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Detection of *Staphylococcus aureus* enterotoxin A and B genes with PCR-EIA and a hand-held electrochemical sensor

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Abstract

Two electrochemical assays for detecting *Staphylococcus aureus* enterotoxin A and B genes were developed. The assays are based on PCR amplification with biotinylated primers, hybridization to a fluorescein-labeled probe, and detection with horseradish peroxidase-conjugated anti-fluorescein antibody using a hand-held electrochemical detector. The limit of detection (LOD) for both assays was approximately 16 copies of the *sea* and *seb* genes. The assays were evaluated in blinded studies, each with 81 samples that included genomic and cloned *S. aureus* DNA, and genomic DNA from *Alcaligens, Bacillus, Bacteroides, Bordetella, Borkholderia, Clostridium, Comanonas, Enterobacter, Enterococcus, Escherichia, Francisella, Haemophilus, Klebsiella, Listeria, Moraxella, Neisseria, Proteus, Pseudomonas, Salmonella, Serratia, Shigella, Streptococcus, Vibrio and Yersinia* species. Both assays showed 100% sensitivity. The specificity was 96% for the SEA assay and 98% for the SEB assay. These results demonstrate the feasibility of performing probe-based detection of PCR products with a low-cost, hand-held, electrochemical detection device as a viable alternative to colorimetric enzyme-linked assays of PCR products.
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Keywords: Staphylococcus aureus; Enterotoxins; SEA; SEB; Electrochemical PCR-EIA

1. Introduction

Staphylococcus aureus is a gram-positive bacterium that colonizes and persists in diverse host habitats. It produces 15 enterotoxins, SEA-SEE, SEG-SEO, and SEU. Based on their antigenic properties, these entertotxins were divided onto three subfamilies: the *sea* sub-family includes *sea, sed, see, seh* and *sej*; the *seb* sub-family includes *seb, sec* and *seg;* and the *sei* subfamily includes *sei, sek* and *se*.[1]. These and other *S. aureas* toxins are known to cause human food poisoning and toxic shock [2–4]. *S. aureus* is also considered to be a potential biological threat agent because it can be produced in large quantities, its toxins are relatively stable to chemical and physical inactivation, and small amounts of toxins (<1 μg) can trigger symptoms of toxic shock.

Various immunoassays based on antigen capture have been developed to identify and assay enterotoxins produced by *S. aureus*. Among these assays are the reversed passive latex agglutination (RPLA), manual and automated enzyme-linked imunosorbent assays (ELISAs), and immuno-magnetic electrochemiluminescence assays [5–7]. With these assays, it was possible to detect 1 pg/ml of *S. aureus* B toxin [5] or 0.2–1.25 ng/ml of *S. aureus* A, B, C, D, E toxins [5]. The issue of cross-reactivity and limited specificity of these assays continue to be a major disadvantage and explain the need for DNA based toxin identification methods.

PCR-based assays with gel electrophoresis, dot blots, or Southern blots were developed to specifically identify genes of *S. aureus* enterotoxins A, B, C, D, E, exofoliative toxins (ETA, ETB) and TSST-1 [8–13]. While these assays are useful as research tools, they do not lend themselves for use in rapid diagnosis. PCR-based colorimetric enzyme-immuno assays (PCR-EIA) for detecting *sea* and *seb* genes have been developed. Becker et al. developed

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14. ABSTRACT
Two electrochemical assays for detecting Staphylococcus aureus enterotoxin A and B genes were developed. The assays are based on PCR amplification with biotinylated primers, hybridization to a fluorescein-labeled probe, and detection with horseradish peroxidase (HRP)-conjugated anti-fluorescein antibody using a hand-held electrochemical detector. The limit of detection (LOD) for both assays was approximately 16 copies of the sea and seb genes. The assays were evaluated in blinded studies, each with 81 samples that included genomic and cloned S. aureus DNA, and genomic DNA from Alcaligens, Bacillus, Bacteroides, Bordetella, Burkholderia, Clostridium, Comanonas, Enterobacter, Enterococcus, Escherichia, Francisella, Haemophilus, Klebsiella, Listeria, Moraxella, Neisseria, Proteus, Pseudomonas, Salmonella, Serratia, Shigella, Streptococcus, Vibrio and Yersinia species. Both assays showed 100% sensitivity. The specificity was 96% for the SEA assay and 98% for the SEB assay. These results demonstrate the feasibility of performing probe-based detection of PCR products with a low-cost, hand-held, electrochemical detection device as a viable alternative to colorimetric enzymelinked assays of PCR products.

