JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.7b04820 • Publication Date (Web): 19 Jan 2018

Just Accepted

ACS Publications

Journal of Agricultural and Food Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Category of paper:

Original Article

Article Title:

A colorimetric DNAzyme biosensor for convenience detection of enterotoxin B harbouring *Staphylococcus aureus* from food samples

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Keywords: Enterotoxin B, DNAzymes, Hemin, Antiprotein A, Colorimetry

Abstract

In the present study, a colorimetric DNAzymes biosensor strategy was devised in combination with immunomagnetic separation for rapid and easy detection of enterotoxin B harbouring *Staphylococcus aureus* from food and clinical samples. The method employs immunocapture of *S. aureus* and amplification of *seb* gene by DNAzyme complementary sequence integrated forward primer and with specific reverse primer. The DNAzyme sequence integrated dsDNA PCR products when treated with hemin and TMB (3, 3′, 5, 5′–tetramethylbenzidine) in the presence of H_2O_2 produce colorimetric signal. A linear relationship of optical signal with the initial template of *seb* was obtained which could be monitored by visually or spectrophotrometrically for qualitative and quantitative detection. The limit of detection for the assay was approximately 10^2 CFU/mL of *seb* gene harboring target. This method is convenient compared to gel based and ELISA systems. Further, spiking studies and analysis on natural samples emphasized the robustness and applicability of developed method. Altogether, the established assay could be a reliable alternative, low-cost, viable detection tool for the routine investigation of *seb* from food and clinical sources.

Keywords: Enterotoxin B, DNAzymes, Hemin, Antiprotein A, Colorimetry

Introduction

Identifying infectious disease and biological threat agents is of utmost priority in food security, armed forces and nation defence application. *Staphylococcus aureus* is considerd as one of the major pathogen associated with food-borne illness worldwide, known as staphylococcal food poisoning (SFP) [1, 2]. SFP caused due to consumption of contaminated food with small heat stable exotoxins commonly known as staphylococcal enterotoxins (SEs). Small amount of SEs (as low as 100 ng) can cause gastrointestinal symptoms and toxic shock syndrome. Among the SEs, Staphylococcal enterotoxin B (SEB) is responsible for severe food poisoning in humans and pose a threat to human life, currently listed as a category B Bio-warfare agent due to its potency (lethal dose 0.02 μg/kg and emetic response 0.4 ng/kg), ease to aerosolize and stability under various environmental conditions. Different strains of *S. aureus* can produce different SEs; however, staphylococcal enterotoxin B (SEB) is most common [2]. Therefore, the detection of this SEB producing *S. aureus* in food and environment is most important.

There are several methods for the detection of staphylococcal enterotoxins B, most common method is traditional culture techniques followed by assessment for the presence of toxins. To optimize the incubation time for production toxins, various factors, such as pH, osmotic pressures and substrates are important [5]. Various immunological methods are available for detection of Enterotoxins such as enzyme-linked immunosorbent assays, chemiluminescence, reversed passive latex agglutination tests and immunosensor are fairly reproducible and selective but selectivity depends on the expression and presence of the toxin in samples and required sophisticated equipment [6-12]. Moreover, highly trained personnel and relatively long analysis time are required as they are not readily available in developing countries and not capable of on-site detection. Antibodies also have some limitations as biorecognition ligands such as time-consuming, high production cost and required animals

for production. In addition, specificity and affinity depends on specific environmental conditions and become unstable or inactive upon modification which can be unfavorable for food and environmental samples analysis [13].

Therefore, nucleic acids based identification has gained attention as part of techniques for diagnosis of pathogens, detection of biomolecules and forensic studies [14-16]. The polymerase chain reaction (PCR) has provided enormous advance in molecular based detection because PCR can amplify very small quantity of target DNA exponentially. Amplicon can be monitor by using gel electrophoresis or real-time PCR [17]. The main disadvantage of real-time PCR is requirement of expensive fluorescence labelled probes and sophisticated equipments which are limited to well-established laboratories. These limitations attract researcher for development of alternative rapid and simple methods for the identification of PCR products. Research was carried out to develop new DNA sensing platforms employing molecular beacon and nanoparticle conjugated probes. However, these assays are time consuming and requirement of costly fluorescence or thiol labelled probe limits their applications [14-16].

Therefore, simple and rapid suitable alternative methods for the identification of PCR products without the use of instrumentation can overcome the existing limitation. On this concern, in recent years DNAzymes (deoxyribozymes or catalytic DNA) have paying much attention for DNA target detection. DNAzymes are G-quadruplex nucleic acid sequence which can mimics the catalytic activity of peroxidase enzymes upon binding with hemin. Particularly, catalyzes oxidation of TMB (3, 3, 5, 5′ tetramethylbenzidine) or ABTS (2, 2-azinobis (3-ethylbenzothiozoline)-6-sulfonic acid) in presence of H_2O_2 and generates colored oxidized form [18]. Using DNAzymes and UV–Vis absorption spectroscopy a number of sensors has been developed [19–21]. Till now, to best of our knowledge there are no reports on the use of DNAzymes assisted PCR base detection of Staphylococcal enterotoxin B are available.

