공학석사 학위논문

Development of Immunoassays using Novel Antibodies for Rapid Detection of Antibiotic-Resistant and Susceptible Pathogens

항생제 내성 및 감수성 병원균의 신속 검출을 위한 신규 항체 기반 면역측정법 개발

2023 년 2 월

서울대학교 대학원

협동과정 바이오엔지니어링 전공

김 주 경

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Master's Thesis

Submitted to the Faculty of Seoul National University

By

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Interdisciplinary Program of Bioengineering

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이 논문을 공학석사 학위논문으로 제출함

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김주경의 공학석사 학위논문을 인준함

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Abstract

Development of Immunoassays using Novel Antibodies for Rapid Detection of Antibiotic-Resistant and Susceptible Pathogens

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Interdisciplinary Program of Bioengineering

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Staphylococcus aureus (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) are major pathogens frequently detected in food and beverage poisoning, and persistent infections. Therefore, the development of a rapid method that can detect these pathogens before serious multiplication is required. In this study, I produced recombinant antibodies

against *S. aureus* and *P. aeruginosa* from mammalian cells with high yield and purity. Then I developed four different detecting methods and compared those assay times and sensitivities.

First, I performed enzyme-linked immunosorbent assay (ELISA) using these recombinant antibodies. High binding affinity and low detection limit in both indirect and sandwich ELISAs were obtained to detect methicillin-resistant *S. aureus* and imipenem-resistant *P. aeruginosa*. Afterward, I developed a rapid ELISA to decrease the assay time of the conventional ELISA. By optimizing the assay conditions, I reduced the antigen immobilization time from 16 h to 2 h and the primary antibody reaction time from 1 h to 10 min. Next, I developed a novel fluorescence-linked immunosorbent assay (FLISA)-based *S. aureus* detection method using fluorescent anti-*S. aureus* antibody. I optimized the FLISA system to reduce the working time in comparison to the conventional ELISA system as well as the rapid ELISA system, resulting in the total assay time of 10 h. Moreover, I established a flow cytometry (FCM)-based detection method that allows rapid acquisition of cell populations in fluid samples by using a fluorescent antibody against *S. aureus*. Using this method, I

detected *S. aureus* with a 10^3-10^5 CFU order of limit of detection value within 1 h. Notably, I represented the efficacy of these four assay methods, ELISA, Rapid ELISA, FLISA, and FCM for detecting *S. aureus* using clinically isolated MRSA samples. As a result, FCM showed the shortest whole clinical sample detecting time, 4 h including pathogen preincubation.

These results indicate that the detecting system using recombinant antibodies-produced herein can be used for the accurate and rapid detection of *S. aureus*, with a wide range of applications in medical diagnosis, food safety, and drug discovery.

Key Words: recombinant antibody, *Staphylococcus aureus, Pseudomonas aeruginosa,* ELISA, FLISA, Flow cytometry

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1. Introduction

Food and beverage poisoning is caused by bacterial pathogens in contaminated foods and drinks that grow in the stomach [1]. *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) are major gram-positive and gram-negative food-borne pathogens, respectively, leading to long-term chronic diseases, typhoid fever, septicaemia, gastroenteritis, and serious infections [2, 3]. Although antibiotics against these pathogens have been developed, such as oxacillin for *S. aureus* and imipenem for *P. aeruginosa*, the evolution of antibiotic-resistant strains is increasing [4]. Moreover, *S. aureus* and *P. aeruginosa* cause infections even at low concentrations. Therefore, rapid detection of these pathogens is crucial for the timely testing of food and beverage poisonings. To date, a culture and colony counting-based method is standard for detecting *S. aureus*. [5] However, practical applications of this conventional method are limited because it requires 3-5 days to obtain results, which is time consuming and inadequate for timely in-situ detection. Polymerase chain reaction (PCR)-based detection using nucleic acid amplification, such as PCR, has been introduced [6]. As PCR is based

