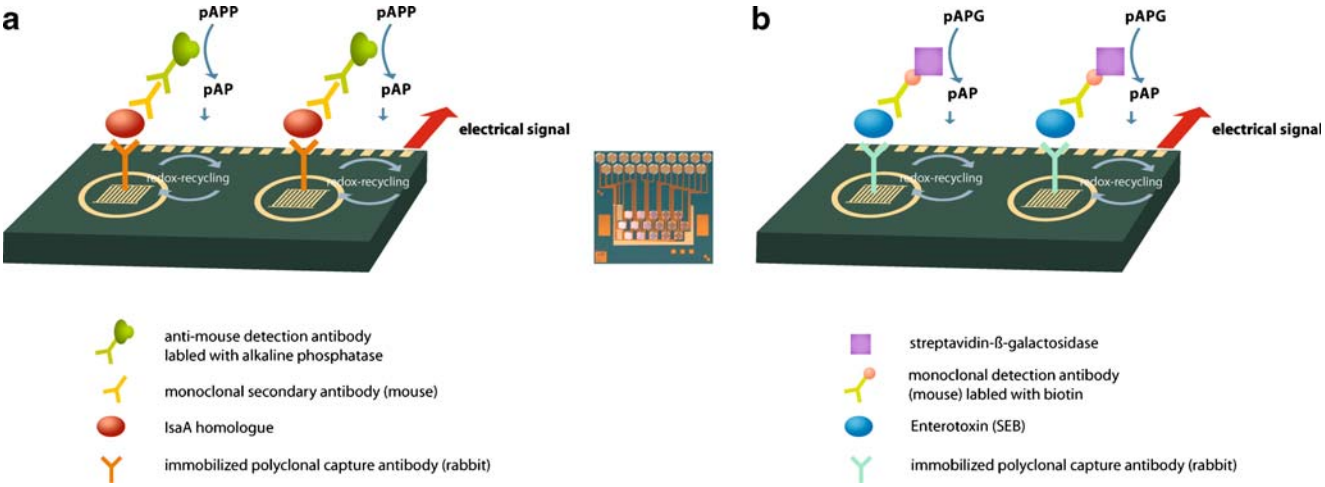

Fig. 1 Schematic presentation of **a** the detection of the IsaA homologue by means of the alkaline phosphatase as reporter enzyme and the **b** streptavidin-β-galactosidase-based assay for the detection of the SEB toxin with the electrical protein array chip. The capture antibody, immobilized on the gold electrodes, facilitates the binding of the target molecule out of the applied sample. Afterwards, an enzyme-coupled detection antibody binds the target molecule and allows for the enzymatic release of *p*-aminophenol (*pAP*) that causes a redox recycling at the electrodes. This results in an electrical signal measured in nanoampere that corresponds to the amount of bound target molecule

program pumps all components sequentially into the flow cell, in which the protein array chip is located.

An automated ELISA program for the detection of staphylococcal proteins on the electrical protein chip was established and optimized (Table 1). The final assay program includes five washing steps with TBS buffer for the alkaline phosphatase (1% BSA, 0.01% Tween 20 in TBS buffer, pH 8) and PBS buffer for the streptavidin-β-galactosidase (0.5% BSA, 0.02% Tween 20 in PBS buffer, pH 7.4). In order to ensure stringent conditions for the binding of the target proteins and of the detection antibodies, 45°C was used (see Table 1). The complete assay takes less than 23 min from loading the sample into the device until the final signal readout. For each measurement with the electrical protein array chips, a detection limit was calculated. The detection limit is the lowest concentration level that can be determined to be statistically different from a blank, in our case, the so-called negative control. For the calculation of the detection limit, the standard deviation of the negative control was multiplied with three, and then the calculated average of the three negative control positions was added.

Table 1 List of the different steps of the electrical protein array chip protocol used for the detection of the staphylococcal virulence factors SEB and IsaA homologue

Step number	Program function	Temperature (°C)	Time (s)
1	Wash	RT	21
2	Sample with virulence factor	45	561
3	Wash	45	11
4	Secondary mouse antibody	45	335
5	Wash	45	8
6	Conjugated enzyme	45	334
7	Wash	45	21
8	Substrate	45	14
9	Stop	45	30
10	Wash	RT	13
Total			22.5 min

Detection of SEB and IsaA homologue in supernatants

For the multiplex protein array chip measurements, polyclonal capture antibodies against SEB and IsaA homologue were spotted in five parallel controls with a concentration of 0.5 mg/ml. The anti-SEB antibody was located on positions 4–8 and the anti-IsaA homologue antibody on positions 9–12 and 14 of the chip. The positive control, a monoclonal mouse antibody (Set 15), was located on positions 1–3 on the protein array chip. The negative control BSA was immobilized on positions 13, 15, and 16. The optimal final concentration of the monoclonal secondary antibodies against SEB toxin and IsaA homologue was 2 µg/ml.

