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High Sensitive Detection of Staphylococcal Enterotoxin B by Silver Enhanced Lateral Flow Assay

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ABSTRACT

Background: *Staphylococcus aureus* is one of the most important microorganisms that can colonize the human body and cause various types of human diseases by secreting virulence factors. The lateral flow assay (LFA) is one of the well-known commercial immunoassay methods that provides a high sensitive and rapid approach to monitoring infectious agents in blood, serum and urine. LFA has been considered as an ideal immunochromatographic test.

Methods: Anti Staphylococcal Enterotoxin B (SEB) antibodies were conjugated to 20 nm colloidal gold nanoparticles and were applied in assembled lateral flow layer. Suitable reagents were prepared and used in silver enhancement method. We designed a single immunochromatographic test strip to detect SEB.

Results: In this study, the smallest amount of SEB identified using sandwich LFA method was 10 ng/mL. We also established a "silver enhanced method". Silver could improve the sensitivity detection of the test 100-fold greater than the previously mentioned sandwich LFA.

Conclusion: Regarding the high sensitivity of the new method for detection and measurement of SEB (0.1 ng/mL), this strip test offers great promise for a rapid technique instead of the other diagnostic SEB tests in laboratories for the first time.

Keywords: Immunochromatographic test strip, Lateral flow assay, Rapid detection, Silver Enhancement, Staphylococcal enterotoxin B, Test strip

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Abbreviations:
SEB: Staphylococcal Enterotoxin B
LFA: Lateral Flow Assay
mAb: monoclonal antibody

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Introduction

Staphylococcus aureus is one of the most important microorganisms that can colonize the human body and cause various types of human diseases by secreting virulence factors which are known as staphylococcal super antigens (SAgs) **(1)**. SAgs are recognized by their ability to make a cross-link between some subsets of T Cell Receptor (TCR) and Major Histocompability molecule class II (MHCII) in a different position from MHC cleft **(2, 3)**. It should be considered that different types of staphylococcal strains could produce different SAgs significantly including TSSToxin-1 (TSST-1), Staphylococcal Enterotoxin B (SEB) and Staphylococcal Enterotoxin C (SEC) **(2)**. Among them, SEB is responsible for staphylococcal food poisoning in human and acts through stimulating the cytokine release and inflammation induction **(4, 5)**.

Staphylococcal food poisoning is usually diagnosed by the clinical symptoms and also the probable presence of SEB in blood, urine, respiratory secretions, etc. The toxin can be detected by Enzyme-Linked Immunosorbent Assay (ELISA), chemiluminescence (CL), Reverse Passive Latex Agglutination test (RPLA-oxide), etc. Despite providing acceptable sensitivity, most of these common tests are time-consuming hence they need to improve in terms of increasing the sensitivity and decreasing the time consumption **(6-9)**.

The Lateral Flow Assay (LFA) is one of the well-known commercial immunoassay methods providing a high sensitive and rapid approach to monitoring the infectious agents in blood, serum, urine, etc. Briefly, conventional LFA comprises capture antibodies embedded in test and control lines which are assembled on a nitrocellulose membrane and a detection antibody conjugated to colloidal gold which is loaded on a conjugation pad. In addition, gold nanoparticles have been used in various immunoassay techniques over the recent years because of their high stability, easy size controlling and high compatibility with biological molecules including antibodies. In this study, gold-conjugated antibodies are designed to develop a one-step immunochromatographic strip test in order to detect analyses.

Moreover, regarding the need for having high sensitive methods to precisely identify infectious compounds such as SEB, it would be indispensable to improve the sensitivity of LFA.

Recent studies have indicated that the sensitivity of conventional LFA can be enhanced approximately from 10 to 100-fold by adding a silver enhancement step to the test procedure hence, our laboratory has developed immunochromatographic rapid test strips similar to LFA to identify SEB. In the present study, the efficacy and sensitivity of conventional LFA is compared with the improved silver enhanced method of sandwich LFA. Thus, this new high sensitive method can be substituted for the previous SEB detection and measurement methods.

Materials and Methods

Reagents and Components

The Staphylococcal Enterotoxin A (SEA), SEB and SEC were purchased from SIGMA Company (S-4881). Colloidal gold was purchased from Arista Biological INC (CGUCG-0000). Two types of Anti-Entrotoxin B Staph (S222 and S624 Hytest) were used as conjugated and test antibodies, respectively. Goat-anti Mouse antibody was obtained from Hytest Company. The Silver Enhancer solution A and B (S5020 and S54145) used in the study were manufactured by Sigma Company.