on nucleic acid amplification, its sensitivity is high and low numbers of bacterial cells can be detected. However, PCR-based detection requires isolation of bacterial DNA, followed by enzyme reaction and agarose gel electrophoresis, which are complex and tedious procedures. Moreover, PCR-based methods are based on single- or double-locus detection; these methods have a high risk of false positive/negative results [7]. Other molecular diagnostics using lectin or aptamer combined with flow cytometry have been developed to detect *S. aureus* [8, 9]. However, these methods suffer from low selectivity and labor intensity. Immunological assays have been developed for detecting *S. aureus* with high accuracy, because of the high specific response of antibody to antigen. For example, surface plasmon resonance (SPR) detects *S. aureus* in a label-free and real-time manner with a LOD of 10^3-10^4 CFU/mL [10, 11]. However, this method requires phage production, isolation, and purification, which have limitations due to complicated handling. Subramanian et al. performed SPR by using an antibody against protein A (PA), which is expressed on the surface of *S. aureus*, but the LOD of the system was high (10^7 CFU/mL) [12]. Additionally, as SPR-based assay needs sensor chips, the technology

needs a high cost. A quartz crystal microbalance with a random antibody shows high specificity for real-time detection of *S. aureus* [13]. However, it requires control over antibody orientation to ensure Fc recognition and the response affected by the antibody content of the sample. Enzyme-linked immunosorbent assay (ELISA) can be used for *S. aureus* detection because of its simplicity, low cost, and reliability. However, as conventional ELISA-based *S. aureus* detection uses hybridoma-based antibodies, the high cost of obtaining antibodies is a major burden [13-15]. Besides, hybridoma clones, which are used for producing monoclonal antibodies, lose their secreting ability over time. A recombinant antibody has attractive attributes compared to the traditional hybridoma-based antibody: (1) It can be overexpressed with high yield, and its production is controlled; (2) It is expressed from a unique gene, so the sequence information allows for subcloning to be modified or to generate fusions with other molecules, such as fluorescent proteins, enzymes, and peptides, without interfering with their antigen binding properties; and (3) Several issues with hybridoma production, such as gene mutations and cell line drift, can be avoided, leading to a high level of consistency between

batches. Escherichia coli (*E. coli*) is one of the systems for producing recombinant proteins including antibody. To produce a functional antibody using *E. coli*, the key to allow the correct formation of disulfide bonds is the secretion of V chains into the periplasmic space of *E. coli*, the oxidizing environment, for assembling into a functional Fv fragment [16]. Therefore, the production of recombinant antibodies using *E. coli* sometimes results in nonfunctional aggregates, and the recovery of functional antibody from inclusion bodies by denaturation followed by refolding is difficult and time-consuming [17]. Small antibody fragments, such as single chain fragment of variable domain (scFv) and the antigen-binding fragment (Fab), can be relatively readily expressed in *E. coli*. However, the yield of a larger full-sized antibody in soluble form is generally low because it requires post-translational modifications (PTMs). This has led to the development of a mammalian expression system. Eukaryotic cells, including mammalian cells, have developed an advanced folding, PTM, glycosylation, and secretion apparatus compared with bacteria [18]. Moreover, antibodies from mammalian cells are indistinguishable from those in humans, with least concerns for immunogenic modifications.

human embryonic kidney (HEK)293 cells and Chinese hamster ovary (CHO) cells are commonly used for producing a high yield of protein that is hard to be expressed in bacteria. HEK293 cells, human-derived cell line, provide the best environment for the expression of human antibodies, and cell lines derived from other species, such as CHO cells, have been found to introduce non-native PTMs not present on human antibodies [19]. These foreign PTMs can cause immunological reactions in humans and may affect the stability and function of recombinant antibodies [19]. Moreover, HEK293 cells can be transfected with recombinant plasmids for antibody production [20-23]. A highly useful derivative of HEK293 cells has been the establishment of HEK293Freestyle (HEK293F) cells, which are a variant of HEK293 cells and have been widely used to produce challenging proteins [20]. The HEK293F cell line has been adapted to suspension growth, and transient antibody expression allows convenient production [24]. Thus, HEK293F cells can offer a rapid method to produce large quantities of recombinant antibodies. In addition, it has been known that HEK293F cells show high transfection efficiency with the use of the inexpensive polymeric reagent polyethylenimine (PEI), and the results can

be evaluated within 48 hours after transient transfection, which provides a fast and economical alternative to produce recombinant antibodies [25-27].