For the simultaneous detection of SEB and IsaA homologue, supernatants of *S. aureus* and *S. epidermidis* cultures were used as samples, which were diluted 1:1 in the corresponding medium. The automated program pumps the supernatant, approximately 230 µl, into the flow cell, in which the protein array chip is located, and the supernatant is pumped 50 times bidirectionally over the chip. After a washing step, the monoclonal secondary antibody was pumped over the chip allowing for binding at the appropriate

target molecules, which were bound to the specific capture antibody on the protein array chip. Subsequently, the anti-mouse detection antibody coupled with the alkaline phosphatase was introduced. Both components were bidirectionally pumped through the flow cell 30 times. For the detection, the substrate *p*-aminophenyl-phosphate was added. This electrode-inactive substrate is hydrolyzed by the alkaline phosphatase into the electrode-active product *p*-aminophenol, which is measured by the electrochemical redox-recycling reaction as illustrated in Fig. 1a.

Detection of SEB in various matrices

The detection limit of recombinant staphylococcal SEB toxin (Toxin Technology, Sarasota, FL) was determined in different matrices (medium, milk, and urine). For this purpose, different concentrations of purified SEB toxin (1–10 ng/ml) were tested. In this protocol, the streptavidin-β-galactosidase was used as the detection enzyme, which converts the substrate *p*-aminophenyl-β-D-galactopyranoside into the product *p*-aminophenol (Fig. 1b).

The polyclonal capture antibody against SEB was spotted on six parallel positions with a concentration of 0.5 mg/ml (positions 7–12) on the chip. The positive control, a biotin-labeled polyclonal antibody was located on positions 1–6 of the chip. The negative control BSA was immobilized on positions 13–16 of the protein array chip. The optimal concentration of the biotinylated monoclonal detection antibody was determined with 5.5 µg/ml final concentration. In order to verify that the signals obtained with the electrical protein array chips are specific for SEB, samples of a non-SEB-producing strain (*S. aureus* 8325) were tested.

Results

In this study, we present a new analytical approach for the detection of virulence factors of *S. aureus* and *S. epidermidis* based on a sandwich immunoassay carried out on electrical protein array chips with the eMicroLISA (AJ eBiochip GmbH, Itzehoe, Germany). The established assay allows for the detection of selected staphylococcal proteins in different liquid matrices.

Detection of SEB and IsaA homologue on multiplex protein array chips

Figure 2 shows the detection of the *S. aureus* enterotoxin SEB (Fig. 2a), the *S. epidermidis* IsaA homologue (Fig. 2b), and the simultaneous detection of both virulence factors on a multiplex protein array chip (Fig. 2c). The alkaline phosphatase was used as the detection enzyme in these assays. In these experiments, the monoclonal anti-

