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Enzyme-linked immuno-strip biosensor to detect *Escherichia coli* O157:H7

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ABSTRACT

A strip-based biosensor using the enzyme-linked immunosorbent assay technique was fabricated to detect *Escherichia coli* O157:H7. Two types of antibody specified to *E. coli* O157:H7 were used to form sandwich-binding complexes. To fabricate an immuno-strip, capture antibody (monoclonal antibody) was immobilized onto signal generation pad and polyclonal antibody conjugated with horseradish peroxidase (HRP) was utilized as detection antibody. Four different functional membranes have been used to fabricate immuno-chromatographic assay strip. A sample application pad was a glass fiber membrane pre-treated with polyvinyl alcohol. A conjugate release pad was fabricated using a glass membrane. A signal generation pad was made on nitrocellulose membrane. Finally, a cellulose membrane was used as an absorption pad. Under optimal conditions of analysis, a color signal in proportion to the *E. coli* O157:H7 concentration was measured using a detector. The measurement range was 1.8×10^3–1.8×10^8 CFU/mL.

1. Introduction

Food poisoning caused by food-borne pathogen occurs in a worldwide and increase public health problem. Economic Research Service (ERS) estimated that the cost associated with five major pathogens was at least US $6.9 billion annually in the United States [1]. About 90% of food-borne illnesses are reported to be caused by pathogenic microorganisms [2]. *Escherichia coli* O157:H7 is one of the most harmful food-borne pathogens and caused an estimated 73,000 infection and 61 deaths in the United States each year [3]. The infection of *E. coli* O157:H7 has been linked to hemolytic uremic syndrome and hemorrhagic colitis in humans, and these illnesses cause diarrhea, kidney failure, seizure, and death [4–6]. The infection of *E. coli* O157:H7 is associated with a contaminated beef, sprouts, iceberg lettuce, salami, milk, juices, and water [7].

The frequent outbreaks of bacterial infection associated with foods and drinks increased the necessity of a rapid analytical instrument. In a conventional microbial measuring method, the contaminated sample is to be cultivated in an enriched media for several hours and a portion of culture broth is plated on agar media to grow and count colony-forming units which may take at least 1 day. Biosensors are analytical devices with sensing biomolecules (microbes, enzymes, antibodies, nucleotide, and artificial receptors). The reaction of sensing biomolecule with a target analyte converts into detectable signal. Major advantages of biosensing devices are its specificity, sensitivity, simplicity, short detection time, and small-integrated instrument [8].

Recently, biosensor technology to measure biological components have been developed using both indirect- and direct-detection method. While indirect methods promise good sensitivity, direct methods (bioluminescence, impedance measurement, acoustic wave detection, quartz crystal microbalance, and surface Plasmon resonance) allow label-free detection which result in drastically decrease the complexity [9,10]. Immunosensors are composed of antibodies as the sensing biomolecule with a various kind of transducer. Biosensors based on optical immunoassays have been reported using colorimetric, fluorescence, and chemiluminescence detection methods [11,12].

Immunochemical methods such as enzyme-linked immunosorbent assay (ELISA), western blotting, and radioimmunoassay have been studied to detect pathogens. Among these methods competitive ELISA and sandwich ELISA is the most widely used to quantitatively measure pathogenic bacteria. Despite of high sensitivity of ELISA technique, it takes relatively long analysis time due to multistage procedure [13,14]. An immuno-chromatography technique using lateral flow along the membrane strip provides the tool for a simple, rapid, and on-site analysis. This methodology can be used either as a qualitative, disposable kit or as a biosensor measuring the analytes [15].

In this study, a colorimetric immuno-sensor based on the sandwich ELISA technology and lateral flow immuno-chromatography

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was fabricated to detect *E. coli* O157:H7, produced colorimetric signal was quantitatively measured by using a digital camera and home-made image analysis software. And the performance of a proposed immuno-sensor was analyzed in water samples. The results showed that the lower limit of *E. coli* O157:H7 detection is 10^3 level of colony-forming units (CFU) per mL, which analysis was completed within 30 min.