15. SUBJECT TERMS
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PCR-EIA assays for the *sea* and *seb* genes and detected as little as 50 and 100 pg of genomic DNA for single and multiplex PCR, respectively [8]. Gilligan et al. developed PCR-EIA assays specific for *sea* and *seb* [14]. Both assays detected about 250 gene copies of the *sea* or *seb*. More recently, quantitative Real-Time PCR assays for the detection of enterotoxins genes *sea* to *sej*and *seu* were reported [15,16a,16b]. The detection limits varied from 100 to 400 copies for the *sea* gene [15] and 250 to $>10^5$ copies per PCR reaction for the *sea* to *sej* genes [16b].

The aim of this study was to develop an electrochemical PCR-EIA as an alternative to a colorimetric PCR-EIA to detect the *sea* and *seb* genes of *S. aureus*.

2. Materials and methods

2.1. Bacterial strains and DNA samples

The bacterial strains and cloned DNA used in this study are listed in Table 1. The DNA from cultures and clinical specimens was isolated by standard phenol-chloroform extraction followed by ethanol precipitation [17] or by using the QIAamp DNA purification kit (Qiagen, Hilden, Germany). The final DNA pellets were suspended in 100 µl of TE buffer (1 mM EDTA; 10 mM Tris-Cl pH 7.5), the concentration was determined with a spectrophotometer and the DNA was stored at −20 °C until use.

Table 1
Bacterial species and strains used for electrochemical PCR-EIA

Organism	Strain/ isolate	Organism	Strain/ isolate
Alcaligens xylosoxidans	19606	*Pseudomonas aeruginosa*	17933D
Bacillus anthracis	Ames	*Salmonella choleraesuis*	9150D
Bacillus anthracis	4728	*Serratia odorifera*	33077
Bacillus cereus	13061	*Shigella flexneri*	12022
Bacillus cereus	10867	*Shigella sonnei*	9290
Bacillus coagulans	7050	*Staphylococcus aureus*	25923
Bacillus licheniformis	2759	*S. aureus*	29247
Bacillus megaterium	8244	*Staph-SEA*	RIID clinic
Bacillus polymexa	4525	Staph-SEB	RIID clinic
Bacillus stearothermophilus	7953	Staph-SEC	RIID clinic
Bacillus subtilis var niger	6633	Staph-SED	RIID clinic
Bacillus thuringiensis	35646	Staph-SEE	RIID clinic
Bacteroides distasonis	8503	Staph-TSST-1	RIID clinic
Bordetella bronchiseptica	10580	*S. hominis*	27844
Burkholderia cepacia	25416	*S. epidermidis*	49134
Clostridium perfringens	13124	*Stenotrophomonas maltophilia*	13637
Comanonas acidovorans	15668	*Streptococcus pneumoniae*	33400
Enterobacter cloaceae	49141	*S. pyogenes*	19615
E. aerogenes	m10822	*Vibrio cholerae*	N16961
Enterococcus durans	6056	*Yersinia entercolitica*	NA
E. faecalis	29212	*Y. kristensii*	33639
Escherichia coli	25922	*Y. pestis*	CO92
Francisella tularensis	NA	*Y. pestis*	K25
Haemophilus influenzae	51 907D	*Y. pestis*	Nairobi
Klebsiella pneumoniae	13883	*Y. pestis*	Java9
Listeria monocytogenes	15313	*Y. pseudotuberculosis*	6904
Moraxella cattaharalis	25240	*Yersinia ruckeri*	29473
Neisseria lactamica	23970	SEAWT	clone
Proteus mirabilis	7002	SEBWT	clone
Proteus vulgaris	49132		

2.2. PCR amplification

The primers used to amplify *sea*and *seb* genes are listed in Table 2. PCR amplifications were carried out in 50 µl volumes. Each reaction contained PCR buffer (10 mM Tris–HCl pH 9.0, 50 mM KCl, 0.1% Triton× 100), 0.5 µM of each primer, 0.250 mM dNTPs (Roche, Indianapolis, IN), 3.125 mM MgCl2 (Promega, Madison, WI), 0.0125 U/µl uracil N-glycosylase (Roche), 0.25 U of Taq DNA polymerase (Promega) and 5 µl of template DNA. Thermal cycling consisted of one cycle at 95 °C for 2 min; 45 cycles of 95 °C for 15 s and 60 °C for 60 s; followed by one final extension cycle at 72 °C for 5 min on a PTC 100 thermal cycler (MJ Research, Waltham, MA).