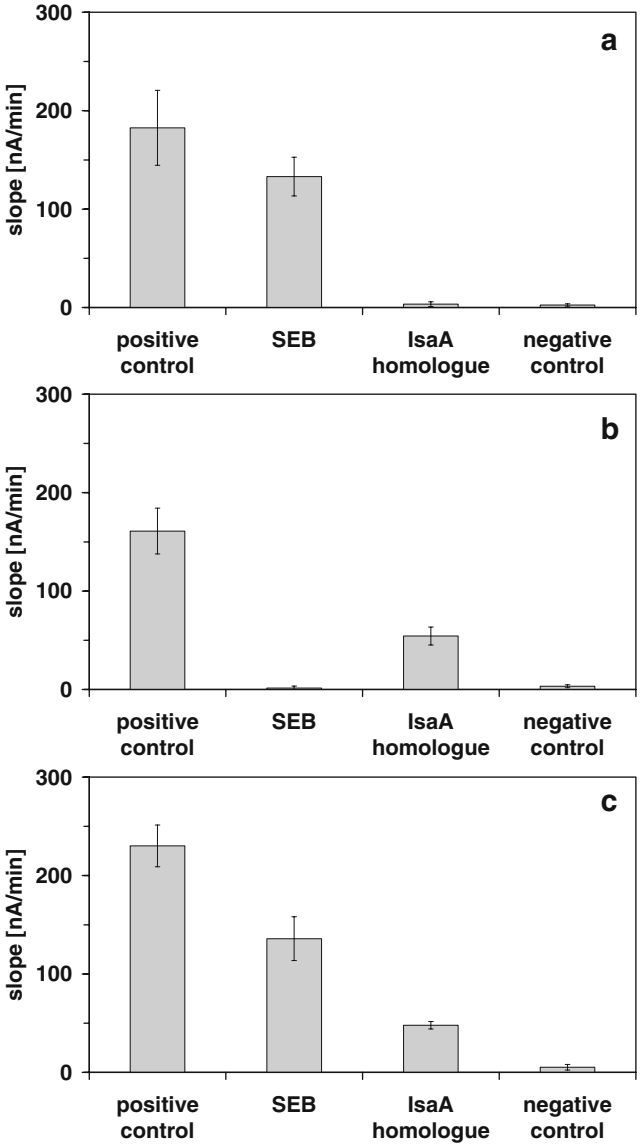

Fig. 2 Detection of SEB toxin in the supernatant of the strain *S. aureus* COL (**a**) and of IsaA homologue in the supernatant of *S. epidermidis* (**b**) with the electrical protein array chips and the simultaneous detection of SEB and IsaA homologue (**c**). All supernatants were diluted 1:1 with medium, and the alkaline phosphatase was used as a reporter enzyme. The *error bars* show the standard deviation of ten independent measurements

body Set15 was used as a positive control for the assay performance. The direct binding of the anti-mouse alkaline phosphatase antibody with the monoclonal antibody Set15 showed a signal in a range of about 180–260 nA/min. The single detection of SEB in the supernatant of the strain *S. aureus* COL with the electrical protein array chip showed specific and reproducible signals of up to 130 nA/min (Fig. 2a). No cross-reactions were observed at electrode positions with capture antibodies for the *S. epidermidis* IsaA homologue. The application of the protein array chips for the detection of the IsaA homologue in *S. epidermidis*

culture supernatant revealed reproducible signals of around 50 nA/min (Fig. 2b). No cross-reaction signals at control electrode positions could be observed.

The simultaneous detection of SEB toxin and IsaA homologue in one assay (Fig. 2c) gave signal intensities comparable to the ones in the individual assays (Fig. 2a, b). The interval of the signal intensity, represented by the electrical current, of SEB toxin was in a range of 130.84 (±19.72) nA/min and that of the IsaA homologue was 53.61 (±8.13) nA/min in the supernatants (1:1 diluted). The simultaneous detection showed signals of 135.88 (±22.29) nA/min for SEB toxin and 47.90 (±3.79) nA/min for IsaA homologue. In all cases, signals were obtained, which were clearly above the detection limit of approximately 7 nA/min.

For all experiments, the standard variations within a protein array chip measurement, the intra-assay variance as well as the reproducibility of independent measurements of protein array chips, and the inter-assay variance were determined. The overall intra- and inter-assay variances were <20%, whereas the general measurements showed variations of <10%.

The streptavidin-β-galactosidase was tested as potential alternative detection enzyme in comparison to the alkaline phosphatase. For this enzyme, a better dynamic range was suggested. The usage of the streptavidin-β-galactosidase as detection enzyme resulted in an around tenfold increase of the detected signals. However, unspecific signals were observed in case of the IsaA homologue (data not shown). These signals were obviously caused by the unspecific binding of the polyclonal capture antibody anti-IsaA homologue to the streptavidin-β-galactosidase. In contrast, the readout of the capture antibody against SEB toxin showed no unspecific binding with the streptavidin-β-galactosidase (data not shown).

Detection of SEB in various matrices

Since the streptavidin-β-galactosidase showed no unspecific binding with the antibody against *S. aureus* SEB but generated about tenfold higher signals, this enzyme was used for establishing an assay for the single monitoring of SEB in liquid matrices and for the evaluation of the detection limits of the established assay. The minimal detection limit of *S. aureus* SEB amounts was analyzed using different concentrations of commercially available, purified SEB toxin ranging from 1 to 10 ng/ml SEB solved in medium (Fig. 3a), milk (Fig. 3b), and urine (Fig. 3c). The protein chip analyses showed a linear increase of the signal intensity by increasing SEB concentrations. For all measurements, the standard deviations of the signals were calculated. This is indicated by error bars in Fig. 3. For the detection of SEB in medium signals of 103.88 (±14.34) nA/min for a concentration of