Absorbent pad, Nitrocellulose membrane (AE98F) and Sample pad were purchased from Schleicher and Schuell Company. Backing card was purchase from G and L Company.

Colloidal Gold Conjugation of Detection Monoclonal Antibody

Based on the standard protocols **(10)**, Anti-Entrotoxin B Staph mAb (S222) was diluted to Borax buffer to make 5, 10, 15, 20, 25, 30 and 40 µg/mL concentrations and the pH was adjusted to 9 with 0.2 M of k_2CO_3, then 1.0 mL of colloidal gold (pH=9) was added to 100 µL of each antibody dilution and incubated for 10 min at room temperature. Then, 100 µL of 10% NaCl was added to each vial and the color alterations were exposed. The lowest concentration with no color change was introduced as optimal concentration for stabilizing the gold solution (Figure 1). In order to stabilize conjugated antibodies, the solution was centrifuged at 8000×g at 4°C for 30 min. Conjugations were stored in Phosphate Buffer (PB) containing 50 mM phosphate (pH=9), 0.1 mm Tween® 20 and 1% Bovine Serum Albumin (BSA) at 4°C.

Production of Immunochromatographic Rapid Test Strip

Conjugated mAbs were diluted in 20 mM phosphate buffer and applied to the conjugation pad, in 2 µL/cm by biojet-quanti dispenser (BioDot, Irvine, CA.). Test line contained 0.5 µL/cm Anti-Enterotoxin B Staph and control line enclosed 0.5 µg/cm Goat-anti Mouse antibody. Various layers including sample pad, Conjugate pad, Nitrocellulose membrane, and absorbent pad were assembled on the backing card with a 2 mm overlap between them. Finally, as shown in Figure 2, all sheets were cut in 5 mm width using an automated cutter.

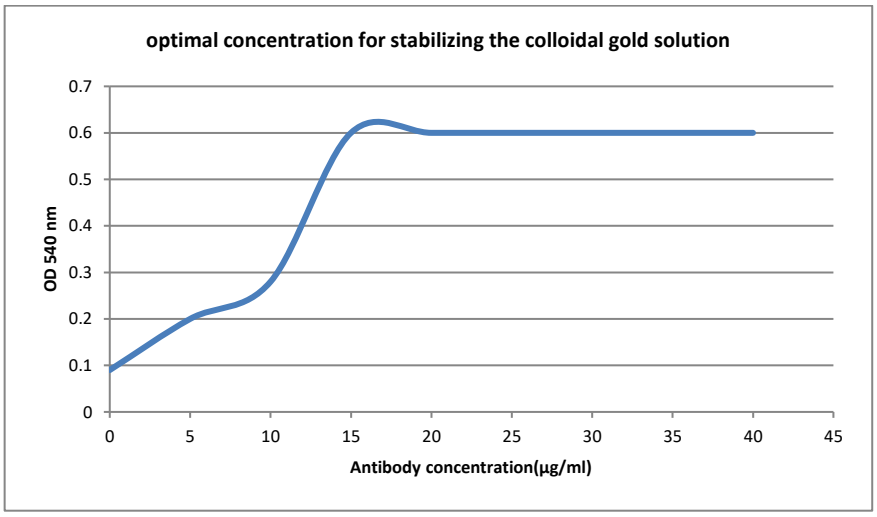

Figure 1. Optimum antibody concentration for colloidal gold conjugation. The minimum antibody concentration that cause no color change after NaCl addition is suitable .as seen in the table 2µg antibody for 1 ml colloidal gold conjugation is suitable.

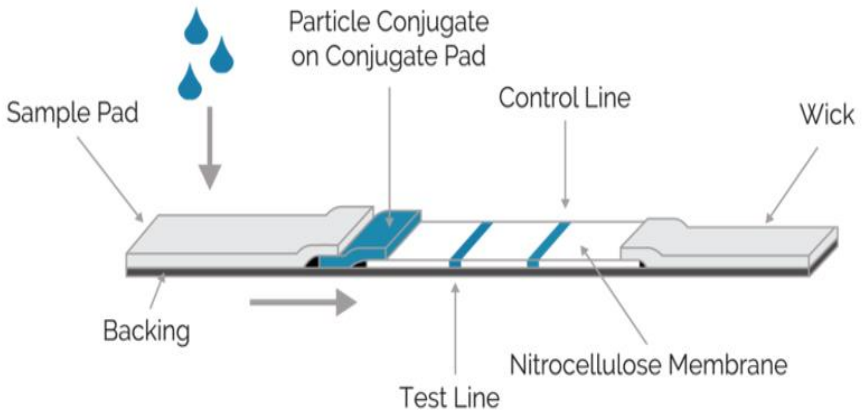

Figure 2. Schematic view of lateral flow assay (LFA)

