Signal Amplification and Detection of *Staphylococcus aureus* Enterotoxins in Foods

Leenalitha Panneerseelan and Peter Muriana.

Story in Brief

Staphylococcus aureus ranks as one of the most important bacteria that can cause foodborne illness. The organism produces a variety of enterotoxins that may cause food borne intoxications. Some staphylococcal enterotoxins are classified as biological select agents. The presence of *Staphylococcus aureus*, and especially its toxins in foods is of great concern to the food industry as it represents a major problem for the health of consumers in addition to potential economic losses. The organism rapidly grows and produces toxins in processed foods due to the absence of microbial competition. It is imperative to detect minute quantities of the toxins because the SE's are potent even in very small quantities and it is possible that the intoxication dose could be underestimated due to the limits of detection methodologies. The sensitivities of many assays are often hindered when presented with environments such as found in many complex food matrices. In order to overcome detection biases and obtain amplified signals from the toxin antigen, a bead-based immunodetection PCR assay was developed and tested. The assay involves immunocapture of the toxin antigen(s) by paramagnetic beads coated with a primary *capture antibody*. A secondary antibody, covalently conjugated with reporter DNA, is then amplified by real-time PCR using SYBR green detection chemistry. Using this assay, the detection of SE had considerably improved over current commercial ELISA detection limits (1 ng ml^{-1}) with a sensitivity of less than 7.5 fg ml^{-1}. The assay is semi-automated, simple, and could be performed within 5-6 h.. Furthermore the method was not hindered by the presence of the food matrix or other microbial flora.

Key Words: Enterotoxins, Immuno PCR, Sandwich ELISA, Signal amplification, *Staphylococcus aureus*

Introduction

Staphylococcus aureus is a Gram-positive microorganism that can produce enterotoxins that is often responsible for causing food poisoning. The intoxication is characterized by enteric responses like diarrhea, abdominal cramps, and vomiting within 1-6 h of consumption of contaminated food (Jorgenson et al., 2005). Different strains of *Staphylococcus aureus* are known to produce at least twenty different types of enterotoxins, the most common ones being SEA-SEE (Nema et al., 2007). The toxins are heat stable proteins with a molecular weight of 16,900- 29,600 (Holeckova et al., 2002). The bacterium is heat labile and does not compete well with other microorganisms and therefore, contamination usually occurs after the food has been processed when there is little competition from other microorganisms. The organism usually gains access to foods from food handlers or other surfaces like the processing equipment. Although Staphylococci are commonly found on animal skins, water, soil etc, bacteria from food handlers and other human sources are considered as the most important contributing factors to intoxications associated with food (Aycicek et al., 2005). Food poisoning is of great concern to food industries and regulatory agencies as it represents massive health and economic losses. The foods that are commonly contaminated by SE are baked dessert items such as cream filled

pastries, cream pies, chocolate éclairs, meat and meat products, potatoes, tuna, chicken, turkey, ready-to-eat salads, eggs, poultry, dairy and milk products. The quantity of enterotoxin present in foods involved in food poisoning may vary from less than 1ng g^{-1} to more than 50 ng g^{-1} (Bergdoll, 1991). The detection limits that have been reported by commercial systems for SE range from 0.5 to 2 ng enterotoxin g^{-1} of food (Wieneke, 1991; Park et al., 1994; Di Pinto et al., 2004). Many biological and immunological assays are often hindered by complex food matrices. This shows that it is also possible that the established intoxication dose is underestimated due to constraining detection limits (Rajkovic et al., 2006). The objective of the study is to obtain amplified signals from staphylococcal enterotoxins, therby improving the sensitivity and detection limits. The results reported herein indicate that a magnetic bead based immunological PCR assay could amplify toxin signals detected in food and greatly improve detection limits. The assay is not hindered by the presence of foodstuff matrix or other microbial flora and could be performed within 5-6 hrs.

Materials and Methods

Bacterial Strains, Media and Conditions. The *Staphylococcus aureus* strain ATCC 51740 was maintained in Tryptic Soy Broth (TSB) (EMD Chemicals Inc, Gibbstown, NJ) with 10% glycerol at -83°C. The strain was resuscitated in TSB by incubating at 37°C for 24 h before use and all reference stocks were made in the same media.