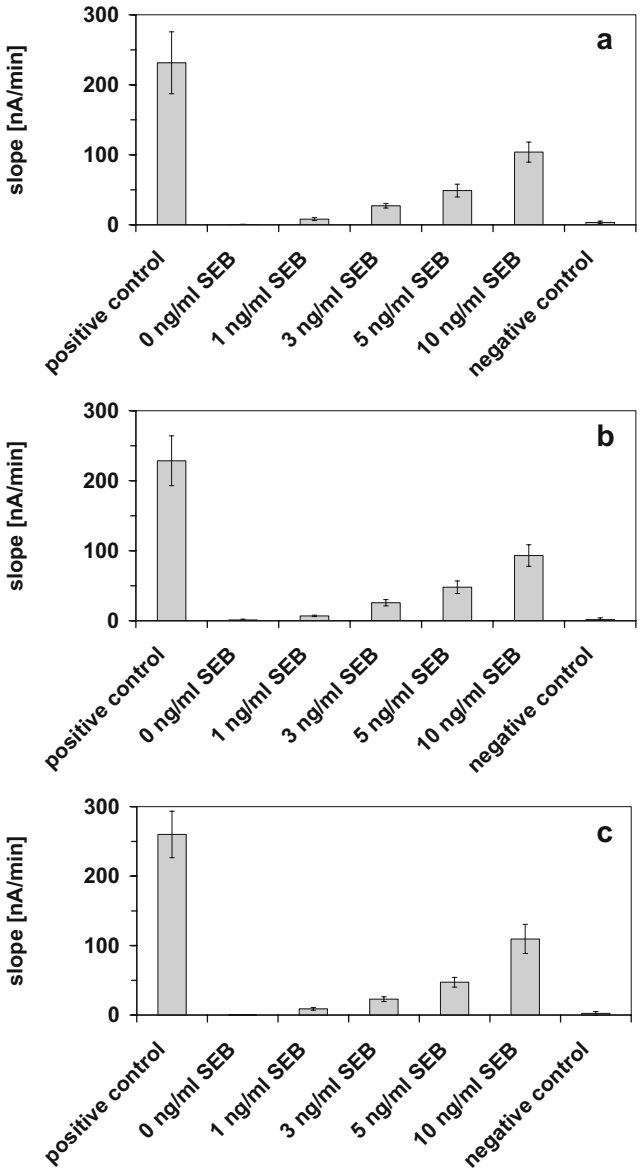

Fig. 3 Detection of different concentrations of SEB toxin in medium (**a**), milk (**b**), and urine (**c**) with the electrical protein array chips and the streptavidin-β-galactosidase as a reporter enzyme. The *error bars* show the standard deviation of 12 independent measurements

10 ng/ml, 48.96 (±9.10) nA/min by 5 ng/ml, 28.20 (±3.30) nA/min for 3 ng/ml, and 8.16 (±2.11) nA/min for 1 ng/ml were achieved. The negative control BSA showed signals of 1.66 (±0.89) nA/min. The overall intra- and inter-assay variances were <20% except for the measurement with the concentration of 1 ng/ml SEB toxin, in which a variance of <30% was determined. The obtained data showed that the limit of detection for SEB in liquid matrices is >1 ng/ml. Although the standard deviations in this experiment were higher compared to other measurements, the detected signals were specific and significantly higher than those of the negative control. In order to verify that the detection is

specific for SEB, a non-SEB producing strain (*S. aureus* 8325) was analyzed. No significant signals were generated at the positions with anti-SEB antibody after incubation with culture supernatant of the SEB-deficient strain *S. aureus* 8325 (Fig. 4a). In addition, pure medium and supernatant of *S. epidermidis* were used as further negative controls (Fig. 4a) to analyze whether the capture antibodies show unspecific cross-reactions with the samples. However, none of these extracts led to a positive signal at the protein array positions with the immobilized specific antibody against SEB.