Conventional and Silver Enhancement Methods of LFA

In the conventional LFA as sandwich-type, SEB was detected by the quantity of the colloidal gold-conjugated mAbs accumulated on the capture antibody-assembled site resulting in appearance of two red lines on a single strip with a sensitivity about 10 ng/mL in purified toxin. After running the sample, sandwich-type strips were immersed in silver enhancer reagents A and B for 5 min. Finally, common washing processes in PB buffer and Tween® 20 solution was applied.

Results

Detection of SEB by Sandwich Type of LFA

In order to detect SEB in the present study, we applied the capture mAb (S624) in the test line (0.5 µL/cm) and conjugated mAbs to the conjugate pad (2 µg/cm) while the control line only contained Goat-anti Mouse antibody (0.5 µL/cm). As shown in Figure 3, the complete white strip including the sample pad and the absorbent pad was able to detect SEB in the range of 120-10 ng/mL displaying two red lines in test and control margins. No reactivity was visualized with the buffer alone as a negative control.

Detection of SEB by Silver Enhanced-Method

Using silver, plays an important role in signal amplification. When the silver solution was added, the detection sensitivity in sandwich-type LFA increased approximately about 100-fold compared to the conventional LFA. As shown in Figure 4, the sensitivity of SEB protein detection was 10 ng/mL before the enhancement and increased to 0.1 ng/mL after silver enhancement.

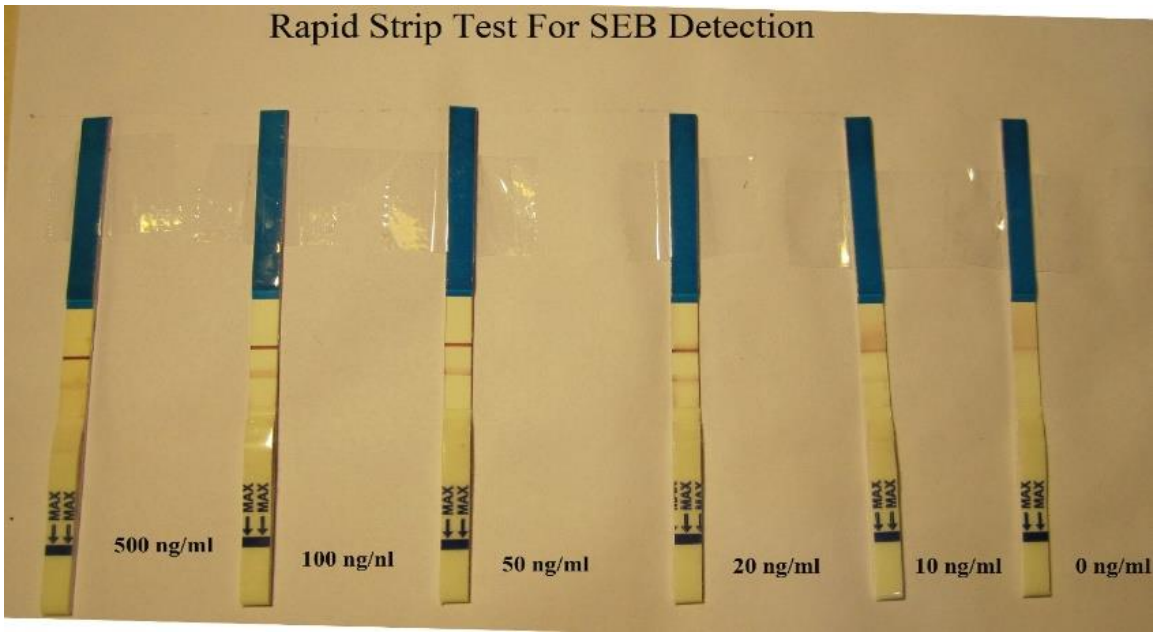

Figure 3. Application of sandwich LFA for detection of SEB. A series of dilutions (500–10 ng/mL) of SEB was prepared in PBS. The detection limit of SEB toxin was 10 ng/mL. False positive was not detected in the absence of SEB.

