Journal of Medical Microbiology and Infectious Diseases

JoMMID
ISSN: 2345-5349
eISSN: 2345-5330

# Detection of *Staphylococcus aureus* Enterotoxin A (SEA) Using Dot-ELISA in Milk Samples

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ARTICLE INFO

**Original Article**

**Keywords:** dot-ELISA, *Staphylococcus aureus* Enterotoxin A (SEA), Raw milk

Received: 20 Jan. 2020
Received in revised form: 01 Dec.2020
Accepted: 06 Dec. 2020
**DOI:** 10.29252/JoMMID.8.4.132

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ABSTRACT

**Introduction:** *Staphylococcus aureus* enterotoxin A (SEA) is one of the most common causes of staphylococcal food poisoning. Due to the simplicity and no requirement for laboratory apparatuses, dot-ELISA is a choice method for detecting Staphylococcal enterotoxins. The present study aimed to develop a dot-ELISA for the detection of SEA. **Methods:** Nitrocellulose membranes were coated with the SEA antibody and blocked by the addition of 3% bovine serum albumin (BSA) blocking buffer. After 1 h incubation and washing the membranes, milk samples and the positive control (SEA, 50 ng/ml) were added to the membranes and incubated for 1 h. The membranes were then washed and incubated for 45 min with HRP-conjugated SEA, followed by the addition of TMB. **Results:** Our dot-ELISA could detect amounts of ≥ 50 ng/ml of SEA in the milk samples. Of the 30 raw milk samples randomly purchased from dairy product stores in District 3, Tehran, 5 (16%) contained SEA ≥ 50 ng/ml by the dot-ELISA. **Conclusion**: The dot-ELISA showed to be a reliable method for the preliminary screening of milk samples for SEA contamination. This method is cost-effective, fast, and does not require an ELISA-reader device.

## INTRODUCTION

Foodborne diseases have become a serious concern due to changes in food consumption patterns, globalization of food markets, and climate change. Today, people demand less processed natural food products containing no preservatives and the least amount of salt, sugar, and fat, but with extended shelf-life. The demand for ready-made food is increasing, and food industries are trying to develop novel techniques for supplying high-quality ready-made foods accordingly.

Ready-made foods provide a suitable environment for toxin-producing bacteria like *Staphylococcus aureus*. This bacteria can grow and produce pathogenic toxins in various foods such as dairy, meat and meat products, eggs, and egg-containing products, e.g., cakes and ice-creams [1-4].

According to the WHO report, approximately two million diarrhea-associated deaths occur due to contaminated foods. The disease caused by *S. aureus* enterotoxins (SAEs) is the second most common cause of foodborne disease [5]. Twenty-three different Staphylococcal enterotoxins (SEs) have been identified so far, including SEA to SElV except for SEF. The SEF, renamed as TSST-1 (toxic shock syndrome toxin 1), belongs to the superantigen family. Since SEB and TSST-1 are known as potent biowarfare toxins, setting up a diagnosis tool is of immense help in isolating them from food or environment samples during the emergence of a biological war or suspected *staphylococcal* food poisoning outbreaks [6]. Classical SEs (SEA-SEE) are responsible for 95% of food poisoning outbreaks associated with staphylococci. Among these, the SEA is the most common cause of Staphylococcal food poisoning [7]. Sensitive, specific, and quantitative immunological assays, including enzyme-linked immunosorbent assay (ELISA), mass spectrometric methods [8], and biosensors [9], are available for diagnosis of SEs. However, these methods are time-consuming and mostly require expensive equipment and sophisticated laboratory setups. Diagnosis of SEs in resource-limited settings under field conditions demands



[ DOI: 10.29252/JoMMID.8.4.132 ]

inexpensive and straightforward methods with no requirement for specific equipment or highly trained human resources. The present study aimed to design and evaluate a rapid dot-ELISA screening test to detect SEA in raw milk samples.

## MATERIALS AND METHODS

**Chemical reagents.** *S. aureus* enterotoxin A (Catalog No. S9399) was purchased from a commercial company (Sigma-Aldrich, Germany). Rabbit anti-SEA-polyclonal capture antibody (7.3 mg/ml, Catalog No. S7656, Sigma, USA) was used as the first antibody. The SEA capture antibody was conjugated with HRP (Horseradish Peroxidase) in the laboratory to obtain a conjugated SEA capture antibody. A 0.22 µm pore size nitrocellulose membrane (Bio-Rad, USA) was used as the substrate for material binding. The 3,3',5,5'-Tetramethylbenzidine (TMB), and horseradish peroxidase (HRP) were purchased from a commercial company (Padtan Elm Co., Tehran, Iran). Other chemical reagents were of analytical grade and obtained from commercial sources.