Comparison of the electrical protein array measurements with a commercially available reference kit

In order to evaluate the protein chip results, a commercially available reference kit for the detection of various enterotoxins (enterotoxins A, B, C, D, and E) of *S. aureus* was used. For this purpose, supernatants of *S. aureus* 8325, *S. aureus* COL, and *S. epidermidis* were analyzed with the electrical protein array chips in comparison to the "Staphylococcal enterotoxin detection kit SIDVIA ID72" of the company TECRA (Frenchs Forest, Australia) (Park et al. 1996). The staphylococcal enterotoxin visual immunoassay kit from TECRA is designed for the detection of staphylococcal enterotoxin types A to E and the sensitivity of the kit for detection of SEB is >1 ng/ml. In the case of the SEB-producing strain *S. aureus* COL, SEB could be clearly detected in parallel samples of *S. aureus* COL supernatant using the electrical protein array chips (Fig. 4a) and the reference kit from TECRA (Fig. 4b). With the TECRA test kit, signals with an extinction of >3.5 $OD_{405/490\ nm}$ were detected. The measurements using the electrical protein array chips resulted in signal intensities of >750 nA/min. Both methods revealed signal intensities that were significantly above the detection limit. In contrast, the analysis of the medium sample revealed that the supernatants of *S. epidermidis* and of *S. aureus* 8325 did not give any positive signals for SEB—neither with the SIDVIA ID72 kit nor with the electrical protein array chips (Fig. 4a, b).

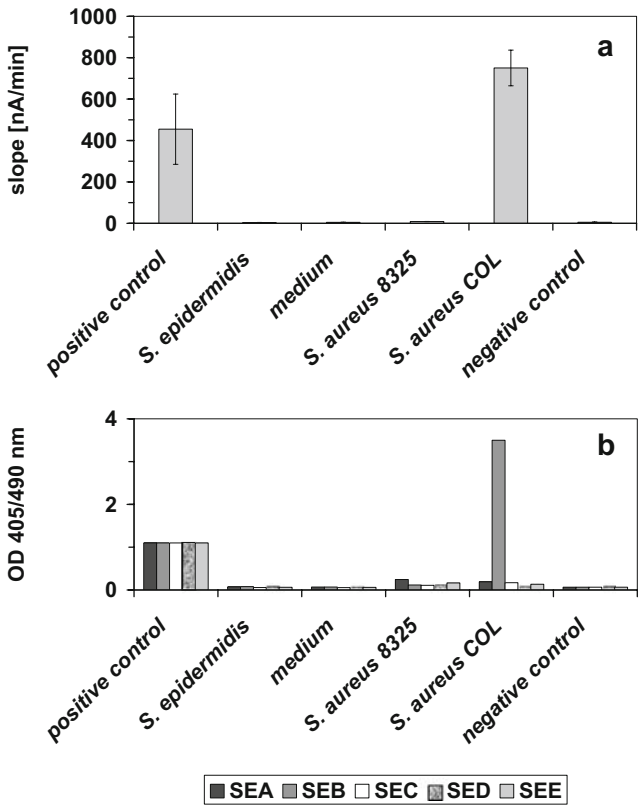

Fig. 4 Comparison of the detection of SEB in supernatants of different staphylococcal strains with the electrical protein array chips using the streptavidin-β-galactosidase as a detection enzyme (**a**) and by means of the commercially available TECRA test kit system (**b**) (SIDVIA ID72, TECRA, Australia)

Discussion

The prevalence of *S. aureus* and *S. epidermidis* in nosocomial infections with their increasing antibiotic resistance and their great importance for food poisoning necessitates a rapid detection. In this study, an alternative assay for the detection of staphylococcal virulence factors by means of electrical protein array chips could be established. This technique is easy to handle and less time consuming than other test systems. The sandwich immunoassay on the protein array chips allows for a fast readout of the electrochemically generated signal within less than 23 min.

For the measurements with the electrical protein array chips, two different detection enzymes were tested in terms of their specificity and sensitivity. The alkaline phosphatase was characterized by a high specificity but, compared to the streptavidin-β-galactosidase, it revealed a lower sensitivity, which allowed a detection limit for SEB of around 10 ng/ml. A clear increase of the signal intensity of around 10-fold was achieved using the streptavidin-β-galactosidase as a reporter enzyme. The increase in signal intensity resulted in a detection limit for SEB of 1 ng/ml. The better signal generation not only appeared to be due to the described higher dynamic range of this enzyme, but was also accompanied by unspecific signals raised from cross-reactions of the streptavidin-β-galactosidase with the capture antibody of the *S. epidermidis* IsaA homologue (data not shown). Both enzymes, the alkaline phosphatase and the streptavidin-β-galactosidase, are useful reporter enzymes for the measurements with the electrical protein array chips. However, the choice of the enzyme crucially depends on the applied antibodies and on potential cross-reactions with components of the assay and has therefore to be tested in advance. The analyses of

multiplex protein array chips have revealed that the simultaneous detection of staphylococcal protein SEB among IsaA homologue is possible without cross-reaction, depending on the capture antibodies and the reporter enzyme.