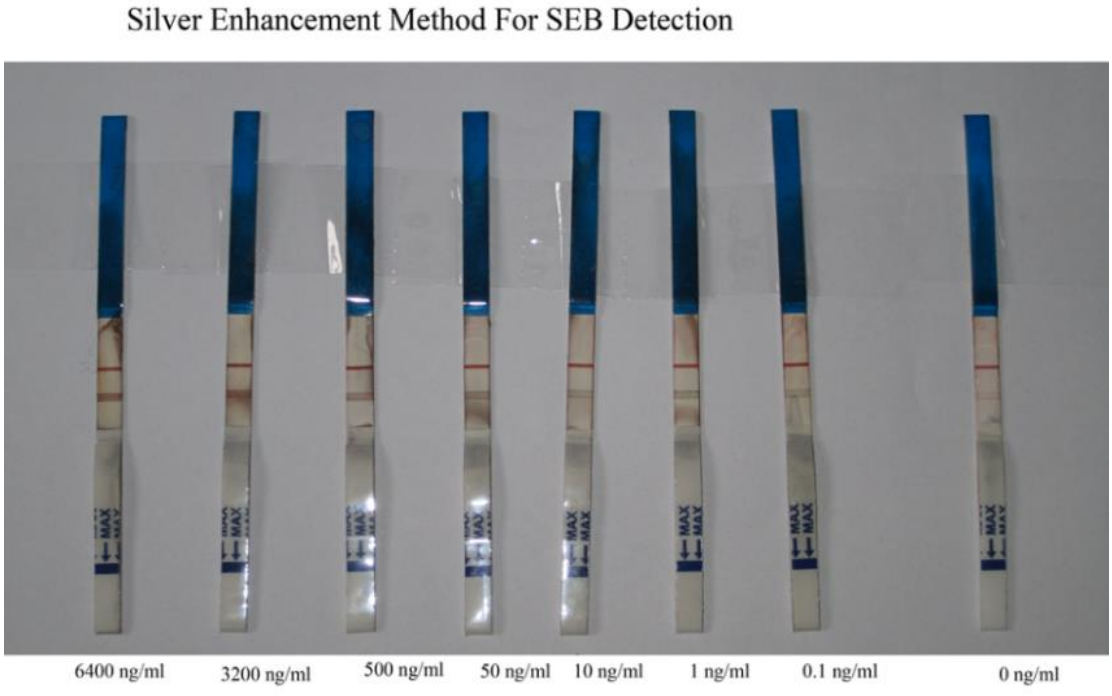

Figure 4. the silver enhanced method. A series of dilutions (6400–0.1 ng/mL) of SEB was prepared in PBS. After running the sandwich LFA, the silver solution was added. the detection sensitivity in sandwich assay increased approximately about100 fold compared with the conventional LFA. The detection limit of SEB toxin was 0.1 ng/mL. False positive was not detected in the absence of SEB.

Study of Immunochromatographic Strip Maintenance

In order to obtain the best consuming time after keeping the strips in the fridge, the quality of stored immunochromatographic strips was investigated. Our investigation suggested that the efficacy of the test strips for effective detection of SEB will remain unchanged for 24 months if they are kept in a dried place at 2-8°C. Moreover, to confirm the specificity of the test strips, other enterotoxins of *S. aureus* including SEA and SEC were tested by new enhanced LFA. Negative results were

observed for both enterotoxins using present models of LFA (unpublished data).

Discussion

SEB has been known as an important member of staphylococci enterotoxin group that causes clinical infection and food poisoning in human. To diagnose such a hazardous agent at the trace level, a number of high sensitive methods of detection have been widely developed **(11, 12)**. Over the past decade, lateral flow assay has been considered as one of the best-known commercial alternatives to the immunoassay methods for detection of various analyses.

This method is based on the specific binding of antibody to antigen with an improved sensitivity and specificity as well as considerable reduction in the test time consumption (less than 5 minutes), sample preparation (~200 µL) and personnel training. These advantages make LFA ideal for initial screening tests but it should be mentioned that these tests are qualitative and other quantitative methods including ELISA and CLA should be used for detection of antigens. **(10, 13-15)**.

