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Lateral flow assay for rapid detection of *Staphylococcus aureus* enterotoxin A in milk

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ABSTRACT

Staphylococcus aureus is closely associated with food poisoning incidences, more, because of intoxication due to contamination of food with its enterotoxins. *Staphylococcus* enterotoxin A (SEA) is highly resistant to heat and other food processing conditions and has been implicated in food borne diseases from dairy products. Here we describe a rapid, gold nanoparticle based lateral flow immunoassay (LFIA) for detecting SEA in milk. Assay is in sandwich format and uses two specific monoclonal antibodies against SEA, one of which is labeled with gold nanoparticles and other is immobilized at control line. We also demonstrate the effect of using a combination of additives in capture antibody in order to enhance assay sensitivity. The developed LFIA is specific and has a lower detection limit of 0.5 μg/ml. LFIA is tested both on spiked buffer and milk samples and display good reproducibility. The assembled strips stable for a month at 4 °C. The method may be suitable for testing SEA in milk samples as it is quick, easy to perform and reproducible.

1. Introduction

Safety of food can be ensured by the timely detection of toxins and pathogens present in food products. According to the World Health Organization (WHO), food borne illness is defined as the state of intoxication caused by the ingestion of food or water contaminated with single or multiple types of enterotoxin [1–2]. *Staphylococcus aureus* produces various enterotoxins (SEs) which are stable stable in adverse environmental conditions such as heating, freezing, drying and wide range of pH (pH 4–10) and, hence, are responsible for majority of food borne illness worldwide [3–4]. These enterotoxins are also resistant to gut proteases, subsequently, maintains their activity even in the digestive tract immediately after its consumption [1]. Food processing methods are unable to destroy SEs completely at concentration range of 0.5–10 μg/100 ml or 100 g which is sufficient enough to cause food poisoning [5].

More than 20 different SEs have been identified till now, out of which SEA, SEB, SEC, SED and SEE are the most antigenic enterotoxins accountable for the major staphylococcal food poisoning syndrome [6]. Staphylococcal enterotoxin A (SEA) is predominant SEs which is comparatively more stable to heat and reported to be a cause of staphylococcus-related food borne diseases in many countries [1,3,6]. SEA is responsible for more than 50% of food poisoning in UK; 77.8% in USA; 69.7% in France [7] and 90% in Korea [8]. Its ingestion induces symptoms like emesis, nausea, vomiting, abdominal cramps, diarrhea and approximately 100 ng of toxin is sufficient to cause intoxication symptoms [9].

It has been reported that approximately 15% of staphylococcal food borne diseases in developed countries are caused by dairy products [10]. Milk serves as an excellent substrate for the growth of *S. aureus* which can contaminate raw milk or at any time during processing. Bacteria itself gets killed during processing but its enterotoxins can withstand the adverse processing conditions and survive [1,4]. This drives the need for detection methods to assess milk quality for SE toxin contamination. Till date, several immunological and molecular assays have been introduced for the detection of SEs in different food matrices [11]. For example, enzyme-linked immunosorbent assay (ELISA) [12–14], radioimmunoassay (RIA) [15], polymerase chain reaction (PCR) [16–18], LC-MS/MS based approach [19] and Western blotting, have been employed for detecting SEs. In addition several kits are also commercially available and have been utilized for SEs detection [20–23]. These techniques are all sensitive and specific; however, the high equipment cost, requirement of highly trained personnel, relatively longer time required to perform these techniques, and the complex analytical operations always limit their use in the field. To overcome these drawbacks and expand the limits of detection, an ideal method is required for rapid, sensitive, accessible, and low-cost detection of low levels of the toxin in routine clinical practice and in foods.

In the recent years, there has been a growing interest in developing low cost lateral flow immunoassays (LFIAs) for the rapid detection of toxins and pathogens [24]. LFIAs draw more attention owing to their simple user-friendly architecture, rapidness, cost effectiveness, easy to interpret results, on-site detection. LFIAs for detecting SEB enterotoxin

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in various matrixes like serum, plasma or food samples [23,25–27], have been reported but rapid assays for SEA toxin detection are scarce [10] particularly in milk samples. Jin et al. 2013 have reported the use of chicken antibodies (IgY) for developing immunopillar chips, ELISA and LFIA for SEA detection in dairy products. The sensitivity of LFIA for milk sample was 0.2 ng/ml. However, the assay has two limitations a) the Gold-Ab conjugate was pre-incubated with SEA sample for 15 min and subsequently used for assembly of LFIA strip. This defeats the very purpose of LFIA i.e. on site detection. Only trained personnel can assemble the strip after preincubation, b) Secondly, the assay employs antichicken anibodies which are less common in use than mice/rabbit antibodies. Hence, it is necessary to design sensitive LFIAs that can be run in single step without need of pre-incubation.

This paper reports a gold nanoparticle based LFIA for detecting SEA toxin in milk samples. Achieving good sensitivity and reproducibility is a daunting task while developing LFIA. In this context we have used various additives in capture antibody and studied their influence on LFIA sensitivity. It is demonstrated that using a combination of additives may significantly improve LFIA sensitivity. Reported LFIA is reproducible and stable at 4 °C.