In the present study, a colorimetric biosensor strategy for detection of Enterotoxin B (SEB) producing *S. aureus* was devised. The assay employ combination of immunomagnetic separation of *S. aureus* cell from food samples using specific antibody followed by polymerase chain reaction with *seb* gene specific forward primer integrated with DNAzyme complementary sequence and normal reverse primer for optical signal generation. The amplified DNAzymes structure form with hemin can mimic peroxidase activity and produce blue color. The colorimetric signal of the catalytic reaction can be monitored spectrophotrometrically or visually. The specificity and sensitivity studies were carried out. The effectiveness of developed assay was evaluated on large number of *S. aureus* isolates and non-staphylococcal bacterial strains. Sensitivity was also tested using artificially spiked milk samples as well as natural samples.

Material and Method

Chemicals and Reagents

Sodium chloride (NaCl), Potassium chloride (KCl), dNTPs, *Taq* DNA polymerase, Hemin, TMB (tetra methyl benzidine) substrate reagent set, DNA ladder (Dye plus) were purchased from Sigma-Aldrich (India). Ethylene diamine tetra acetic acid (EDTA) and other reagents were obtained from Himedia (India). The primers were synthesized and purchased from Xcelris Bioscience (Ahmadabad, India). Analytical grade reagents, solvents and ultrapure water (Mill-Q, Millipore, India) were used throughout the study.

Bacterial strains and culture

The reference bacterial cultures used in the study are listed in table 1. In addition, *S. aureus* isolates (food and clinical isolates confirmed by biochemical and molecular characterization) from laboratory repository were also used. Staphylococcal and non-staphylococcal cultures were cultured in brain heart infusion broth (BHI) (Himedia, India) under aeration at 37 ºC for 18-24 h for genomic DNA preparation. The cultures were grown overnight at 37 °C shaking

incubator in Luria–Bertani broth (Himedia, India). The bacterial cultures were cryopreserved in 15 % glycerol at -80 °C.

Antigens, antibodies, and kits

Antiprotein A antibody antibodies for capturing of *S. aureus* purchased from Sigma, India. Antibody Biotinylation kit was purchased from Bangalore Genei, Bangalore, India. Two in house developed assays for comparison with assays developed in this study: PCR based detection kit and ELISA Kit.

Generation of biotinylated antibody

Anti protein A (5 mg /mL) antibody was prepared in 1× PBS and labeled with biotin using biotinylation kit (Bangalore genei, India) as per manufacturer's instructions. Further, for the confirmation of biotinylation, dot-ELISA was performed by coating 10 µL of different elutions of biotinylated antiprotein A antibody and normal antiprotein A antibody as control on a nitrocellulose membrane (Millipore, India). The membrane was air dried and blocked with 1 % BSA. Then the membrane was probed with streptavidin-HRP (Invitrogen, India) and chromogenic reaction was carried out using TMB/H_2O_2. After each step, the membrane was washed thoroughly with PBST (Tween 20-0.05 %) solution.

Preparation of antibody/Dynabeads conjugates

For binding of streptavidin dynabeads to the biotinylated antibodies, dynabeads solution was added to 10 µL of biotinylated antiprotein A antibodies (5 mg/mL). The solution was incubated in shaker (130 rpm) at room temperature for 30 min (Fig. 1). Then magnetic separation was carried out by placing the mixture tube in a magnetic separator for 15 min. The supernatants were removed by gently pipetting and pellets were re-suspended in 200 µL of BSA (0.1%) in 1× PBS. Consequently, the re-suspended dynabeds-antibody conjugates were centrifuged at 6000 rpm at 4 °C for 20 min. The centrifugation and re-suspension

procedure was carried out twice and the ensuing mixture of the antibody/dynabeds conjugates was stored at 4 ^{0}C before use.

Immunomagnetic Separation (IMS) Procedure

S. aureus cells (1.5×10^{6} CFU/mL) stock was prepared in 1× PBS for capturing by IMS. The antigen–antibody interaction between *S. aureus* and antibody/dynabeads conjugates was carried out by mixing 30 µL of antibody-dynabeads solution with the stock culture in the 1.5 mL eppendrof tubes. After incubation for 30 min with moderate shaking (130 rpm) at RT, the tube was placed in a magnetic particle concentrator stand (MPC-S) for 5 min. Cell bound dynabeads-antibody conjugates were magnetically separated from the solution and the supernatant was discarded without removing the tube from the MPC-S (Fig. 2). The supernatant collected and plated on the trypticase soy agar medium using spread plate method to enumerate unbound *S. aureus* to the dynabeads. Bacteria-dynabeads was monitored by scanning electron microscopy analysis. Captured cells enumeration was carried out by re-suspending the immune magnetically separated cells in 100 µL of 1× PBS and plated on agar plate. The bead-*S. aureus* complex was washed with 1× PBST and the tube was placed in the MPC-S for 5 min. The supernatant was discarded and this step was repeated thrice. Later the bead-*S. aureus* complex was resuspended in 100 µL of sterile water. The suspension was subjected to boil lysis for extraction of the genomic DNA. For control experiments, the equal amount of *S. aureus* cells was added to 90 µL of 1× PBS and cells separation carried out as discuss earlier.

Standardization of PCR amplification condition

PCR amplification was carried out using isolated DNA as template employing specific primer sets **(Table. 2, Supporting Information1**). Amplified DNA was analyzed in 2 % agarose gel electrophoresis and the concentration of PCR product was determined by NanoDrop 2000 (Thermo Scientific, India). The control PCR was carried out without the target template DNA

(absence of target bacteria). The specificity and reliability of the primer was assessed employing other related and non-target bacterial DNA as template for PCR amplification. To authenticate the amplified DNAzymes sequence is responsible for colorimetric signal, the PCR product was purified using PCR purification kit (sigma, India) and then confirmed by colorimetric sensing method.