The current LFA is based on micro fluidic mobility of molecules through the test strip and their accumulation on the test and control lines. Therefore, the time of passing the reagents and consequently, formation of red lines in the strips would be one of the important parameters likely affecting the sensitivity of our described method **(16)**. In order to improve the performance of the test and shorten the time of immunoreactions, we stored the conjugated antibodies in PB containing 50 mM phosphate (pH=9), 0.1mm Tween® 20 and 1% BSA. This buffer contains BSA which offers specific features to the strips including prevention of nonspecific bindings of antibodies to the antigens as well as providing a higher viscosity to reagents leading to a longer SEB and gold-conjugated antibody interaction time **(17)**.

Additionally, the proper selection of nitrocellulose paper sheet support layers with the smaller pore sizes led to decreased solution flow rate across the paper and subsequently increased interaction time of antigen-antibody. Thus, we chose a paper with the smallest pore size

(AE98F) and the slowest flow rate among the other different porous papers (AE98F, 99F and

100F). Moreover, using two monoclonal antibodies increased the sensitivity and specificity of antigen-antibody interaction in comparison with other immunoassay methods **(18-21)**.

Finally, in the present study, silver enhancement was performed for signal amplification to maximize the sensitivity of immunochromatographic strips.

During silver enchantment, the colloidal gold which had been attached to the antibody, played the role of a nucleation site for the metallic silver deposition. The silvers layer increased the size of the gold and imparted a block color to the stained line in order to improve the optical extinction. As we found out, using the silver enhancement method, the sensitivity of the strip tests could be improved from 10 ng/mL to 0.1 ng/mL.

It should be noted that the minimal detection limits of the strip tests ranged between 1 to 5 ng/mL for antigen detection. In our strip tests, minimal detected limit was about 0.01 ng/mL. This sensitivity is considered advantageous for strip tests and a development in quick detection methods in this diagnostic range. It can be introduced as a suitable alternative for high sensitive Immunoassay tests. Based on technical information, minimal detection limits for most of the the ELISA kits ranges from 0.1 to 0.5 ng/mL. The mentioned limit can be lowered to 0.01 ng/mL only using some technical improvements such as Avidin-Biotin method. Therefore, sensitivity improvement by silver enhancement method (SEM) got these kits categorized in high sensitive immunoassays.

In the previous study, we produced colloidal gold based lateral flow immunoassay for SEB detection and compared its sensitivity and specificity with PCR method in *S. aureus* contaminated solutions (22). The kits used in the mentioned study were much less sensitive and had a minimum detection limit of approximately 10 ng/mL. Application of SEM significantly increased the sensitivity of the lateral flow immunoassay and lessened the toxin minimal detection concentration to 0.1 ng/mL. This method was used to increase the sensitivity of prostate specific antigen (PSA) detection kits by Rodríguez and the results suggested that it is very effective in increasing visual signals and subsequently effective in lowering detection limits (23). One of the important items in the test procedure is reaction time. By adding the resonator solution, the color of the lines become gradually bolder and bolder. Therefore, at a certain time, the reaction have to be stopped.

By increasing the reaction time, we had a dark background and test lines could not be distinguished from each other. Therefore, in cases where this method was used to detect toxins, the test procedure was stopped at a specific time and then results were documented. Since the test time was variable in different runs, it was very difficult to determine a reliable protocol for good precision and accuracy. To eliminate this problem, it is necessary to evaluate a negative test at the same time in all experiments in order to decide about the best reaction time.

In this study, we tried to simplify the test procedure by adding a silver enhancer pad on the test strip. The results

indicated that the process was made much easier and the kit was more applicable. Therefore, a negative control line (containing neutral proteins such as IgG) was used in addition to the test line and the control line in this type of kits, so that the results of the test line and the control line could be compared simultaneously with the negative control for better decision about the test ending point.

Conclusion

Using rapid strip tests is broadly applicable for detecting various antigens and increasingly dispersed throughout the world. The increased sensitivity of these types of tests will allow them to be used in more important diagnostic cases. Using the silver enhancement method can increase rapid strip test sensitivity by resonating visual gold signals. In the present study, we used the colloidal gold-conjugated antibodies and the silver enhancement method for detecting SEB toxin increasing the test sensitivity relatively. Our results were indicative of usefulness of this method for rapid antigen detection present in nanogram scale.

Acknowledgment

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Conflict of Interest

Authors declared no conflict of interests.

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