2. Experiment

2.1. Materials

E. coli O157:H7 (ATCC 43895) was purchased from the American Type Culture Collection (USA). Monoclonal antibody (mAb; mouse IgG3, ab20976) against *E. coli* O157:H7, horseradish peroxidase (HRP)-conjugated polyclonal antibody (HRP-pAb; rabbit IgG, ab20425) against *E. coli* O157:H7, and polyclonal antibody (pAb; duck IgY, ab31193) against rabbit IgG were obtained from Abcam Plc. (Cambridge, UK). Nitrocellulose (NC) membrane for signal generation pad (10 μm pore size, CNPF-SN12), cellulose membrane for absorption pad (AP045), glass fiber membrane for detection antibody release pad (PT-R5), and glass fiber membrane for sample application pad (GFB-R4) were purchased from Advanced Microdevices Pvt. Ltd. (Ambala Cantt., India). Casein buffer solution, ascorbic acid, trehalose solution, bovine serum albumin (BSA) and Tween 20 were purchased from Sigma-Aldrich Co. (MO, USA). Enzyme substrates, 3,3′,5,5′-tetramethylbenzidene (TMB) and SuperSignal West Femto substrate were obtained from BioFx Laboratories Inc. (MD, USA) and Pierce Biotechnology (IL, USA), respectively.

2.2. Sensor strip preparation

Four different functional membranes were used to prepare immuno-chromatographic assay sensor strip [16]. A sample application pad was a glass fiber membrane (4 mm × 15 mm). A conjugate release pad was manufactured by dropping 8 μL of a conjugate solution onto a glass membrane (4 mm × 5 mm). The conjugate solution was prepared by adding the HRP-pAb (2 μL, rabbit IgG) in casein solution (12 μL) containing Tween 20 (0.5 wt%) and trehalose (20 w/v%). A signal generation pad was fabricated by dispensing a mAb solution (0.1 μL, mouse IgG3) diluted in PBS buffer onto NC membrane (4 mm × 25 mm) using a non-contact type microarrayer (Versa 100, Aurora Biomed Inc., Vancouver, Canada) to obtain detection signal. On the same membrane, pAb (duck IgY) against rabgit IgG (0.1 mg/mL) in PBS buffer was also dispensed onto a different site to obtain control signal. A cellulose membrane (4 mm × 15 mm) was used as an absorption pad. The membrane strip was arranged to be sample application pad, conjugate release pad, signal generation pad, and absorption from bottom. After drying at 37 °C for 1 h, a membrane strip was kept in a desiccator at a room temperature.

2.3. Analytical procedure

Sample solutions were prepared by a serial dilution of *E. coli* O157:H7 culture medium with PBS buffer solution containing 3% BSA. A prepared sensor strip was placed into each sample solution (100 μL) for 15 min to absorb the solution into the strip in the vertical direction. After immuno-reaction, two pads, glass fiber membrane (13 mm × 13 mm, GFB-R4) to apply TMB substrate solution and cellulose membrane (13 mm × 13 mm) to absorb substrate flow in horizontal direction, were arranged on each lateral side of the signal generation pad of the sensor strip, respectively. The TMB substrate solution was added onto the substrate application pad and allowed the enzyme reaction for 5 min. The color signal generated by enzyme reaction was captured using a digital camera (DSC-W5, Sony, Japan). The control and detection signal were analyzed by separation of the three primary color and averaging intensity using home-made image-analyzing software.

3. Results and discussion

3.1. Optimization of immuno-strip fabrication

Immuno-chromatography assay method with enzyme (HRP) reaction was used to simplify the proposed detection system and to enhance the detection sensitivity. Immuno-reaction which is antigen–antibody affinity was occurred by in vertical sample flow, and HRP reaction was then accomplished by horizontal enzyme substrate flow as shown in Fig. 1. Colorimetric signal was produced by HRP reaction, which can visualize and enhance the signal of immuno-reaction, at two specific positions on the signal generation pad. A detection signal at a lower position can be used to analyze qualitatively and quantitatively target microorganisms (*E. coli* O157:H7), and a control signal at an upper position represents the available working of immuno-strip. Samples were prepared with buffering substances to control pH and inert proteins (3% BSA) to prevent non-specific bindings between immuno-reagents and solid surface. Also, a nonionic detergent component (Tween 20), which makes a hydrophilic environment, was examined to reduce the background staining. As a result of experiments, 0.5 wt% Tween 20 was more effective compared to other concentration (data are not shown).

3.2. Performance of enzyme-linked immuno-strip analysis

The enzyme-linked immuno-strip analysis procedure in cross-flow [16] as was achieved to detect *E. coli* O157:H7. After sample injection on sample application pad, the primary immuno-reaction between HRP-pAb and *E. coli* O157:H7 was occurred on a conjugate release pad, and the secondary immuno-reaction between immobilized mAb and *E. coli* O157:H7/HRP-pAb complex