Detection of Enterotoxin B positive *S. aureus* using colorimetric DNAzymes biosensor

The assay protocol for the colorimetric detection of Enterotoxin B positive *S. aureus* is illustrated in Fig. 2 [21]. Immunomagnetic Separation of *S. aureus* cell and DNA extraction carried out as mention above. PCR was performed according to the standardized condition. We have adopted the DNAzymes based assay for qualitative and quantitative detection of enterotoxin B producing *S. aureus* by visually and UV-vis spectroscopically, respectively. In brief, The amplified PCR products were mixed in the solution containing of 50 µL HEPES buffer, 6 µL KCl (70 mM) and 2 µL hemin (5 µM) and incubated at 37 °C for 15 min. Then, the solution was incubated with 50 µL of freshly prepared TMB/H_2O_2 for colorimetric signal generation and the qualitatively results was determined by naked-eyes. To terminate the reaction 50 µL H_2SO_4 (0.2 M) was added and quantitative result was interpreted by measuring the absorbance at 450 nm by using a spectrometer (TECAN, India).

Colorimetric DNAzyme biosensor specificity and sensitivity

To evaluate the specificity of developed biosensor various staphylococcal and non-staphylococcal cultures were tested mentioned in Table. 1. Briefly, the bacterial cultures were grown in respective media and tested directly through Immunomagnetic separation based colorimetric biosensor. For comparison, staphylococcal isolates were subjected to PCR analysis to examine the presence of *seb* gene using *seb* specific primers. Sensitivity of colorimetric biosensor was evaluated by employing tenfold serially diluted overnight *S. aureus* culture. Results were monitor by using agarose gel electrophoresis and colorimetric

assay. In order to assess the practicability of the assay for direct detection in food matrix, enterotoxin B positive *S. aureus* cell was spiked into milk samples (10^6 to 10^1 CFU/mL) and IMS colorimetric biosensing was performed.

Evaluation of colorimetric biosensor on food samples and clinical isolates

To evaluate the field applicability, IMS Colorimetric biosensor was evaluated employing *S. aureus* isolates (Table. 1) from different sources which were characterized earlier and naturally contaminated samples, including milk (n = 24), cheese (n =22), ice-cream (n= 10), chicken (n =10), and pastries (n =10), were pre-enriched with BHI broth and colorimetric assay perform as described previously [22]. Isolates of *S. aureus* (n = 91) from our laboratory repository were also included in this study. Reference strains of *S. aureus* were used as positive controls.

Comparison of colorimetric biosensor with available kits

The sensitivity and feasibility of the newly described method was evaluated by comparing with in-house developed PCR and ELISA kits. Various dilutions of *seb* positive *S. aureus* cells were assessed by the above mentioned detection systems (as per instruction) and colorimetric assay in culture broth as well as in spiked food samples. The absorbance measurement carried out at 450 nm by ELISA reader (TECAN, India) and the results were interpreted.

Statistical Analysis

The experiments were performed in triplicates. Data collected from three different experiments were then analyzed and interpreted statistically with the aid of GraphPad prism version 6.0.Standard deviation (SD) was determined and graphs were constructed. The level of significance was set at $p < 0.05$ and the sensitivity was considered at the values having at least three times more SDs above the blank.

Result and Discussion

Generation and characterization of biotinylated antibody

Staphylococcal protein A (SpA), a major cell wall associated surface protein is present nearly in all *S. aureus* strains. In our study monoclonal anti protein A antibody was considerd as a suitable capturing ligands to concentrate *S. aureus* from sample matrix. Antiprotein A antibody was biotinylated for conjugation with streptavidin modified dynabeads. Ligands and dynabeads interaction help in target capturing and concentration. To confirm biotinylation of antiprotein A antibody, dot ELISA was performed. Different elution of biotinylated anti protein A along with normal anti protein A antibody in carbonate–bicarbonate buffer, was fixed on the membrane and probed with streptavidin-HRP. After developing with TMB/H_2O_2, blue strong dots appeared on nitrocellulose membrane corresponding place of biotinylated antibody and no visualized dot was observed at position of normal Antiprotein A antibody (Fig. 3).

Immunomagnetic Separation (IMS) Procedure of *S. aureus* cell

One of the major challenges in detecting foodborne pathogens is in dealing with sample complexity. Although many rapid methods are available, most of them focus on assay development but complex sample enrichment and preparation steps limits their application. The major challenge with immunomagnetic separation of whole cell is availability of good-quality antibody against surface receptor of bacterium. In our study we have used Antiprotein A antibody a major surface protein present in all *S. aureus*. As per our result, we have found IMS resulted in effective isolation of *S. aureus* cells which is characterized by a distinct pellet of beads on the sides of the tubes. In this study we investigated the feasibility of using IMS for sample concentration for target detection. Scanning electron microscopy imaging confirmed the binding of antibody-dynabeads complex with *S. aureus* cells (Fig.4). Moreover, the cell capturing efficiency of Antiprotein A antibody coated dyna beads was evaluated using agar plating and colony counting. By enumerating the bound and unbound

bacteria, the capturing efficiency was determined to be almost 100 % for concentrations ranging between 1 and 10^2 cells, 99 % for 10^3–10^5 cells/mL, and 90 % for 10^6 cells/mL (Fig. 5).