Commercial kits, Antigens, and Antibodies. Two commercial detection systems, SETVIA (TECRA, Australia) and ELISA kit for SEB detection (Toxin Technologies Inc., Sarasota, Fla), were used for comparison with IPCR results. Staphylococcal enterotoxins SEA, SEB, and sheep polyclonal antibodies (SLB1202) (Toxin technologies) were employed in ourIPCR assays.

Coating of Paramagnetic Beads. The coupling of COOH-modified magnetic beads (2.8 µm, Dynal Biotech, CA) with sheep polyclonal antibodies was performed as described elsewhere (Alefantis et al., 2004). The coating was performed using the semi-automated bead retriever (Dynal Biotech, CA).

Generation of Reporter DNA. A DNA template encoding the luciferase gene (Promega, Madison, WI) was used for generation of reporter DNA as described previously (Wu et al., 2001). The forward primer was amino-modified, in order to covalently conjugate the reporter DNA to the secondary (reporter) antibody and the reverse primer was unmodified. A 563 bp amino-modified reporter DNA was generated by PCR.

Synthesis of Conjugate. Briefly, the amino-modified reporter DNA (in 60 µl, containing approximately 20 ng/µl) was activated with succinimidyl-4-[N-maleimidomethyl]-cyclohexane-1-carboxylate (SMCC) (Geno Tech., St.Louis, MO). The amine group on the DNA was covalently bound to the carbonyl group of SMCC via an ester reaction. The anti-SE sheep polyclonal antibody (300µl of $4x10^9$ molecules/µl) was reduced with dithiothreitol (Promega, Madison, WI). The DNA-SMCC molecules were combined with the reduced antibody and allowed to form a covalent bond between the maleimide group of the SMCC and the sulfhydryl groups of the antibody molecules in the dark at 25°C (Hendrickson et al., 1995). The conjugate generated from this reaction was ready for use without further purification.

Toxin Detection in Broth by IPCR. Real-time PCR SYBR green chemistry was used to quantify the reporter DNA bound to the secondary antibody. Magnetic beads coated with the primary antibody were incubated with the 10-fold serial dilutions of the antigen. The starting concentration of the toxin was either 750 or 75 ng ml^{-1} and dilutions were made up to 10^9 fold corresponding to 7.5 fg ml^{-1}. The incubation was at 25°C for 1 h with mild rotation, after which the beads were washed four times with wash buffer containing phosphate buffered saline. Washing was performed using the Bead Retriever (Dynal). Blocking buffer (100 µl) was added and the mixture was incubated at room temperature with rotational agitation for 15 min. The particles were washed, collected in 200 µl of distilled water and 50 µl of conjugate (secondary antibody - reporter DNA) was added and incubated with shaking at 37°C for 1 h. The samples were then washed three times with wash buffer and three times with distilled water and finally collected in 200 µl of distilled water. The collected beads were incubated at 85°C for 5 min and centrifuged at 5000 x g for 5 min. Following centrifugation, 5µl of the supernatant was added to the PCR master mix containing the SYBR green dye (1x) and the 100 nM of each of the primers. A 183 bp amplicon obtained in the test samples and positive controls and was confirmed by melting curve analysis and agarose gel analysis (1.5% gel).

Toxin Detection in Foods. Pure toxins SEA and SEB were spiked in foods including milk, lemon meringue pie, and deli tuna salad. The starting concentrations were 75 ng ml^{-1} and serial dilutions were carried up to 10^9 fold to a concentration of 7.5 fg ml^{-1}. The results obtained with IPCR were compared with the kits.

Enterotoxin Production by S. aureus ATCC 51740. An overnight culture of *S. aureus* ATCC 51740 was tested for production of enterotoxin by IPCR and also by commercial systems. The organism was also inoculated and tested for enterotoxin production in foods commonly incriminated in intoxications such as milk, ice cream, lemon meringue, tuna salad, and processed turkey meat. The toxin was extracted from the culture media and foods prior to the analysis. All of the foods had a pH of above 5.8 and water activity greater than 0.98. All tests were done in duplicate or triplicate including the controls.

Comparison with Commercial Systems. The results obtained with the IPCR were compared with the commercial systems. The manufacturer's protocol was followed for performing the tests and the absorbance was measured at 405 nm using an ELISA plate reader (Tecan GENios, Carlsbad, CA).