In this study, I generated novel recombinant antibodies against *S. aureus* through mammalian cell culture. I produced large amounts of antibodies from HEK293F cells and purified them in a convenience manner. Then I developed four different detecting methods and compared those assay times and sensitivities. First, I revealed the binding efficiency of the produced antibodies to MSSA and MRSA by performing both indirect ELISA and sandwich ELISA, indicating the usefulness of these antibodies for sensitive detection of *S. aureus*. Afterward, I developed a rapid ELISA to decrease the assay time of the conventional ELISA. By optimizing the assay conditions, I reduced the antigen immobilization time from 16 h to 2 h and the primary antibody reaction time from 1 h to 10 min. Next, I developed a novel fluorescence-linked immunosorbent assay (FLISA)-based *S. aureus* detection method using fluorescent anti-*S. aureus* antibody. I optimized the FLISA system to conspicuously reduces the working time in comparison to the conventional ELISA system as well as

the rapid ELISA system, which I additionally conducted in this study. Notably, I presented the efficacy of the FLISA for detecting *S. aureus* using clinically isolated MRSA samples and compared the results to the whole clinical sample detecting time using conventional ELISA and Rapid ELISA.

Another detecting method, Flow cytometry (FCM), is a high-throughput analysis method that enables the measurement of the properties of individual cells in a flow [28]. FCM using fluorescent antibodies is one of the reagentless immunoassays that require no immobilisation steps. Namely, by reacting the fluorescent antibodies to the antigens on the cells and then applying them to the fluid system, the positive signal from an antigen-bound antibody can be separated from the extra signal from a free antibody, which does not bind to the antigen. FCM is highly automated because cells pass in a flow and are focused by laser beam, then each cell is characterised by its scattering and fluorescence properties [29]. Moreover, FCM allows the analysis of cells at a rate of over 50,000 per minute [30]. This high speed of FCM is an advantage over culture-based methods, which requires time-consuming dilution procedures and a waiting period until the formation of colonies

on a selective agar plate. FCM provides information regarding the cells present in the culture, which can be collected within several minutes [31].

Bacterial cells are more difficult to analyse using FCM than eukaryotic cells because the volume of bacteria are 1,000-fold smaller than those of eukaryotes [29]. Moreover, because prokaryotes do not have organelles, their cellular complexity is lower than that of eukaryotes, resulting in the former having weaker scattering signals than the latter [32]. Therefore, FCM is typically used in immunoassays for eukaryotic cells, such as cancer and immune cells, but its use for the detection and analysis of pathogens has limitations. Despite this difficulty, rapid FCM-based methods, which can detect pathogens in water, food, and clinical samples, have been developed [33-36]. Some methods using fluorescent antibodies combined with FCM have been reported to detect *S. aureus* or *P. aeruginosa*. [37-40] However, these methods were not optimised to detect low concentrations of pathogens, because the studies have focused on the application of pathogens in real samples. For example, Kennedy *et al*. detected the food-borne pathogen *S. aureus* by cultivating cultures in broth until the exponential phase, followed by mixing the cultures with

fluorescent antibodies [41]. However, the cells were grown until their optical density at 600 nm reached 0.2, with a cell population of 1.0×10^7 CFU/mL, and a high concentration of cells was used to evaluate the detection method.

Therefore, I also generated fluorescent antibodies against *S. aureus* and *P. aeruginosa* by conjugating a fluorescent dye to commercially available or recombinant antibodies. I then established an FCM-based assay method using these fluorescent antibodies for the rapid detection of these pathogens.

2. MATERIALS AND METHODS

2.1. Materials

96-well Maxi-binding and Uni-binding black plates were obtained from SPL (Seoul, Korea). Goat anti-mouse IgG HRP was obtained from Pierce (Seoul, Korea). Mouse anti-*S. aureus* monoclonal IgM and rabbit anti-*S. aureus* polyclonal IgG were obtained from Thermo. HRP-conjugated goat anti-rabbit IgG (H+L) and HRP-conjugated goat anti-mouse IgM were obtained from Invitrogen (Waltham, MA, USA). HRP-conjugated anti-DYKDDDDK antibody was obtained from (Biolegend, CA, USA). Alexa 647-NHS ester was obtained from Broad Pharm (San Diego, USA). Hydroxyl amine-HCl was obtained from Daejung (Cheongju, Korea). *BL21* cells were obtained from New England Biolabs (Seoul, Korea). *KCTC1682*, *Bacillus subtilis*, and *Shigella flexneri* cells were obtained from KCTC (Seoul, Korea). Other chemicals and reagents, unless otherwise indicated, were from Sigma.

2.2. Gene construction of recombinant anti-*S. aureus* antibody

DNA cloning was performed by co-worker, Ms. Eun-Jung Kim [42].