**Dot-ELISA design.** Sandwich dot-ELISA was performed on nitrocellulose membrane cut into 1×1 $cm^2$ pieces. Each piece was placed inside a well of a 24-well ELISA plate. Then, 3 µl of rabbit anti-SEA-polyclonal antibody diluted 1:1000 in phosphate-buffered saline (PBS) was added to the wells onto the papers. After 30 min, when the capture antibody was well-dried on the membrane, 500 µl of 3% bovine serum albumin (BSA) in PBS was added to the wells, and the plate was kept at room temperature (RT) for 1 h in order to block the parts of the membrane where no antibody was present. The plate was washed with phosphate-buffered saline (PBS) once, and 300 µl of commercial enterotoxin A (containing 12.5 to 100 ng/ml) in PBS (as the positive control) or milk samples were added to the wells. The plate was kept at RT for 1 h, washed with wash buffer (10 mM sodium phosphate, 0.15 NaCl, 0.05% Tween-20, pH 7.5), and then 300 µl HRP-conjugated rabbit anti-SEA-polyclonal antibody, diluted 1:500 or 1:2,000 in PBS was added to the wells. The plate was incubated for 45 min and subsequently was washed twice with the wash buffer. The nitrocellulose membrane pieces were then removed from the wells and allowed to dry. Amounts of 20 µl of TMB were poured on the nitrocellulose membrane pieces, placed in the dark at room temperature (20–25 °C), and finally, the reaction was stopped 30 min later by adding 20 µl of 1.0 M sulfuric acid.

## RESULTS

**Development of SEA dot-ELISA.** Two dilutions, 1:500 and 1:2000, of HRP-conjugated rabbit SEA antibody were tested in SEA dot-ELISA to detect 50 ng/ml of SEA (Fig. 1). Color development was observed only at 1:500 dilution of the conjugated antibody. Control wells containing ddH2O did not show any specific signals.

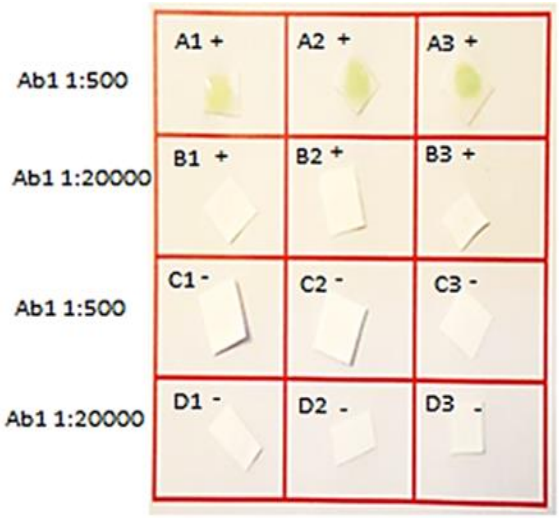


**Fig 1**. Optimization of SEA dot-ELISA with two dilutions, 1:500 and 1:2000, of HRP-conjugated rabbit SEA antibody (Ab1). Rows A and B were incubated with commercial enterotoxin A (50 ng/ml, positive controls), rows C and D were incubated with ddH2O (negative controls). Color development was observed only in the positive control nitrocellulose membrane containing the HRP-conjugated rabbit SEA antibody at 1:500 dilution (row A). The negative controls remained colorless too.

**SEA dot-ELISA Sensitivity**. The SEA sandwich dot-ELISA sensitivity was evaluated using the optimized dilutions of the capture and conjugated antibodies to detect SEA at concentrations 12.5 to 100 ng/ml (Fig. 2). The results showed that color development was visible at SEA concentrations of 50 and 100 ng/ml. Hence, the sensitivity of our dot-ELISA was≥50 ng/ml of SEA.