Primers used in the study

Generation of reporter DNA for tethering to antibody.

Primer A - Amino 498: 5' GTT CGT CAC ATC TCA TCT AC 3'

1061(rev): 5' TCG GGT GTA ATC AGA ATA GC 3' Product size - 563 bp

Real time amplification of reporter DNA conjugated to secondary antibody.

Primer B - 5' CCA GGG ATT TCA GTC GAT GT 3'

(rev) - 5' AAT CTG ACG CAG GCA GTT CT 3' Product size - 183 bp

Results and Discussion.

Detection of pure toxin in broth and food. The bead based IPCR assay detected pure SEA and SEB diluted 10^9-fold in tryptic soy broth. The amplification plots (Fig. 1& 2) shows the detection of enterotoxins SEB and SEA respectively in TSB. The lowest concentration of toxin detected was approximately 7.5 fg ml^{-1}. The threshold cycle (C_t) range which allows the quantification of the toxins was between 15 and 28 cycles. The method was about 1000 times more sensitive than the microtitre plate based iqPCR methods reported. Quantification of the toxin was based on comparing the C_t of the samples with that of a standard. The sensitivity of the IPCR was similar in the various foods tested. The amplification plots in Fig 2 & 4 shows the detection of SEB in deli tuna salad and SEA in lemon meringue respectively. The detection limits were similar to the detection of toxins in broth, with the lowest being 7.5 fg ml^{-1} . The data shows that the procedure was not inhibited by the presence of any complex food matrix.

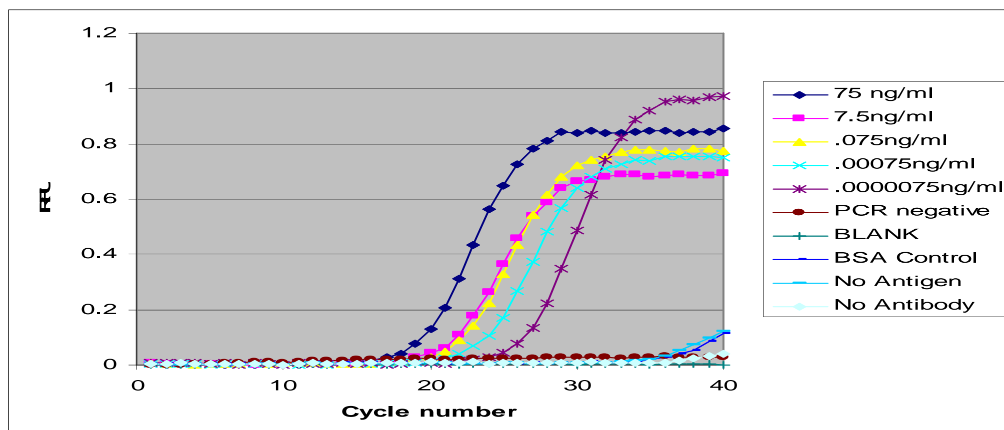

Figure 1. Enterotoxin B in tryptic soy broth.