In order to validate the feasibility of the electrical protein chip assay, the commercially available reference kit from TECRA was used in this study. Although a direct comparison of the signal intensity of both methods is not possible, since the signals were read out with different technologies, it could be shown that a comparable sensitivity in the detection of the SEB toxin can be achieved with the protein chips. However, in contrast to the electrical protein array chips, the application of the staphylococcal enterotoxin visual immunoassay kit from TECRA takes about 4 h.

Besides the visual immunoassay kit from TECRA, a number of assays for the detection of staphylococcal enterotoxins with a comparable detection limit have been suggested. A previously described piezoelectric crystal immunosensor obtained a detection of SEB in liquid samples with 2.5–60 µg target protein per milliliter. However, this promising assay requires several preparation steps in advance, and the storage life of the prepared immunosensor is only about a few days (Lin and Tsai 2003). A further method for the detection of SEB, called piezoelectric-excited millimeter-sized cantilever, which also uses a polyclonal capture antibody against SEB, attained a detection limit between 12.5 and 50 pg/ml (Campbell et al. 2007). However, this procedure is time consuming. Alefantis et al. (2004) described a magnetic-bead-based immunoassay combined with an optical read-out. The method is highly sensitive and allows for a detection of 100 pg target protein, but the method is associated with a high cost factor.

In contrast to many of the time-consuming and laborious test systems that are available to date, the assay established in this study is characterized by an improved assay time of only 23 min from loading of about 230 µl sample into the device until the final signal readout. The process is fully automated and does not require any additional steps. The inexpensive protein array chips with the immobilized antibodies can be manufactured in advance and can subsequently be stored over a time period of at least 3 months. Furthermore, the 16 independent gold-electrode positions on the array chips facilitate the simultaneous detection of different target molecules in one sample.

The food industry demands a detection limit of enterotoxins in the range of around 125–260 ng per 100 g or per 100 ml food (Jay 2000), since enterotoxin-originated food poisoning causes gastrointestinal symptoms even at exposure levels as low as 20–100 ng per person (Asao et al. 2003). The electrical protein chip assay of this study allowed so far a detection limit of *S. aureus* SEB in liquids, e.g., medium, milk, or urine, of >1 ng/ml. A further increase of the sensitivity of this methodology for the detection of staphylococcal toxins could be ensured by an optimized antibody design and improved spotting techniques in the future. Nevertheless, the presented protocol provides a fast method with a promising potential for the application in the detection of other toxins, antigens, or organisms.

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References

Albers J, Grunwald T, Nebling E, Piechotta G, Hintsche R (2003) Electrical biochip technology—a tool for microarrays and continuous monitoring. Anal Bioanal Chem 377:521–527

Alefantis T, Grewal P, Ashton J, Khan AS, Valdes JJ, Del Vecchio VG (2004) A rapid and sensitive magnetic bead-based immunoassay for the detection of staphylococcal enterotoxin B for high-through put screening. Mol Cell Prob 18:379–382

Alladyce RA, Hill AL, Murdoch DR (2006) The rapid evaluation of bacterial growth and antibiotic susceptibility in blood cultures by selected ion flow tube mass spectrometry. Diagn Microbiol Infect Dis 55:255–261

Archer GL (1998) *Staphylococcus aureus*: a well-armed pathogen. Clin Infect Dis 26(5):1179–1181

Asao T, Kumeda Y, Kawai T, Shibata T, Oda H, Haruki K, Nakazawa H, Kozaki S (2003) An extensive outbreak of staphylococcal food poisoning due to low-fat milk in Japan: estimation of enterotoxin A in the incriminated milk and powdered skim milk. Epidemiol Infect 130:33–40

Balaban N, Roosley A (2000) Staphylococcal enterotoxins. Int J Food Microbiol 61:1–10

Bryan CS, Kirckhart B, Brenner ER (1984) Staphylococcal bacteraemia: current patterns in nonuniversity hospitals. South Med J 77:693–696

Campbell GA, Medina MB, Mutharasan R (2007) Detection of *Staphylococcus* enterotoxin B at picogram levels using piezoelectric-excited millimeter-sized cantilever sensors. Sens Act B 126:354–360

Crossley KB, Archer GL (1997) The staphylococci in human disease. Churchill Livingstone, New York

Elsholz B, Wörl R, Blohm L, Albers J, Feucht H, Grunwald T, Jürgen B, Schweder T, Hintsche R (2006) Automated detection and quantitation of bacterial RNA by using electrical microarrays. Anal Chem 78:4794–4802