2.3. Cell culture and production of recombinant antibody

Production of recombinant IgGs was performed by co-worker, Ms. Eun-Jung Kim [42]. Production of scFv was performed by co-worker, Mr. Hanool Yun, Department of Biological and Chemical Engineering, Hongik University. Details can be found from his Master's thesis, 2023 Feb.

2.4. *S. aureus* culture

The cells of two *S. aureus* strains, MSSA (*S. aureus WKZ-1*, NR-28984) and MRSA (*S. aureus WKZ-2*, NR-28985) [43], were cultured by growing cultures overnight to log phase at 37°C at 200 rpm. The sample was centrifuged (3500 rpm, 10 min, 4°C) and the supernatant was discarded. The diluted cells with PBS reached an OD_{600} of 0.5 with a cell density of 10^8 CFU/mL.

2.5. Confirmation of the antigen-binding activity

The antigen-binding activities of recombinant and commercial antibodies were tested via indirect ELISA as follows; 100 μL of series diluted LTA or *S. aureus* was immobilized on a 96-well plate for 37 h at 2°C. After that, the well was filled with 350 μL of TBST that contains 3% BSA for 1 h at 37°C, then washed three times with TBST. Subsequently, 500 ng/mL of antibody in 100 μL of PBS with 0.1% BSA was added and incubated for 1 h at 25°C. After washing three times with 350 μL of TBST, bound protein was probed with 5000-fold diluted HRP-conjugated goat anti-mouse IgG antibody in PBS with 0.1% BSA for 1 h at 25°C. The well was washed three times with 350 μL of PBS and developed with 50 μL of TMB solution. After incubation for 10 min, the reaction was stopped with 50 μL of 1 N H2SO4, and the absorbance was read at 450 nm using a microplate reader. Dose−response curves were constructed by fitting the intensities at the maximum emission wavelength of spectra using the Graphpad Prism. The EC50 value was calculated from the curve fitting to a 4-parameter logistic equation. The LOD value was calculated based on the equation of Y = Bottom + (Top − Bottom)/(1 + 10((LogEC50-X) × HillSlope)) using GraphPad Prism version 8.0.

2.6. ELISA for scFv

The antigen-binding activity of recombinant anti-*P. aeruginosa* scFv was tested via indirect ELISA as follows; 100 μL of series diluted *P. aeruginosa* was immobilized on a 96-well plate (SPL, maxi binding plate) for 16 h at 4°C. Afterwards, 350 μL Blocking buffer (PBS buffer with 3% BSA) was added to the well and incubated 2 h at 25°C. The well was washed three times with ELISA buffer (PBS buffer with 0.1% BSA). Subsequently, 10 μg/mL of purified scFv in 100 μL of ELISA buffer was added to the well and incubated for 1 h at 25°C. After washing three times using 350 μL of ELISA buffer, 10000-fold diluted HRP-conjugated anti-DYKDDDDK antibody in ELISA buffer was added to the well and incubated for 1 h at 25°C. The well was washed three times with 350 μL of ELISA buffer and developed with 50 μL of TMB solution for 10 min. The reaction was stopped by adding 50 μL of 1 N H2SO4 to the well, and the absorbance was read using a microplate reader. Dose−response curves were constructed by fitting the absorbance at 450 nm using the GraphPad Prism software (GraphPad Software, San Diego, CA).

2.7. Sandwich ELISA

300 ng of recombinant or commercial antibody was immobilized on the 96-well plate for 16 h at 4°C, and the well was filled with 350 μL of PBS buffer that contains 3% BSA for 1 h at 37°C and washed three

times with 350 μL of PBS buffer that contains 0.1% BSA. Subsequently, 100 μL of *S. aureus* was added and incubated for 1 h at 25°C. After washing three times with 350 μL of 0.1% BSA in PBS, bound protein was probed with 300 ng recombinant or commercial antibody for 1 h at 25°C. After washing three times with 350 μL of 0.1% BSA in PBS, HRP-conjugated rabbit anti-human Fc antibody for recombinant antibody, anti-mouse IgM for monoclonal antibody, or anti-rabbit IgG for polyclonal antibody in 0.1% BSA in PBS for 1 h at 25°C. The well was washed three times with 350 μL of 0.1% BSA in PBS and developed with 50 μL of TMB solution. After incubation for 15 min, the reaction was stopped with 25 μL of 1N H2SO4, and the absorbance was read at 450 nm using a microplate reader Model 680 (Bio-Rad). Dose−response curves were constructed by fitting the intensities at the maximum emission wavelength of spectra using the Graphpad Prism. The EC50 value was calculated from the curve fitting to a 4-parameter logistic equation. The LOD value was calculated based on the equation of Y = Bottom + (Top − Bottom)/(1 + 10((LogEC50-X) × HillSlope)) using GraphPad Prism version 8.0.