2.3. Electrochemical detection

The fluorescein-labeled probes used for detection were purchased from Invitrogen (Carlsbad, CA) and are listed in Table 2. Five microlitre of PCR product were added to individual wells of a microtiter plate containing 40.5 µl of lambda-exonuclease (0.06 U/µl) (Roche) and incubated at room temperature for 5 minutes to generate single-stranded PCR products. Sixty microlitre of fluorescein labeled probe (40 nM) were added, mixed, and incubated for 5 min at 80 °C, then for 5 min at room temperature. Fifty microlitre were transferred to wells of neutravidin-coated microtiter plate (Roche) and incubated at room temperature for 10 min. The plate was then washed five times with washing buffer that contained 24 mM Tris, 1 mM EDTA, 75 mM NaCl and 0.05% Tween 20. Fifty microlitre of horseradish peroxidase (HRP)-conjugated anti-fluorescence antibody (0.75 U/µl) were added to each well and incubated for 10 min at room temperature. The plate was washed five times. The electrochemical reaction was initiated by adding 50 µl of 3,3′,5,5′-tetramethylbenzidine (0.4 g/µl) and HRP peroxide (0.02%) (Pierce, Rockford, IL). The current resulting form the enzyme-substrate reactions was measured with an electrochemical reader using intermittent pulse amperometry. For comparison, additional readings at 410 nm were recorded by using an ELISA reader (Dynatech, Chantilly, VA).

Table 2
Primers and probes sequences

Primers/ probes	Nucleotide sequence	Reference
SEAU	Biotin-5′-ATGGTAGCGAGAAAAGCGAA-3′	Gilligan et al. [14]
SEAL	PO4-5′-GCCATAAATTGATCGGCACT-3′	Gilligan et al. [14]
SEAP	Fluorescein-5′-CTAAAGCTGTTCCCTG-CAATTCA-3′	Gilligan et al. [14]
SEBU	Biotin-5′-TGTATGTATGGTGGTGTAAC-3′	Sharma et al. [13]
SEBL	PO4-5′-ACAAATCGTTAAAAACGGCG-3′	Gilligan et al. [14]
SEBP	Fluorescein-5′-ATAGTGACGAGTTAGGTA-3′	This work

2.4. Data analysis

Student's *t*-test and regression analysis were used to evaluate statistical significance and assay linearity. To determine positivity and negativity, three negative controls were run in each assay and a threshold value was calculated from the mean electrochemical signal of the negatives controls plus two standard deviations. Sensitivity was defined as $(TP/(TP+FN)\times 100)$ and specificity as $((TN/(TN+FP)\times 100)$, where TP, TN, FP and FN represents true positive, true negative, false positive, and false negative, respectively.