DNAzymes biosensor approach for Rapid Detection of enterotoxin B producing *S. aureus*

DNAzymes biosensing approch for detection of Enterotoxin B positive *S. aureus* is outlined in Fig. 2 [21]. As shown Table.1, a specific forward primer was designed for amplification and signal generation which contains, a complementary sequence segment of *seb* gene for probing with template DNA, segment of poly A linker sequence for flexibility and a DNAzyme complementary sequence for signal generation. PCR amplification was carried out in presence of target DNA to incorporate DNAzyme sequence in double-stranded PCR products. In presence of hemin the PCR products form G-quadruplex/Hemin complex and catalyze the oxidation of TMB in presence of H_2O_2 to generated blue colour solution. The colorimetric signals due to catalytic reaction could be monitored with the naked eye or spectrometrically.

PCR amplification and viability of colorimetric method

We evaluated the practicability of the biosensor strategy for detection of Enterotoxin B positive *S. aureus*. PCR amplification ability and signaling property was compared using DNAzymes modified primers and normal primers sets. Sharp band in agarose gel indicated that both the primer pairs could successfully amplify *seb* gene without any non-specificity. The amplified product with DNAzymes modified primer is 58 bp larger in comparison with the amplicon size with normal primer (Fig.6). This amplicon size variation is due to the specific integration of DNAzyme sequences. Hence, the results validates that the both primer set could be useful for detection of *seb S. aureus*. Further, the signaling property of both primer sets was confirmed through the colorimetric assay. Obtain result indicates, in

comparison with negative control, amplified product with normal primer could not able to generate colorimetric signal while the PCR product with DNAzyme primer set able to generate signal.

Optimization of the Detection Conditions

We have devised DNAzymes biosensing platform for detection of Enterotoxin B positive *S. aureus*. To achieve best performance, some important assay parameter (assay tempareture, hemin and KCL concentration) which can influence the sensing performance were optimized. The colorimetric responses of DNAzymes modified PCR product was compared with control under different assay conditions. Firstly, the optimal temperature for the colorimetric sensing platform was investigated; strongest optical intensity was obtained at 37 ^{0}C, hence 37 ^{0}C was considered as optimal temperature for colorimetric sensing (data not shown). Similarly, concentration hemins also influence the catalysis reaction. As shown in Fig. 7a, the optimal colorimetric signal obtains at 5 μM of hemin. The KCl concentration is also an important parameter for the catalysis efficiency. Result clearly indicates the maximum optical signal was obtained at 70 mM of KCl (Fig. 7b). Based on our observation, the colorimetric sensing ability of dsDNA PCR products is slightly lower in comparison with ssDNA DNAzyme product. So, heat denaturation of PCR product before performing the assay can improve the assay sensitivity.

Colorimetric biosensor for detection of Enterotoxin B positive *S. aureus*

In our study, colorimetric detection depends on the PCR amplification of *seb* gene. The amplification of different cell concentrations of enterotoxin B positive *S. aureus* DNA was monitored by agarose gel electrophoresis and DNAzymes sensing method. Sharp band in agarose gel at different cell concentrations, clearly indicates successful amplification *seb* gene of *S. aureus* (Supporting fig.1). Moreover, Colorimetric biosensing with the PCR-amplified products validates the same. These qualitative results indicate that PCR products

300 with G-quadruplex/hemin complex could able induce the catalytic reaction to produce color
301 solution in contrast with negative controls. The limit of detection (LOD) was as low as 1 ×
302 10^2 CFU/mL Enterotoxin B positive *S. aureus* in 1× PBS (Fig. 8a). Furthermore, the
303 quantitative study also shown a linear relationship between the Enterotoxin B positive *S.*
304 *aureus* cell concentration and optical absorbance was found to be exponential (Fig. 8b). This
305 quantitative result of the newly described colorimetric biosensor is comparable to available
306 kit for detection of Enterotoxin B positive *S. aureus* in terms of rapidity, sensitivity and
307 specificity.

308 **Colorimetric biosensor Specificity and reliability**

309 Various Staphylococcal and non Staphylococcal organisms were tested in colorimetric assay,
310 colorimetric signals were observed only in SEB producing *S. aureus* strains. Other
311 staphylococcal and non-staphylococcal bacterial cultures showed weak absorbance similar to
312 blank (Fig. 8c). The PCR products of the *seb* gene harboring *S. aureus* group could induce
313 the colorimetric signal and produce blue color while other related organisms are not. The
314 result obtained in comparison with available kit validated the same (Table.1). Altogether the
315 colorimetric protocol with functional DNAzymes primer set could be useful for rapid,
316 specific and easy and detection of Enterotoxin B positive *S. aureus* without any interference.
317 Since milk and milk based products are the main sources of enterotoxigenic *S. aureus* strains,
318 spiking studies were carried out in milk. The assay was efficient to detect Enterotoxin B
319 positive *S. aureus* as low as 10^2 CFU/mL (Fig.8d). Insignificant lower absorbance values
320 were observed in spiked milk tested when compared to pure culture in1× PBS. This is due to
321 the imprecise interaction of antiprotein A antibody with the milk components could also
322 inhibit capturing efficiency. The above said insignificant variation could be rectified by
323 keeping a blank from extracts having similar composition to the sample to be analyzed.