2.8. Fluorescent dye conjugation

Fluorescent antibodies were prepared and confirmed by the following method. 10 μg of proteins in PBS buffer reacted with x20 mol of Alexa647-NHS ester in the dark at 25°C for 2 h.

2.9. Rapid ELISA

Cell cultures were diluted in PBS and transferred into microtubes. 100 μL of serially diluted *WKZ-1* or *WKZ-2* was immobilized on a 96-well plate (SPL, maxi binding plate) for 16 h at 4°C, 2 h at 30°C, 1 h at 30°C, or 0.5 h 30°C. Afterwards, 350 μL Blocking buffer was added to the well and incubated 2 h at RT. The well was washed three times with PBSB. Subsequently, 10 μg/mL of non-fluorescent dye conjugated 6DW2 in 100 μL of PBSB was added to the well and incubated for 1 h at RT. After washing three times using 350 μL of PBSB, 5000-fold diluted HRP-conjugated anti-human IgG Fc antibody in 100 μL of PBSB was added to the well and incubated for 1 h at RT. The well was washed three times with 350 μL of ELISA buffer and developed with 50 μL of TMB solution for 10 min. The reaction was stopped by adding 50 μL of 1 N H2SO4 to the well, and the absorbance at 450 nm was read using a microplate reader.

2.10. FLISA

Cell cultures were diluted in PBS and transferred into microtubes. 100 μL of serially diluted *WKZ-1* or *WKZ-2* was immobilized on a 96-well black plate for 2 h at 30°C. Afterwards, 350 μL Blocking buffer (PBS buffer with 3% BSA) was added to the well and incubated 2 h at RT. The well was washed three times with PBSB (PBS buffer with 0.1% BSA). Subsequently, 10 μg/mL of AF488-conjugated 6DW2 in 100 μL of PBS was added to the

well and incubated for 1 h at RT. After washing three times using 350 μL of PBSB and the fluorescence was read using a fluorescent microplate reader (TECAN). Dose−response curves were constructed by fitting the fluorescence at 524 nm using the GraphPad Prism software (GraphPad Software, San Diego, CA). The EC50 value was calculated from the curve fitting to a 4-parameter logistic equation of Y = Bottom + (Top − Bottom)/(1 + 10((LogEC50-X) × HillSlope)) using GraphPad Prism. The LOD value (EC10) was calculated using GraphPad Prism software.

2.11. Flow cytometry

Cell cultures were diluted in PBS and transferred into microtubes. 2 μg of fluorescent full-sized antibody and 500 ng recombinant scFv was added to serially diluted pathogen samples in 0.3 mL PBS. After incubation for 1 h at RT, 1% methanol was added. Fluorescent antibody-stained samples were analyzed using a FACS CantoII cytometer (BD Biosciences) and data were processed using Flowjo software. Either over 5000 or 10000 events were recorded for each sample. Acquisition was visualized in a cell cell count/FITC histogram in which regions were created for the blue cell-unit population (FITC). The positive signal population of the samples was obtained by gating based on negative control containing only *BL21* and fluorescent antibodies, and the positive ratio for the total number of cells was calculated. Dose−response curves were constructed using the

GraphPad Prism software. The EC50 value was calculated from the curve fitting to a 4-parameter logistic equation of Y = Bottom + (Top − Bottom)/(1 + 10((LogEC50-X) × HillSlope)) using GraphPad Prism software. The LOD was calculated using following equation: LOD = mean blank + 1.645 × SD blank + 1.645 × SD low concentration sample [44].

2.12. Preincubation of clinical cells

Clinical MRSA cells were isolated from patients. Ten *S. aureus* cells were randomly collected at Hallym University Kangdong Sacred Heart Hospital, Seoul, Korea. Single *S. aureus* colony was isolated by streaking cells on LB agar plate and cultivated for 16 h at 37°C. Single colony was inoculated into 3 mL of LB media and incubated at 37°C with 200 rpm shaking until the OD_{600} reached to 0.5. Nine fractions were obtained from the cultivate by collecting the samples every 1 h (from 0 to 8 h).