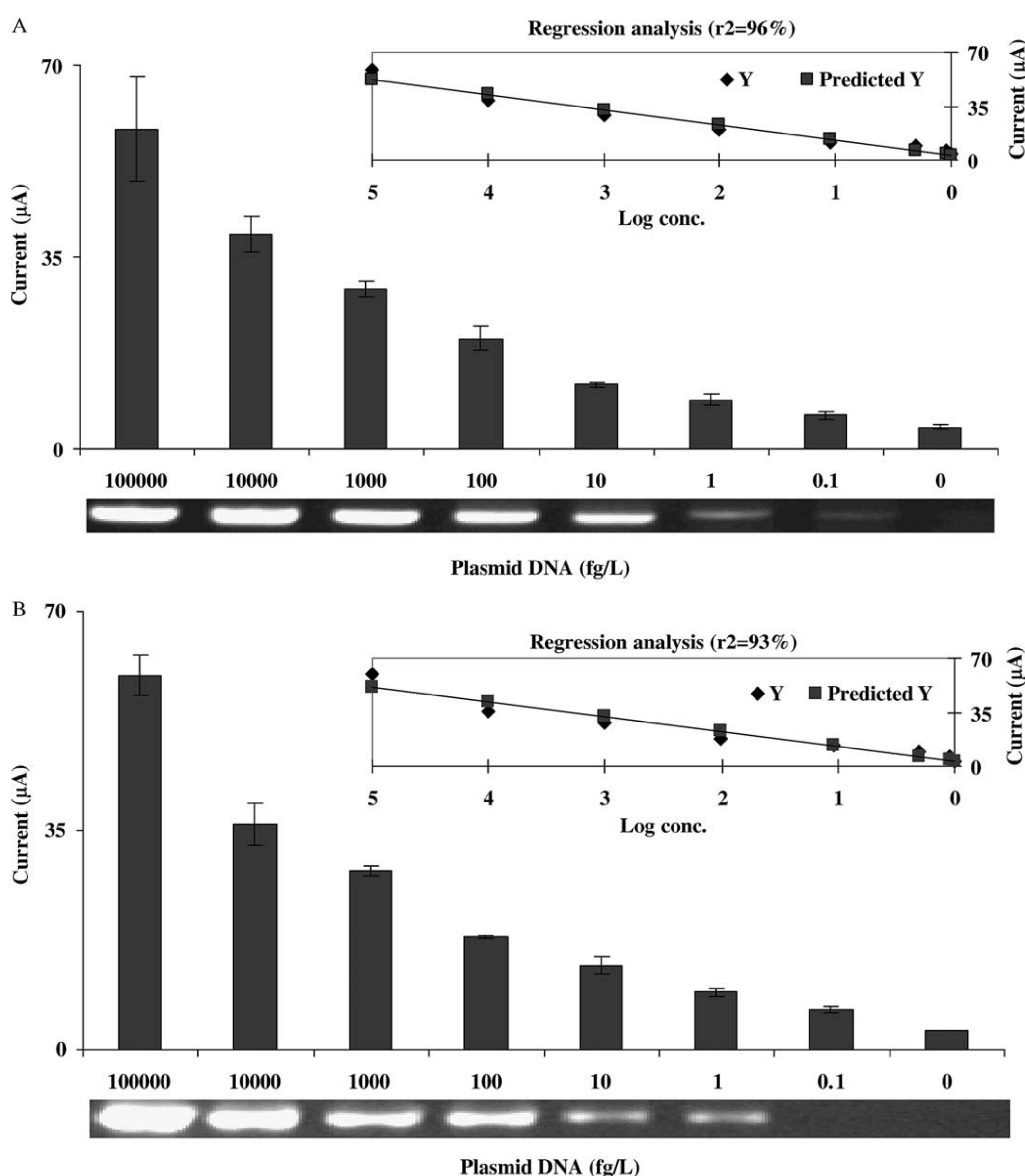

Fig. 1. Detection limits of SEA (A) and SEB (B) assays. Serial 10-fold dilutions of plasmid DNA ranging from 1,000,000 to 0.1 fg/µl were used. Each bar represents the mean of three replicates. Plasmid DNA was detected at 0.1 fg/µl which represents 0.5 fg per PCR reaction (about 12 copies). Regression analysis and the coefficient of correlation are indicated. Agarose gel analysis of PCR product obtained with 10-fold serial dilutions of plasmid DNA is shown below each graph.

3. Results

To determine the assay's limit of detection (LOD), 10-fold serial dilutions of cloned DNA ranging from100 pg/μl to 0.1 fg/μl were made and the assays were carried out according to optimized protocols. The LOD of the SEA and SEB assay was 0.5 fg of plasmid DNA (12 copies) per PCR reaction (Fig. 1). The electrochemical signals obtained with 0.5 fg of plasmid DNA (23 replicates) were significantly higher ($p < 0.001$) than those obtained with the negative controls (23 replicates) for both assays. Regression analysis of electrochemical signals obtained with the SEA and SEB assays showed a linear correlation with DNA concentrations spanning seven orders of magnitude ($r^2 \geq 93\%$) indicating the utility of the assays as quantitative tests (Fig. 1).

When a 10-fold dilution series of *S. aureus* genomic DNA was used, the LOD of each assay was 10 fg/μl (50 fg per PCR reaction) or 16 copies of template. Each assay was tested by colorimetric EIA detection and compared with the electrochemical detection assay. Similar sensitivity levels were obtained with the colorimetric assays (data not shown). Tests with spiked DNA showed that addition of non-enterotoxigenic staphylococcal DNA or human DNA to a cloned DNA had no effect on the amplification and detection limits. This indicates the absence of PCR inhibition and interference with ELISA detection (data not shown) and that the performance of our hand held electrochemical detection system was comparable to the colorimetric PCR-EIA methods.