324 **Analysis of complex food and clinical samples and comparison with available kits**

Colorimetric biosensor was assessed for its efficiency and practicability to detect the targets from direct food samples. Naturally contaminated samples were tested for the presence of *seb* harbouring *S.aureus*. As shown in Table. 3, the assay is able to detect *seb* positive *S. aureus* without any food matrix inhibition from natural samples within 2 to 3 hours (15 min for sample enrichment, 2 hours for PCR amplification and 15 min for assay).The conventional isolation and biochemical assays confirmed *S. aureus* (data not shown) from laboratory repository also tested by colorimetric biosensor. The assay able to detect *seb* hourbering *S. aureus* (Supporting Table. 1). The comparisons of result with available kit validate the same (Table.4). These experimental results demonstrated that DNAzymes based colorimetric sensing device could be a rapid, easy and low-cost platform for routine laboratory food sample analysis, which could detect seb gene harboring *S. aureus* by the naked eye or the UV-vis spectrometer. Moreover, the designed strategy can be adopted as a useful tool for other pathogens as an alternative detection system.

Acknowledgments:

The first author is thankful to Department of Science and Technology (DST, New Delhi, India), for financial support through INSPIRE program. The funders had no role in study design, data collection and analysis, preparation of the manuscript, or decision to publish. All authors are thankful to the Director, DFRL for providing necessary facilities to conduct the study.

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423 **Table 1: List of bacterial strains used in the study**

Sl. No.	Bacterial strains	Source	PCR	ELISA	Colorimetric biosensor
1.	*Staphylococcus aureus*	FRI 722	+	+	+
2.	*Staphylococcus aureus*	NCIM 2122	+	+	+
3.	*Staphylococcus aureus*	NCIM 2120	+	+	+
4.	*Staphylococcus aureus*	TSST JAP	+	+	+
5.	*Staphylococcus aureus*	NCIM 2127	+	+	+
6.	*Bacillus cereus*	ATCC 10876	-	-	-
7.	*Burkholderia pseudomallei*	NCTC 10274	-	-	-
8.	*Clostridium perfringens*	Isolate	-	-	-
9.	*E. coli*	ATCC 10536	-	-	-
10.	*Enterococcus* spp.	Isolate	-	-	-
11.	*Klebsiella pneumoniae*	ATCC 13883	-	-	-
12.	*Lactobacillus* spp.	Isolate	-	-	-
13.	*Listeria monocytogenes*	ATCC 19114	-	-	-
14.	*Proteus vulgaris*	ATCC 33420	-	-	-
15.	*Pseudomonas* spp.	Isolate	-	-	-
16.	*Salmonella paratyphi A*	ATCC 9150	-	-	-
17.	*Shigella flexneri*	ATCC 9199	-	-	-
18.	*Streptococcus* spp.	Isolate	-	-	-
19.	*Yersinia enterocolitica*	ATCC 23715	-	-	-
20.	*Vibrio parahaemolyticus*	ATCC 17802	-	-	-

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426 **Table 2: Primers used for PCR amplification**

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Primer	**Oligonucleotide Sequence (5′-3′)**	**Amplicon size (bp)**
Normal *seb*	F – GAGAGTCAACCAGATCCTAA R – CTTTTTCTTTGTCGTAAGAT	719
DNAzymes modified *Seb* For	F-**TTTACCCAACCCGCCCTACCC**AAAAAA**TTTACCCAA CCCGCCCTACCC**AAAAAA GAGAGTCAACCAGATCCTAA	771
Seb Rev	R – CTTTTTCTTTGTCGTAAGAT	

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429 The bold letters indicate bases coding for modification of sequences and red colour are linker

430 sequences.

431 **Table 3: Analysis of natural samples for enterotoxin B through Colorimetric biosensor**

Sample type	**No. of samples *seb* gene positive for colorimetric biosensor**
Milk (n=24)	6
Cheese (n=22)	6
Ice cream (n=10)	3
Chicken (n=10)	4
Pastries (n=10)	3
Total (n=76)	**22**

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435 **Table 4: Comparison of PCR and ELISA results**

Culture	No. of isolates positive (%)		No. of isolates positive for colorimetric biosensor (%)
	PCR	ELISA	
S. aureus Isolates (n=91)	58 (63.7%)	57 (62.6%)	59 (64.8%)