3. RESULTS AND DISCUSSION

3.1. Binding efficiency to LTA on the surface of *S. aureus*

I Prior to the confirmation of the antigen-binding activity of recombinant antibodies, I performed ELISA using commercial anti-*S.*

aureus antibodies to optimize an ELISA system. As Lupardus's group revealed that the three Fabs showed efficient WTA binding [45], I first tried to use WTA as an antigen for confirming the binding activity of antibodies. However, as WTA was not commercially available, I used LTA as an antigen instead. Among the four types of LTA, LTA type I of *S. aureus* composed of 1,3-polyglycerolphosphate repeating units that are substituted at the C2 position (X in Figure 1A) with hydrogen proton (-15%), D-alanyl ester (-70%), or N-GlcNAc (-15%) (Figure 1A). Although LTA is anchored to the cell membrane, whereas WTA is covalently linked to peptidoglycan, their constructs are structurally similar, and both are N-GlcNAc-modified [46]. Therefore, I estimated that the three recombinant antibodies, whose sequences were from Fabs against β-1,4-GlcNAc as well as commercial anti-*S. aureus* antibodies had binding affinity to LTA. To this end, I performed indirect ELISA using LTA as an antigen, commercial anti- *S. aureus* antibodies, rabbit anti-*S. aureus* polyclonal IgG or mouse anti-*S. aureus* monoclonal IgM, as a primary antibody, and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG or HRP-conjugated anti-mouse IgM as a secondary antibody (Figure 1). The median effective

concentration (EC50) of the polyclonal antibody was 4.49 ± 0.64 x 10^3 ng/mL whereas the EC50 of monoclonal antibody was not detectable (Table 1). These results indicated that the commercial polyclonal antibody can be used for detecting LTA in the ELISA system. This information was revealed for the first time in this study, whereas the detailed experimental conditions had not been exactly presented in the commercial information. As expected, the antigen-binding efficiency of the polyclonal antibody was superior to the one of monoclonal antibody. Generally, as a monoclonal antibody recognizes a single epitope of an antigen, its affinity and selectivity to the target antigen are higher than those of a polyclonal antibody. The two commercial antibodies were generated by immunizing not LTA but *S. aureus*. This can be the reason for the result, regarding to the monoclonal antibody showed lower binding efficiency against LTA than polyclonal antibody.

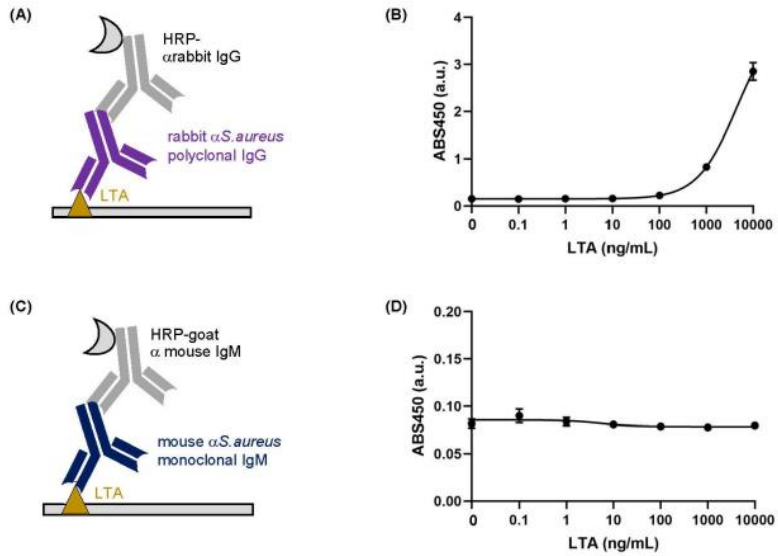

Figure 1. LTA-binding efficiency of commercial antibodies. (A) Schematic representation of indirect ELISA for confirming the dose-dependent LTA-binding efficiency of the polyclonal anti-*S. aureus* antibody, (B) ELISA signal of the polyclonal antibody at different concentrations of LTA, (C) Schematic representation of indirect ELISA for confirming the dose-dependent LTA-binding efficiency of the monoclonal anti-*S. aureus* antibody, and (D) ELISA signal of the monoclonal antibody at different concentrations of LTA. Error bars represent ±1 SD (n =3).