Elsholz B, Nitsche A, Achenbach J, Ellerbrock H, Blohm L, Albers J, Pauli G, Hintsche R, Wörl R (2009) Electrical microarrays for highly sensitive detection of multiplex PCR products from biological agents. Biosens Bioelectron 24:1737–1743

Freeman J, Epstein MF, Smith NE, Platt R, Sidebottom DG, Goldman DA (1990) Extra hospital stay and antibiotic usage with nosocomial coagulase-negative staphylococcal bacteraemia in two neonatal intensive care unit populations. Am J Dis Child 144:324–329

Gabig-Ciminska M, Holmgren A, Andresen H, Barken KB, Wümpelmann M, Albers J, Hintsche R, Breitenstein A, Neubauer P, Los M, Czyz A, Wegrzyn G, Silfversparre G, Jürgen B, Schweder T, Enfors SO (2004) Electric chips for

rapid detection and quantification of nucleic acids. Biosens Bioelectron 19:537–546
Giletto A, Fyffe JG (1998) A novel ELISA format for the rapid and sensitive detection of staphylococcal enterotoxin A. Biosci Biotechnol Biochem 62(11):2217–2222
Gill SR, Fouts DE, Archer GL, Mongodin EF, Deboy RT, Ravel J, Paulsen IT, Kolonay JF, Brinkac L, Beanan M, Dodson RJ, Daugherty SC, Madupu R, Angiuoli SV, Durkin AS, Haft DH, Vamathevan J, Khouri H, Utterback T, Lee C, Dimitrov G, Jiang L, Qin H, Weidman J, Tran K, Kang K, Hance IR, Nelson KE, Fraser CM (2005) Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. J Bacteriol 7:2426–2438
Hwang SY, Kim SH, Jang EJ, Kwon NH, Park YK, Koo HC, Jung WK, Kim JM, Park YH (2007) Novel multiplex PCR for the detection of the *Staphylococcus aureus* superantigen and its application to raw mest isolates in Korea. Int J Food Microbiol 117(1):99–105
Jay JM (2000) Modern food microbiology, 6th edn. APAC, Singapore, pp 445–453
Jürgen B, Barken KB, Tobisch S, Pioch D, Wümpelmann M, Hecker M, Schweder T (2005) Application of an electric DNA-chip for the expression analysis of bioprocess-relevant marker genes of *Bacillus subtilis*. Biotechnol Bioeng 92:299–307
Khan AS, Cao CJ, Thompson RG, Valdes JJ (2003) A simple and rapid fluorescence-based immunoassay for the detection of staphylococcal enterotoxin B. Mol Cell Prob 17:125–126
Khreich N, Lamourette P, Boutal H, Devilliers K, Creminon C, Volland H (2008) Detection of *Staphylococcus* enterotoxin B using fluorescent immunoliposomes as label for immunochromatographic testing. Anal Biochem 377:182–188
Lin HC, Tsai WC (2003) Piezoelectric crystal immunosensor for the detection of Staphylococcal enterotoxin B. Biosens Bioelectron 18:1479–1483
Lorenz U, Ohlsen K, Karch H, Hecker M, Thiede A, Hacker J (2000) Human antibody response during sepsis against targets expressed by methicillin resistant *Staphylococcus aureus*. FEMS Immunol Med Microbiol 29:145–153
Louie L, Majury A, Goodfellow J, Louie M (2001) Evaluation of a latex agglutination test (MRSA-Screen) for detection of oxacillin resistance in coagulase-negative staphylococci. J Cli Microbiol 39(11):4149–4151
Martin MC, Gonzalez-Hevia MA, Mendoza MC (2003) Usefulness of a two-step PCR procedure for detection and identification of enterotoxigenic staphylococci of bacterial isolates and food samples. Food Microbiol 20:605–610
Mc Cormick JK, Yarwood JM, Schlievert PM (2001) Toxic shock syndrome and bacterial superantigens: an update. Annu Rev Microbiol 55:77–104
Mc Lauchlin J, Narayananan GL, Mithani V, O'Neill G (2000) The detection of enterotoxins and toxic shock syndrome toxin genes in *Staphylococcus aureus* by polymerase chain reaction. J Food Prot 63(4):479–488
Medina MB (2006) Development of a fluorescent latex microparticle immunoassay for the detection of staphylococcal enterotoxin B (SEB). J Agric Food Chem 54:4937–4942
Muraille E, De Smedt T, Andris F, Pajak B, Armant M, Urbain J, Moser M, Leo O (1997) Staphylococcal enterotoxin B induces an early and transient state of immunosuppression characterized by V beta-unrestricted T cell unresponsiveness and defective antigen-presenting cell functions. J Immunol 158:2638–2647