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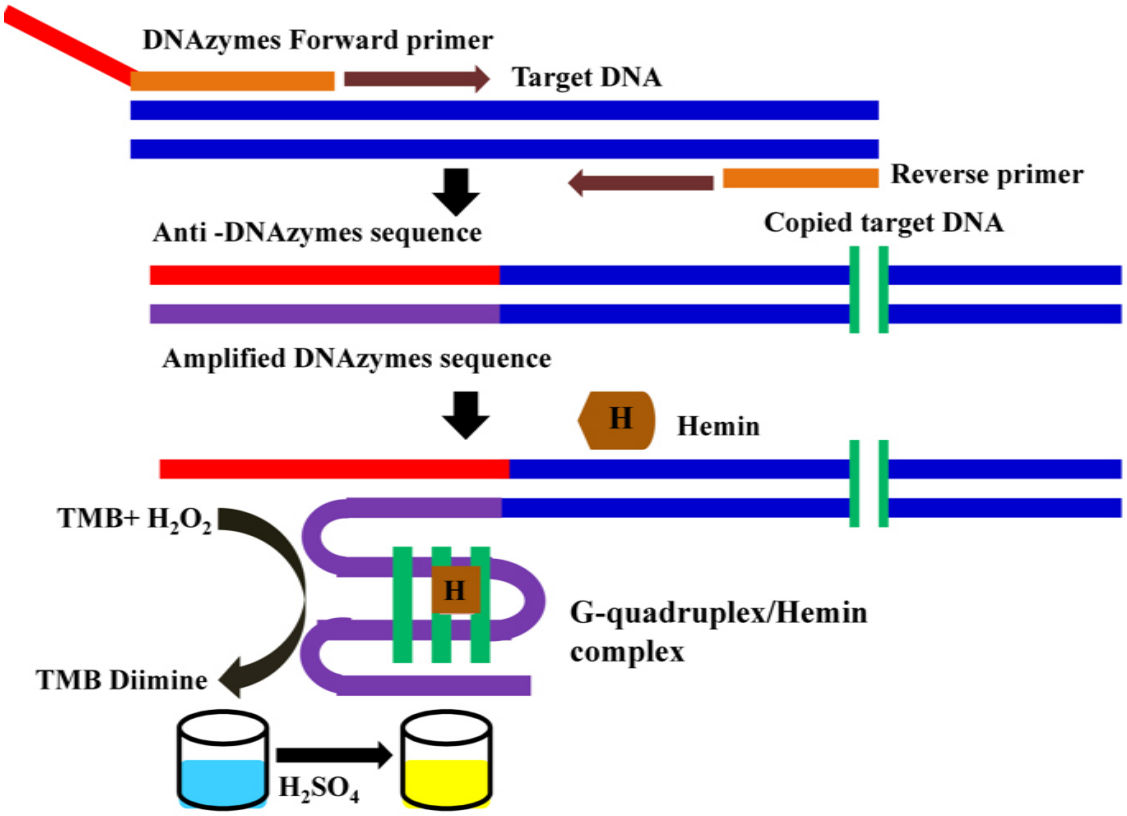
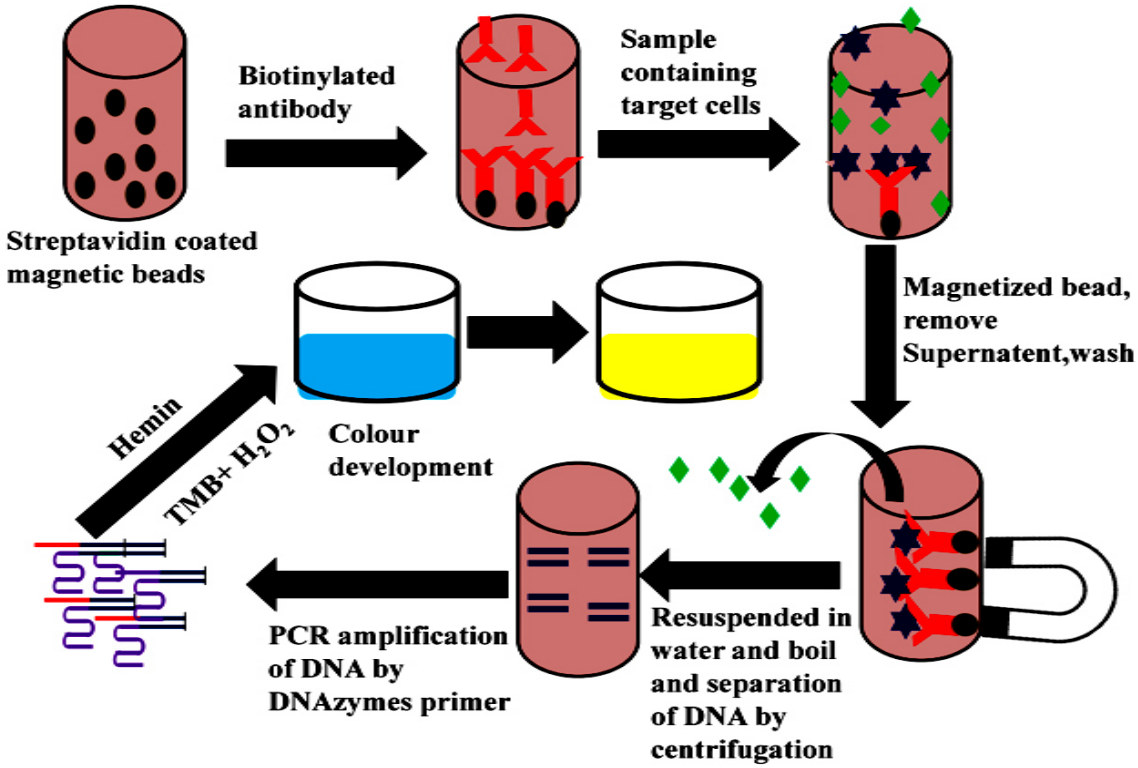
Figure:

Figure 1:



The schematic illustration PCR integrated DNAzyme Biosensing strategy for detection of Enterotoxin B hourbering *S. aureus*

469 **Figure 2:**



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472 **The schematic illustration of magnetic bead assisted DNAzyme colorimetric biosensing**