Table 1. EC50 and LOD values of anti-*S. aureus* antibodies that were determined from the titration curves of indirect ELISA.

Antigen	Antibody	EC50 (CFU)	LOD (CFU)
LTA	Polyclonal Ab	$4.49 \pm 0.64 \times 10^3$	5.32
LTA	Monoclonal Ab	n.d	n.d
MSSA	Polyclonal Ab	$9.0 \pm 6.68 \times 10^3$	1.0×10^4
MSSA	Monoclonal Ab	$2.7 \pm 0.63 \times 10^3$	n.d
MSSA	Recombinant Ab (6DW2)	$6.6 \pm 0.45 \times 10^4$	1.5×10^3
MSSA	Recombinant Ab (6DWC)	$4.7 \pm 0.12 \times 10^4$	1.1×10^3
MRSA	Polyclonal Ab	$7.2 \pm 7.91 \times 10^3$	7.5×10^3
MRSA	Monoclonal Ab	$2.0 \pm 2.64 \times 10^3$	5.4×10^1
MRSA	Recombinant Ab (6DW2)	$2.3 \pm 0.09 \times 10^4$	3.6×10^2
MRSA	Recombinant Ab (6DWC)	$2.1 \pm 0.17 \times 10^4$	n.d.

3.2. Binding efficiency of commercial antibody to *S. aureus*

Next, I confirmed the binding efficiency of the two commercial antibodies against *S. aureus*. I cultured MRSA and MSSA, then seeded the series-diluted cells on each well of a plate and performed indirect ELISA (Figure 2).

The titers increased with an antigen-concentration dependent-manner, and the EC50 and LOD of the two antibodies against MRSA and MSSA were calculated (Table 1). The monoclonal antibody showed a broader detection range than the polyclonal antibody against both pathogens. Moreover, the EC50 and LOD of the monoclonal antibody were higher than those of the polyclonal antibody, suggesting a relatively better usefulness of the monoclonal antibody than polyclonal antibody for *S. aureus* detection. In particular, the LOD of the monoclonal antibody against MSSA and MRSA was under 100 CFU order, indicating high sensitivity.

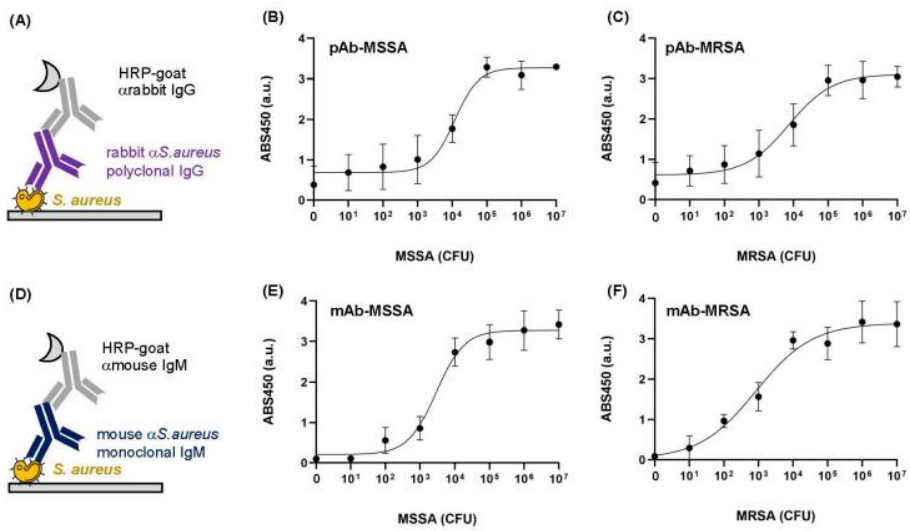

Figure 2. *S. aureus*-binding efficiency of commercial antibodies. (A) schematic representation of the indirect ELISA for confirming the dose-dependent *S. aureus*-binding efficiency of polyclonal antibody, (B) ELISA signal of polyclonal antibody with various concentrations of MSSA, (C) ELISA signal of polyclonal antibody with various concentrations of MRSA, (D) schematic representation of the indirect ELISA for confirming the dose-dependent *S. aureus*-binding efficiency of monoclonal antibody, (e) ELISA signal of monoclonal antibody with various concentrations of MSSA, (f) ELISA signal of monoclonal antibody with various concentrations of MRSA,. Error bars represent ±1 SD (n =3).