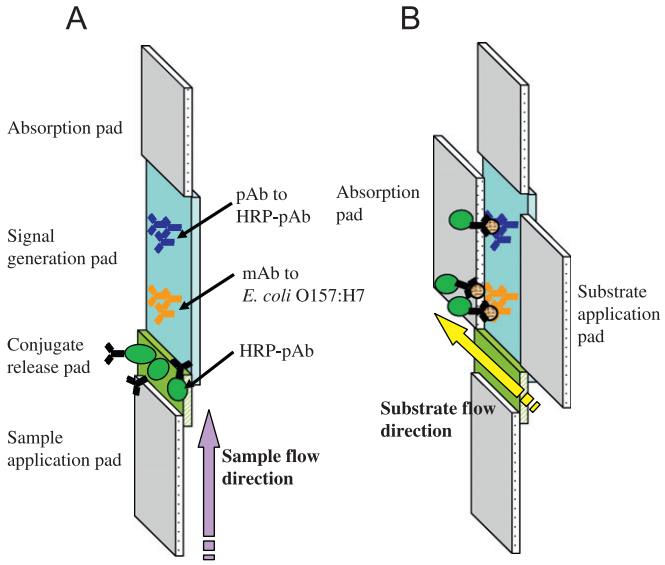

Fig. 1. Schematic diagram of cross-flow immuno-sensor strip. The analytical procedure composed of (A) immuno-reaction in vertical direction and (B) enzymatic reaction in horizontal direction.

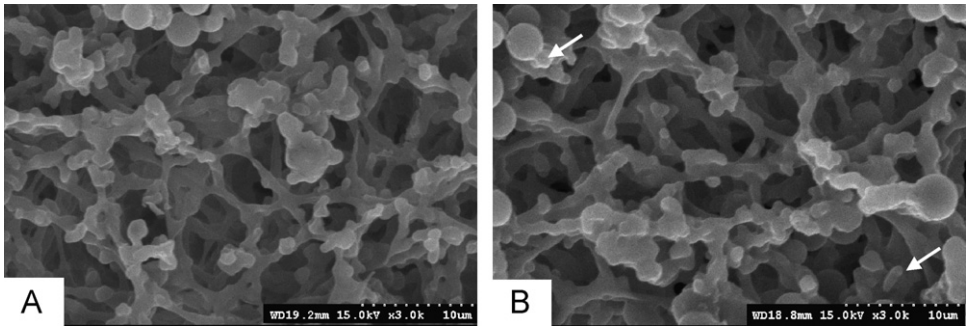

Fig. 2. SEM images of (A) bare nitrocellulose membrane and (B) *E. coli* O157:H7/HRP-pAb complex bound to mAb immobilized on nitrocellulose membrane.

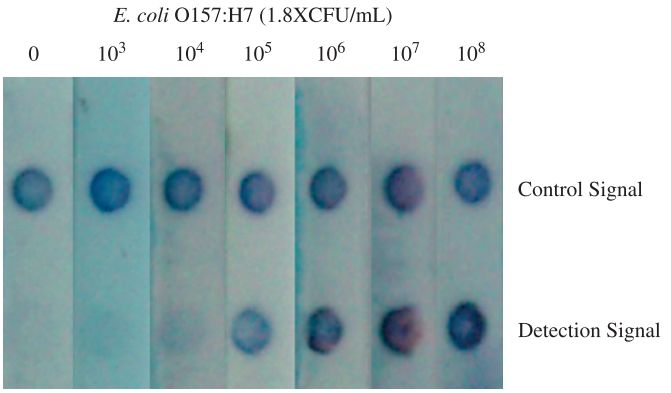

Fig. 3. Images of colorimetric signal change according to various concentration of *E. coli* O157:H7.

was then accomplished on a specific site (a dot of detection signal) of signal generation pad. To verify the immuno-reaction between mAb and *E. coli* O157:H7/HRP-pAb complex, the surface morphology of the membrane was observed using a scanning electron microscope (SEM; S-3500N, Hitachi Ltd., Japan) at 15 kV. Fig. 2 shows the morphology of bare NC membrane (Fig. 2A) and that of *E. coli* O157:H7/HRP-pAb complex bound to mAb immobilized on NC membrane (Fig. 2B). The bare NC membrane showed a smooth surface consisted of fiber and randomly distributed particles. In contrast, *E. coli* O157:H7/HRP-pAb complex bound to mAb immobilized on NC membrane showed some rod type microorganisms on particles and fibrous structure.

Free HRP-pAbs, which had not combined with *E. coli* O157:H7, were bound to immobilized pAb (against HRP-pAbs) on a specific site for control signal genenration. After the sandwich immunoreaction, enzyme substrate was injected on substrate application pad to occur HRP reaction in cross-direction. As a result of enzyme reaction, the dark blue colored signal caused by product of HRP reaction appeared at a control dot. In this study, various concentrations of *E. coli* O157:H7 in a range of 1.8×10^3–1.8×10^8 CFU/mL were examined to obtain detectable signal. After a few minutes, the color images were captured using a digital camera, and these results were represented in Fig. 3. The colorimetric signals varied proportionally to pathogen concentration were obtained.