473 **for detection Enterotoxin B positive *S. aureus***

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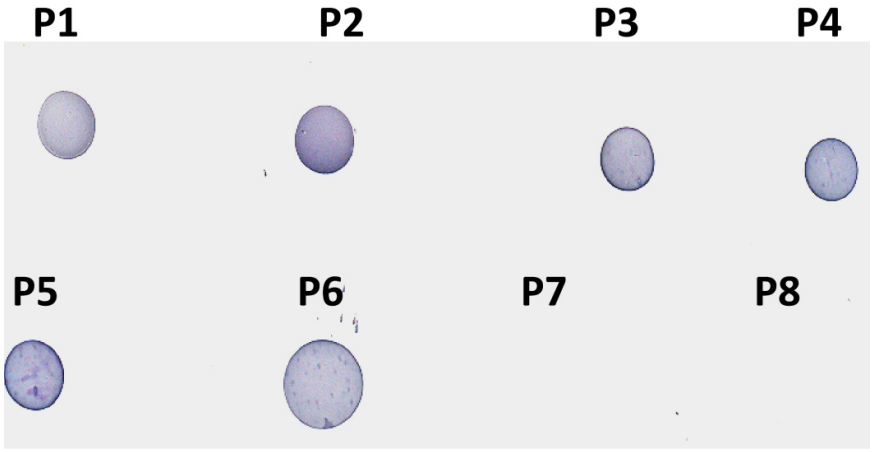
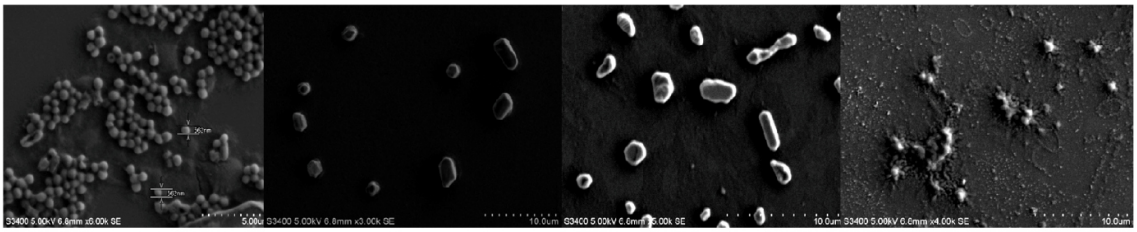
Figure 3:



Dot ELISA for confirmation of biotinylation of antiprotein A antibody. P1-P6: biotinylated antiprotein A antibody various dilution, P7-P8: Normal antiprotein A antibody.

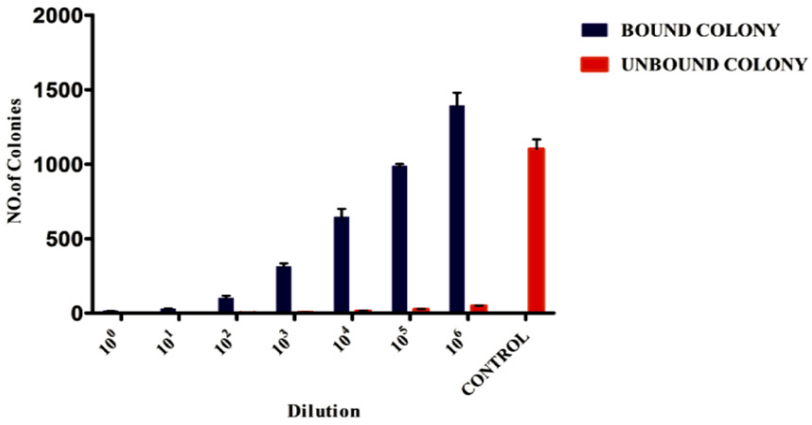
Figure 4:



***S. aureus* cell** | **Streptavidin magnetic bead** | **Antibody conjugated with streptavidin magnetic bead** | **Immunocapture of *S. aureus* cells**

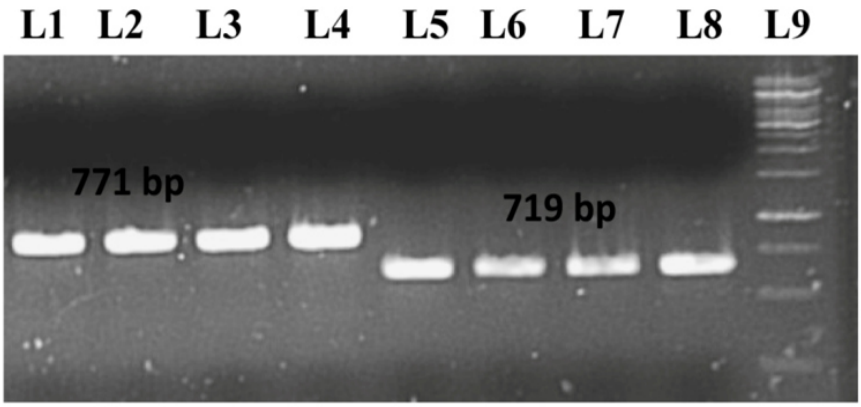
Scanning Electron Microscopy imaging for binding of Antibody-Dynabeads complex with *S. aureus* cells.