[ DOI: 10.29252/JoMMID.8.4.132 ]

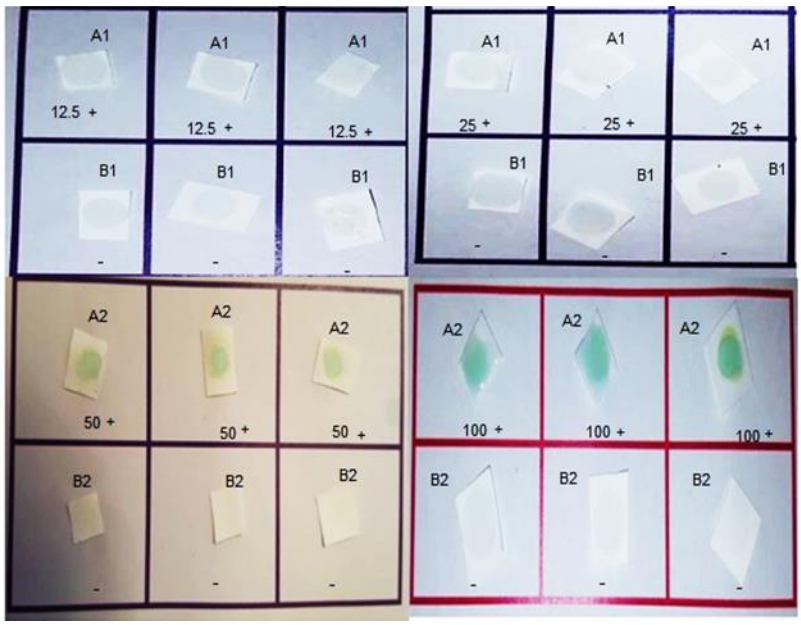


**Fig 2.** Sandwich dot-ELISA results with different enterotoxin concentrations (12.5, 25, 50, and 100 ng/ml) in manually contaminated milk (row A1 row A2), rows B1 and B2 are negative samples. The development of a specific signal was observed only at 50 and 100 ng/ml of SEA.

**Evaluation of SEA dot-ELISA**. Thirty cow milk samples were purchased from traditional dairy stores in District 3 of Tehran, Iran, and transferred immediately to the Mabna Tashkhis Laboratory, Tehran, Iran, and stored at -20 ºC until use. The Dot-ELISA detected SEA in five (16%) samples (samples No. 15, 9, 4, 2, and 25), indicating the SEA amounts of ≥ 50 ng/ml (Fig. 3).

## DISCUSSION

The present study aimed to design a dot-ELISA assay using small nitrocellulose membranes to detect SAE A in milk samples via visualizing a color change. Various methods are available for detecting SAE A in raw milk. The culture method is one of the routine methods for the diagnosis of toxin-producing bacteria species. Johler et al. (2015) reported food poisoning caused by white cheese consumption made from raw milk in a school pension in Switzerland, which occurred seven hours after eating the cheese; culturing identified three SEA and SED producing *S. aureus* species [10].

Molecular methods can also detect the encoding genes for *S. aureus* toxins. Gadyari et al. (2011) and Sharif et al. (2012) used PCR to detect SEA-producing bacteria in the patients' clinical samples; however, the test could not detect staphylococcus SEA genes.[11, 12]. Ahmadi et al. (2010) investigated the *S. aureus* SEA producing gene in milk samples using PCR [13]. Abbassi et al. (2015), in a descriptive-cross-sectional study, investigated the spread and frequency of the Methicillin-resistant *S. aureus* producing A and B genes in 110 samples from the patients hospitalized in Shahr-e Kord Hospitals. After culturing and approving the isolates biochemical tests, PCR showed *S. aureus* enterotoxin A gene in 26 samples (23.6%) [14]. In another study, Barati et al. (2006) used multiplex PCR and detected *S. aureus* SEA genes in the clinical samples [15].