Niwa O, Morita M, Tabei H (1990) Electrochemical behavior of reversible redox species at interdigitated array electrodes with different geometries: consideration of redox cycling and collection efficiency. Anal Chem 62(5):447–452
Novick RP (1967) Penicillinase plasmids of *Staphylococcus aureus*. Fed Proc 26:29–38
Ohlsen K, Lorenz U (2007) Novel targets for antibiotics in *Staphylococcus aureus*. Future Microbiol 2:655–666
Paeschke M, Wollenberger U, Köhler C, Lisec T, Schnakenberg U, Hintsche R (1995) Properties of interdigital electrode arrays with different geometries. Anal Chim Acta 305:126–136
Palomares C, Torres MJ, Torres A, Aznar J, Polamares JC (2003) Rapid detection and identification of *Staphylococcus aureus* from blood culture speciemens using real time fluorescence PCR. Diagn Microbiol Infect Dis 45:183–189
Park CE, Warburton D, Laffey PJ, Collaberators (1996) A collaborative study on the detection of Staphylococcal enterotoxins in food with an enzyme immunoassay Kit (TECRA). J Food Protect 59 (4):390–397
Peruski AH, Johnsonn LH III, Peruski LF Jr (2002) Rapid and sensitive detection of biological warfare agents using time-resolved fluorescence assays. J Immunol Method 263:35–41
Petti CA, Fowler VG (2003) *Staphylococcus aureus* bacteremia and endocarditis. Cardiol Clin 2:219–233
Pioch D, Jürgen B, Evers S, Maurer KH, Hecker M, Schweder T (2008) Improved sandwich-hybridization assay for an electrical DNA-chip-based monitoring of bioprocess-relevant marker genes. Appl Microbiol Biotechnol 78:719–728
Proft T, Fraser JD (1998) Superantigens: just like peptides only different. J Exp Med 187(6):819–821
Proft T, Fraser JD (2007) Streptococcal superantigens. Chem Immunol Allergy 93:1–23
Rucker VC, Havenstrite KL, Herr AE (2005) Antibody microarrays for native toxin detection. Anal Biochem 339:262–270
Sabet NS, Subramaniam G, Navaratnam P, Sekaran SD (2007) Detection of methicillin- and aminoglycoside-resistant genes and simultaneous identification of *S. aureus* using triplex real-time PCR Taqman assay. J Microbiol Method 68:157–162
Sellman BR, Howell AP, Kelly-Boyd C, Baker SM (2005) Identification of immunogenic and serum binding proteins of *Staphylococcus epidermidis*. Infect & Immun 73(10):6591–6600
Shafer WM, Iandolo JJ (1979) Genetics of staphylococcal enterotoxin B in methicillin-resistant isolates of *Staphylococcus aureus*. Infect Immun 25:902–11
Soloaga R, Corso A, Gagetti P, Faccone D, Galas M (2004) Methicillin resistance detection in *Staphylococcus aureus*: comparison between conventional methods and MRSA-screen latex agglutination technique. Rev Argent Microbiol 36(1):36–40
Stuhlmeier R, Stuhlmeier KM (2003) Fast, simultaneous, and sensitive detection of staphylococci. J Clin Pathol 56:782–785
Summers WC, Brookings ES, Waites KB (1998) Identification of oxacillin-susceptible and oxacillin-resistant *Staphylococcus aureus* using commercial latex agglutination tests. Diagn Microbiol Infect Dis 30:131–134
Tenover FC, Gorwitz RJ (2006) The Epidemiology of *Staphylococcus* infections. In: Fischetti VA, Novick RP, Ferretti JJ (eds) Gram positive pathogens. ASM, Washington, pp 526–534
Todd EC (1997) Epidemiology of foodborne diseases: a worldwide review. World Health Stat Q 50:30–50
Vuong C, Otto M (2002) *Staphylococcus epidermidis* infections. Microbes Infect 4(4):481–489
Wellinghausen N, Wirths B, Essig A, Wassill L (2004) Evaluation of the hyplex bloodscreen multiplex PCR-linked immunosorbent assay system for direct identification of gram-positive cocci and gram-negative bacilli from positive blood cultures. J Clin Microbiol 42(7):3147–3152