3.3. Quantitative analysis of E. coli O157:H7

A significant color change according to pathogen concentration was observed as shown in Fig. 3. In order to assess the quantitative performance of proposed analytical tool, the colorimetric signals in captured images were analyzed using home-made image analysis software. Pixel size was adjusted to focus on a colored dot, and then were separated the three primary colors (RGB) and the intensity of each primary color of selected pixels was averaged and evaluated as a relative numerical value. Digitized blue color intensities at various *E. coli* O157:H7 concentrations were plotted to obtain a calibration curve (Fig. 4A). Furthermore, the digitized values of the three primary colors were averaged, and these whole color intensities were

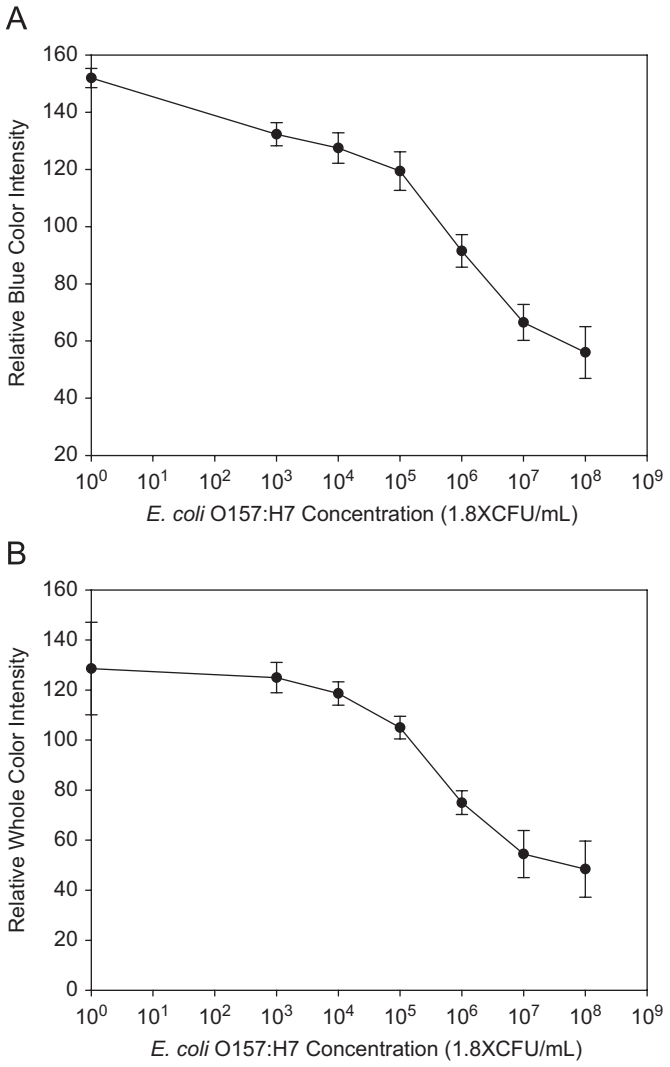

Fig. 4. Calibration curve of (A) blue color intensity and (B) whole color intensity by HRP reaction against the *E. coli* O157:H7 concentration.

plotted against *E. coli* O157:H7 concentrations as shown in Fig. 4B. Two calibration curves showed a similar trend that the signal intensity decreased slightly below 1.8×10^5 CFU/mL and decreased rapidly above 1.8×10^5 CFU/mL. However, the declining slope of calibration curve using blue color was more rapid than that of calibration curve using whole color. Such phenomena might be caused by the colorimetric property of signal, which is dark blue color. It might be that a sharp change of blue color intensity was offset by a smooth change of other two primary color change. In any case, the colorimetric signal of proposed sensing system was useful to analyze quantitatively *E. coli* O157:H7 concentrations. And image-analyzing method using blue primary color was more effective to monitor *E. coli* O157:H7 concentrations in contaminated samples.

4. Conclusion

The enzyme-linked immuno-strip biosensor was developed using immuno-chromatography and sandwich ELISA methods. A proposed analytical procedure was successfully applied to detect *E. coli* O157:H7 with high sensitivity. The enhanced colorimetric signal produced by HRP reaction increased the performance of biosensor. Therefore, the proposed simple analytical system can detect quantitatively 1.8×10^3 CFU/mL of *E. coli* O157:H7, which analysis was completed within 30 min. Furthermore, our system has some advantages; an easy-to-use, a relatively cheap manufacturing cost, and a qualitatively confirmation of signal by bare eyes. And the concept of this study can be easily applied to develop simple biosensor to detect clinical, environmental, and hazardous analytes.

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