Sandwich ELISA is another diagnosis method for determining the amount of toxin in milk samples. In recent years, several ELISA methods have become available for detecting enterotoxins. Kuang et al. (2016) could detect that amounts of SEA equals 0.0282 ng/ml in milk samples; the most sensitive ELISA assay reported so far [16]. Nouri et al. (2018), using direct ELISA, detected enterotoxin A in 23% of the milk samples with the sensitivity of approximately 15.6 ng of toxin and the detection time of 15 min [17]. Moreover, several commercial ELISA kits for the detection and quantification of classical SEs are also available. Due to the food poisoning outbreak caused by milk powder consumption in Japan in 2006, a VIDAS® Staph enterotoxin II (SET2) kit (bioMerieux, USA) that used a polyclonal antibody was deployed. The kit could detect seven enterotoxin types simultaneously with the detection level of 20-100 ng/ml for enterotoxin A [18]. In 2003 following a food poisoning breakout in Osaka-Japan caused by low-fat milk and milk powder, the enterotoxin A level showed to be 20-100 ng/mg [19]. In a similar study, the mini VIDAS kit could detect the lowest amount of SEA (0.1 ng/ml) in milk samples [19]. In Isfahan, Iran, a ready-made RIDASCREEN ELISA kit was used to determine the classic enterotoxins in foods.



[ DOI: 10.29252/JoMMID.8.4.132 ]

Unlike the dot-ELISA method, this assay merely detected the enterotoxins and did not measure the amounts [20]. Molecular and ELISA assays, contrary to the dot-ELISA, require special and costly devices and skilled operators to detect the bacterial enterotoxins.

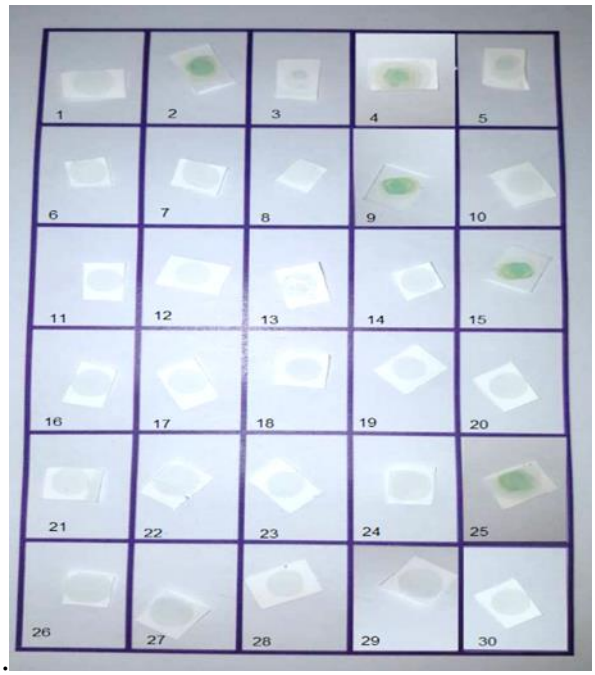


**Fig 3.** SEA dot-ELISA results on 30 raw milk samples purchased from dairy shops in District 3, Tehran city. Samples 2, 4, 9, 15, and 25 contained an enterotoxin amount of ≥ 50 ng/ml, as exhibited by color change.

As a simple test, dot-ELISA can be utilized for preliminary screening of SEA in foods in resource-limited settings under field conditions. Unlike ELISA, dot-ELISA does not require an ELISA reader machine, and results can be observed and interpreted by the naked eye quickly.

Our dot-ELSA could detect SEA at a concentration of ≥ 50 ng/ml. In our dot-ELISA, the capture anti-enterotoxin A antibody was unconjugated; after the incubation of the membranes with the samples, the first antibody was conjugated with HRP and was added as the second detector antibody. However, in the dot-blot method utilized by Singh et al. (2017), there was an extra step. After sample incubation, an unconjugated antibody was added to the membranes as the detector antibody, and after washing by TBS buffer containing Tween 20, the goat anti-rabbit detector IgG peroxidase was added [21]. Hence, in our dot-ELISA method, SEA can be detected much faster by excluding one step and a lower cost. Moreover, our sandwich dot-ELISA can be employed for enterotoxin A diagnosis in food matrices other than milk, and it can be designed for other *S. aureus* subtypes and the toxins of other microbes as well.

## CONFLICT OF INTEREST

The authors declare that there is no issue related to this article to be conceived as a conflict of interest.

## ACKNOWLEDGEMENTS

The authors acknowledge with grateful appreciation the kind assistance provided by the Immunology Laboratory, Faculty of Veterinary Medicine, Tehran University.

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**Cite this article:**

Gholafrouz H, Ahari E, Anvar A, Shahbazzadeh D. Detection of *Staphylococcus aureus* enterotoxin A (SEA) using dot-ELISA in milk samples. J Med Microbiol Infect Dis, 2020; 8 (4): 132-136. DOI: 10.29252/JoMMID.8.4.132