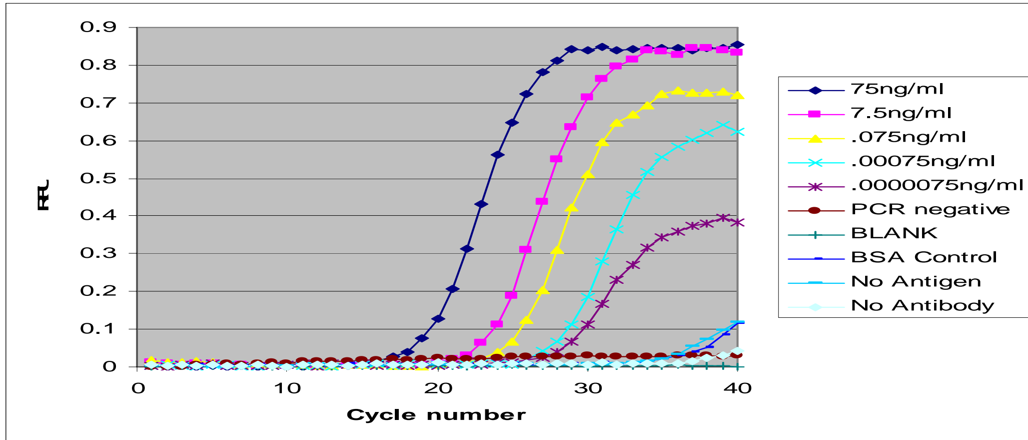

Figure 2. Enterotoxin B in ready to eat tuna salad

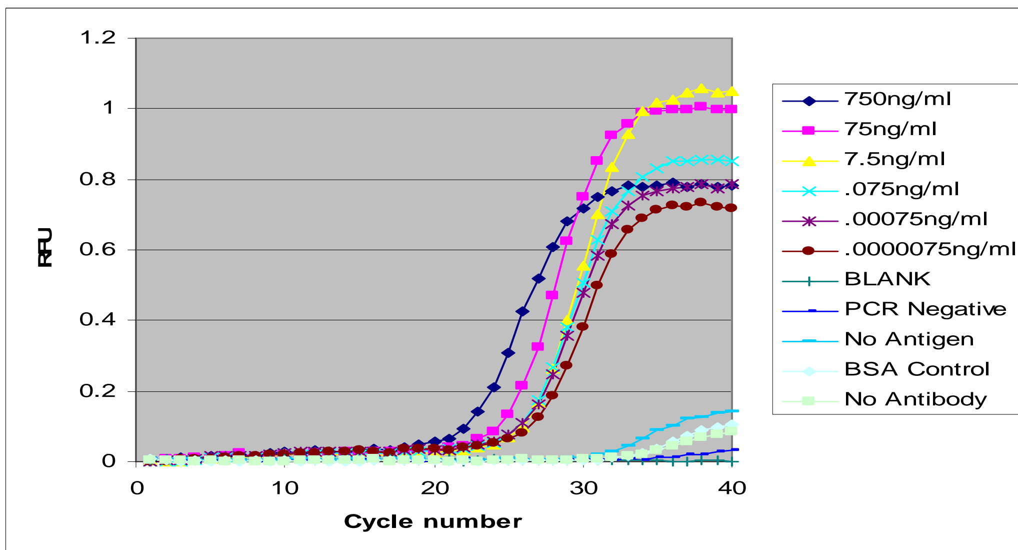

Figure 3. Enterotoxin A in tryptic soy broth.

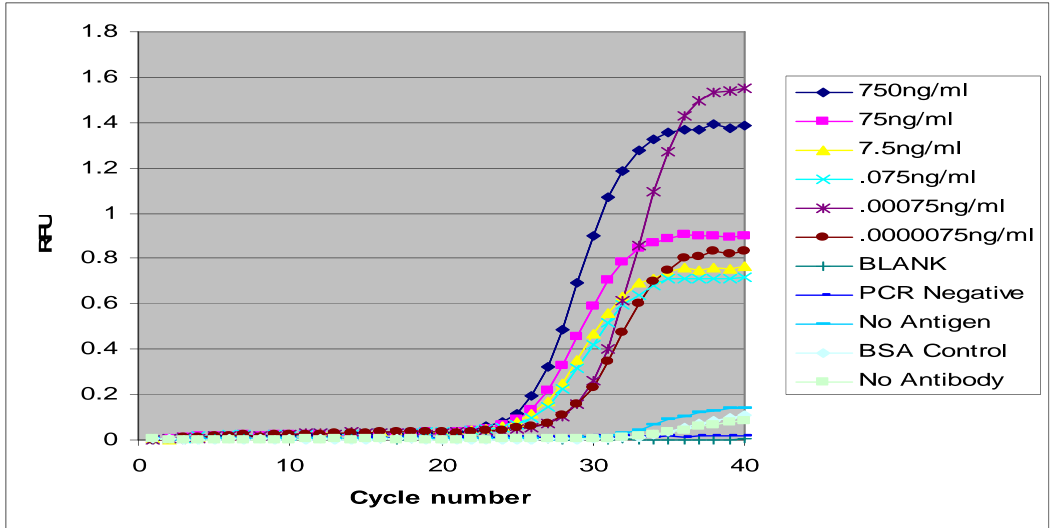

Figure 4. Enterotoxin A in lemon meringue. The change in C_t may be due to minor loss of toxin during extraction.