Figure 5:



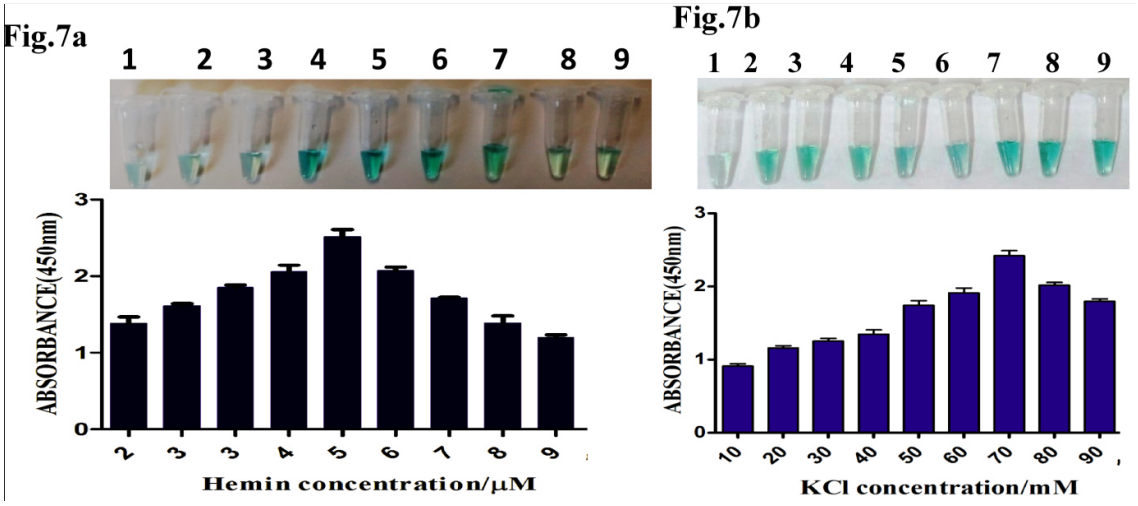
Standardization of bound and unbound cells by for immunomagnetic separation.

Figure 6:



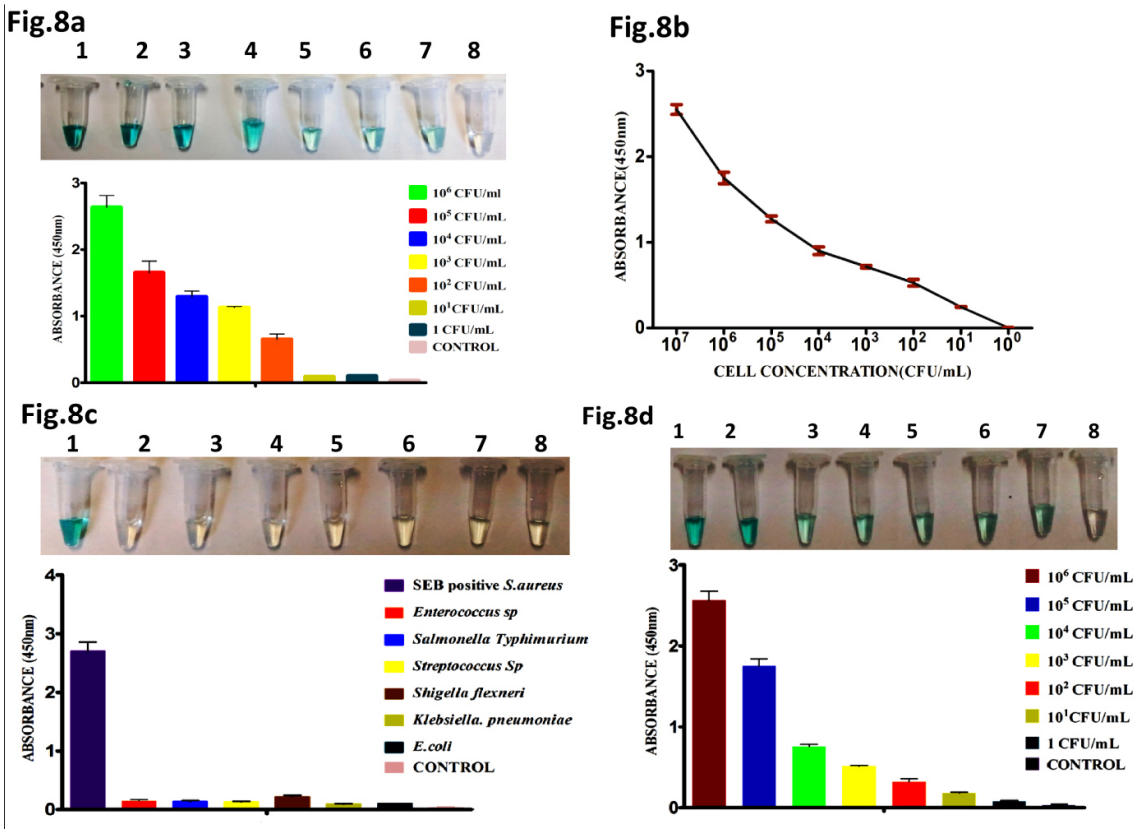
Agarose gel analysis (L1-L4) amplified PCR product with DNAzymes modified primer, (L4-L8) amplified PCR products with normal primer.

Figure: 7



Standardization of (7a) hemin and (7b) KCL concentrations for colorimetric biosensing.

Figure: 8



Evaluation of the specificity and sensitivity of DNAzymes based biosensing (8a) Sensitivity in 1×PBS, (8b) specificity, (8c) sensitivity in milk and (8d) quantitative analysis of Enterotoxin B positive *S. aureus* cells.

520 **ASSOCIATED CONTENT**

521 **Supporting Information**

522 The supporting information is available free of charge on the ACS publication website.

523 **TOC Graphic:**

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