2. Materials and methods

2.1. Reagents and materials

All reagents and chemicals used in this study are of analytical grade. Chloroauric acid ($HAuCl_4$) and staphylococcal enterotoxin A (SEA) was purchased from Sigma-Aldrich, USA. Bovine serum albumin (BSA, fraction V, 96–98%, pH 6–7) and Sodium dodecyl sulfate (SDS) were purchased from Sisco research laboratories (SRL) India. Tween 20 and Methanol were purchased from Merck, India. Trisodium citrate, glycerol and sucrose were purchased from Fisher Scientific, India. Nitrocellulose (NC) membrane laminates (CNPF-SN12-L2-H50), conjugate pad (PT-R5), sample pad (GFB-R7L) and absorbent pads (AP045) were purchased from Advanced Microdevices Pvt. Ltd., Ambala Cantt India. SEA monoclonal antibodies (C86107M and C86104M) were purchased from Meridian Life Science, Inc., USA. Goat Anti-mouse IgG (GX8301) was purchased from Puregene, Genetix, India. Milk samples from different sources were collected in clean, dry containers and stored at 4 °C.

2.2. Gold nanoparticles synthesis

Gold nanoparticles (AuNP) were synthesized using trisodium citrate reduction described elsewhere [28]. Briefly, 0.8 mM of aurochloric acid ($HAuCl_4$) in 45 ml distilled water was brought to boil under reflux with continuous stirring at 1000 rpm. To this solution, 5 ml of 1% trisodium citrate trihydrate was added at once under stirring. The reduction of aurochloric acid is visible with a transition in color of the solution from light yellow to purple, black and finally wine red. The solution was further boiled under continuous stirring for 20 min and then allowed to cool at room temperature (RT i.e. 28 °C). The obtained gold nanoparticles were further stored at 4 °C till use.

2.3. Characterization of AUNPs

The synthesized gold nanoparticles were characterized for their plasmon peak (λ_{max}) by scanning between 400 and 600 nm on a CARRY60 UV–Vis spectrometer from Agilent, India. The size and polydispersity of the nanoparticles was determined using Dynamic light scattering based particle size analyzer from Metrohm, India. Size and shape of these nanoparticles was further confirmed using transmission electron microscopy (TEM) on a HITACHI (H-7500) electron microscope at 120 kV by drying a drop of sample on Cu grid.

2.4. Coupling of AUNPs with SEA antibody

pH of gold nanoparticle solution and minimum amount of antibody required to stabilize the surface of nanoparticles are crucial for obtaining a stable antibody gold conjugate. These two parameters were optimized using NaCl flocculation assay described elsewhere [29]. Following optimization, conjugation was carried out at pH 9.0 using 15 μg/ml SEA antibody. Briefly, monoclonal primary antibody against SEA toxin was added to 1 ml of gold nanoparticle (pH 9.0) in an eppendorf tube, with constant stirring. The AUNPs-Ab solution was incubated at RT with mild shaking at 200 rpm for 1 h to allow the electrostatic interactions between AUNP surface and antibody. To this, 10 μl of Tween 20 (0.01%) and 100 μl of BSA (10%) were added to for stabilization and blocking of the nonspecific sites of AUNPs-Ab conjugate and stirred for additional 30 min. The AUNPs-Ab solution was then centrifuged at 12000 rpm for 20 min at 4 °C. The supernatant was discarded and pellet was washed with PBS buffer (10 mM pH 7.4), resuspended in 50 μl of resuspension buffer (10 mM PBS, 0.01% Tween 20, 0.1% BSA) and stored at 4 °C till use. The success of conjugation reaction was tested by challenging the AuNP-antibody conjugate to high NaCl concentration, UV–Vis analysis and analyzing the conjugate binding to anti-species antibody coated at control line of LFIA strip.

2.5. Preparation of LFIAs strips

2.5.1. Coating of antibody on NC membrane

The NC membrane adhered on a plastic backing card also known as membrane laminate was cut into sections (6.5 cm L × 0.5 cm W). Second monoclonal antibody against SEA toxin (capture antibody) is immobilized on NC membrane using drop coat method approx. 0.5 cm far from the reservoir matrix. A control line was formed using goat anti-mice antibody at the far end of NC membrane near adsorbent pad. The capture and control line antibodies are dispensed at test and control zones of NC membrane respectively at a rate of 2 μl/cm of membrane such that a single strip has 1.8 μg of antibody in the coated zone. Antibody coated strips were incubated in a vacuum desiccator at 37 °C for 2 h with appropriate humidity to allow maximum antibody adsorption on the membrane and minimize loss of capture reagents through sweeping (flowing along with buffer) during the assay run.

2.5.2. AUNP-Ab coating on reservoir matrix

Reservoir matrix is made of glass fibers and loosely holds the Ab-AUNP conjugate. Matrix allows facile release of conjugate after wetting with the sample. Approximately, 10 μl of AUNPs-Ab conjugate is dispensed on 0.5 cm × 0.5 cm reservoir matrix per strip. The matrix was allowed to air dry for 20 min. To increase the stability of conjugate and facilitate the conjugate release from matrix, 5% sucrose is added in the Ab-AUNP conjugate.

2.5.3. Assembly of LFIA strip

The LFIA strip was then assembled by overlapping various components of LFIA viz., sample pad, conjugate pad, nitrocellulose membrane and adsorbent pad as shown in Fig. 1. Reservoir matrix is first overlapped on NC membrane slightly followed by placing the sample pad such that it overlaps the reservoir matrix. Reservoir matrix and sample pad are placed towards that end of NC membrane where test zone is coated. On the other side of membrane, adsorbent pad is placed by removing the peel from adhesive backing of membrane laminate such that it overlaps the NC membrane. Overlapping of 0.2 mm is sufficient to allow a continuous capillary run of fluid across the length of assembled LFIA strip.

2.6. LFIA procedure

The principle of the sandwich LFIA is illustrated in Fig. 1. LFIA was carried out on an assembled LFIA strip by adding approximately 70 μl