Enterotoxin Production by S. aureus. The enterotoxin B production by *S. aureus* ATCC 51740 tested in commonly contaminated foods (Fig. 5) showed that the organism was able to produce considerable amount of toxin (> 75 ng ml^{-1}) in turkey meat. The toxin production in culture media incubated at an optimal growth temperature of 37°C for 24 h was approximately 52.5 ng ml^{-1}. The toxin production in tuna and pie was less than 7.5 fg ml^{-1}. However in ice cream and milk the toxin production was not detectable by the method, which indicates that either the toxin was not produced or it was produced in very low quantities which were undetectable by the method. The results were compared with the commercial systems.

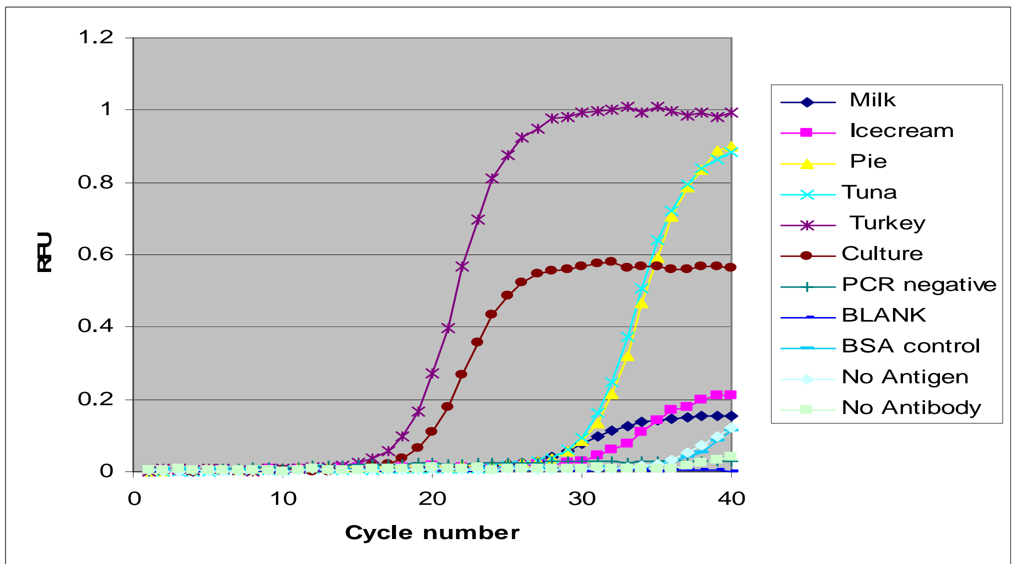

Figure 5. Enterotoxin production by *S.aureus* ATCC 51740 in food samples

Detection by Commercial Systems. Two-fold serial dilutions of pure toxins SEA and SEB encompassing the range covered by IPCR were tested in broth and in foods such as milk, lemon meringue, and tuna salad. Both the commercial systems were able to detect the toxin concentrations up to 1 ng ml^{-1}, but not lower. Enterotoxin production by *S.aureus* ATCC 51740 in culture media was detected by both kits. Enterotoxin production by the organism in foods was undetectable by the commercial kits except in processed turkey meat samples. The microtitre plates were read using an ELISA plate reader and absorbance was measured at 405 nm.

Conclusion

The bead-based immuno PCR was very sensitive in detecting minute quantities of toxins present at even less than 7.5 fg ml^{-1}. The method proved to be at least 10^3 times more sensitive than the reported microtitre plate based iqPCR and 10^6 times more sensitive than commercial kits. The assay was simple and had a processing time of only 5-6 h. The commercial kits had a processing time of 4-5 h, however the detection limits of the commercial ELISA kits was about 10^6 times less than the IPCR assay. The method could also be easily modified to detect other types of enterotoxins in foods. The method involved immunocapture using paramagnetic beads, followed by amplification by PCR, and has the potential for detecting even lower amounts of toxins if the need arises.

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Authors

Panneerseelan, Leena. Ph.D. - Student, Department of Food Science, Oklahoma State University

Muriana, Peter. - Associate Professor, Department of Animal Science & the Food and Agricultural Products Center, Oklahoma State University