To determine the sensitivity and specificity of the assays, we tested DNA samples from 44 different bacterial species in a blinded study. A total of 81 samples plus 11 positive controls and four negative controls were tested. Included in this panel were DNA from *Streptococcus pyogenes* strains containing *spea* or *spec* genes that exhibit a high amount of sequence similarity to *seb* and *sea* genes, respectively. All eight samples positive for *sea* and 18 samples positive for *seb* genes were correctly identified (Table 3). Thus the sensitivity was 100% for both assays. Of 56 DNA samples that did not contain *sea* or *seb* genes, two were considered false positive by SEA assay and one was considered false positive by the SEB assay (Table 3). Thus the specificity of the SEA and SEB assays were 96 and 98%, respectively. However, upon repetition, these samples were negative, and agarose gel analysis did not show any PCR products. These results indicate that the assays are specific for *sea* and *seb* genes.

Table 3
Cross reactivity results of SEA and SEB electrochemical PCR-RIA

Organism	Toxin	Number of positive/total	
		PCR-EIA *sea*	PCR-EIA *seb*
Staphylococcus aureus	–	0/1	0/1
S. aureus	SEA	7/7	0/7
S. aureus	SEB	0/17	17/17
S. aureus	SEA/SEB	1/1	1/1
Staph-SEC	SEC	0/2	0/2
Staph-SED	SED	0/2	0/2
Staph-SEE	SEE	0/1	0/1
Staph-TSST-1	TSST-1	0/1	0/1
Other bacterial species[a]	–	2/49	1/49
Sensitivity	–	100%	100%
Specificity	–	96%	98%

[a] See Table 1.

4. Discussion

The objective of this study was to develop an assay that determines the presence or absence of *S. aureus* harboring enterotoxin A and B genes. We have documented in the forgoing results a highly sensitive and specific electrochemical assay that quantitates PCR products using microtitre strips with wells equipped with immunosensors. The strips were applied for testing a large number of sample DNAs with both increased sensitivity and specificity. The results were automatically acquired and no gel-based detection was required.

Identifying infectious disease and biological threat agents relies on a variety of microbiological, serological, and molecular techniques, each has advantages and disadvantages. Microbiological techniques provide reliable information on viability but they are not necessarily specific. Serological techniques can rapidly detect antigens or antibodies, but they have limited sensitivity and specificity. Molecular techniques are highly sensitive and specific, but do not provide information on viability. In a naturally occurring epidemic or a bioterrorism attack scenario, it may be necessary to use a battery of assays and platforms that when used in tandem can provide reliable information on the quantity, identity, and viability of the agent. In this report we described PCR-EIA methods for detecting *S. aureus sea* and *seb* genes using a hand-held electrochemical detector. Although the detector is used here for DNA detection, it can also be used for antigen or antibody detection using ELISA techniques.

When the assays were tested with a reference panel of 81 samples, all eight samples positive for *sea* and 18 samples positive for *seb* were correctly identified. Thus the sensitivity for both assays was 100%. Of the 56 samples that lacked *sea* or *seb* genes, two false positives (*Enterobacter aerogenes* and *Yersinia rukeria*) were observed with the SEA assay and one false positive (*Yersinia rukeria*) was observed with the SEB assay. These false positive results were not due to non-specific amplification or non-specific probe hybridization. Upon repetition, these samples were negative, and there was no visible PCR products by agarose gel analysis. There was 100% correlation between phenotypic and genotypic identification and the results were reproducible and statistically significant. Furthermore, there was no cross-reactivity with streptococcal enterotoxins, which are genetically related to staphylococcal enterotoxins. However, we elected to state the specificity as observed in the first run to illustrate the fact

that carry-over contamination is always a risk in all PCR-based assays. Therefore, it was important to repeat questionable results at least three times to verify the results.

The same underlying enzymatic reactions are used in electrochemical and colorimetric PCR-EIA assays, and the two approaches show similar sensitivity and specificity. The results reported here show that electrochemical PCR-EIA assays can reliably carry out assays previously done with colorimetric PCR-EIA. In addition, the electrochemical detection procedure can be carried out at ambient temperature with a hand-held device.

The utility of electrochemical PCR-EIA as a screening tool for analyzing organic molecules such as steroids, protein, and antibiotics was previously reported [18,19a, 19b]. Although electrochemical detection scores are based on peroxidase activity, phosphatase and catalase activities measured by electrochemical sensors could also be used as an alternative. There is an amperometric immunosensor reported to detect and assay levels of *S. aureus* electrically in pure cultures and in foods [19] The assay measures amperometrically the catalase activity that was conjugated to an anti-protein A antibody. The use of phosphatase substrate in this format has yet to be tested.

Although TaqMan® assays offer Real-Time detection, PCR-EIA assays are more affordable in some laboratories. Moreover, the electrochemical detection platfrom can be used for antigen or antibody detection as well as for DNA asssays.

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