3.3. Binding efficiency of recombinant antibody to *S. aureus*

I confirmed the antigen-binding efficiency of the newly generated three recombinant antibodies. First, I seeded 10^7 CFU of MRSA or MSSA on each well of a plate. As a control, I added PBS instead of *S. aureus*. After blocking, I added each recombinant antibody, followed by HRP-conjugated anti-human Fc antibody, which binds human Fc that is produced from HEK cells (Figure 3A). As a result, all the three antibodies showed binding efficiency to MRSA and MSSA, and among them, 6DW2 and 6DWC showed higher signal to the one of 6DWI (Figure 3B). Therefore, I move forward to the next step using 6DW2 and 6DWC. I seeded 10^1-10^7 CFU of series-diluted MRSA or MSSA on each well of a plate and performed indirect ELISA. As a result, the signals increased antigen-concentration dependent manner (Figure 3C-3F), and the EC50 and LOD of each antibody was calculated as Table 1.

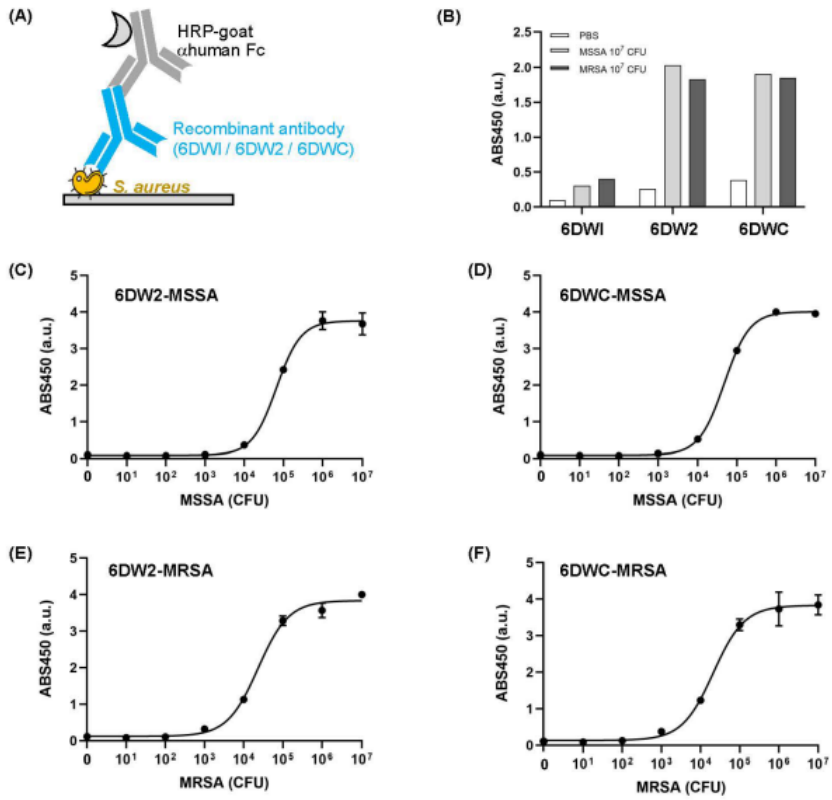

Figure 3. *S. aureus*-binding efficiency of recombinant antibodies. (a) schematic representation of the indirect ELISA for confirming the dose-dependent *S. aureus*-binding efficiency of recombinant antibodies, (b) ELISA signals of 6DWI, 6DW2, and 6DWc with 10^7 CFU of MSSA or MRSA. (c-f) ELISA signals of 6DW2 or 6DWC with various concentrations of MSSA or MRSA. Error bars represent ±1 SD (n =3).

Surbhi et al. revealed that the diagnostic criterion of MRSA is 10^3 CFU/mL [47]. Although the strain is slightly different, the diagnostic criteria of MRSA Pneumonia is 10^4 CFU/mL [48]. Therefore, although the antigen-binding properties of recombinant antibodies were relatively higher than those of commercial antibodies, the LOD values of recombinant antibodies were high enough to detect MRSA and MSSA in clinical samples. It is worth mentioning that the information about the commercial antibodies, including their DNA sequences and production methods, has not been revealed. On the other hand, the sequence information of the recombinant antibodies described herein can be widely used for the future development of various modifications, including fluorescence probes or peptide tagging, and for further DNA cloning for making mutants for improving their properties. [42] In addition, the detailed methods demonstrated in this study, including mammalian cell-based production of antibodies and confirmation of their binding efficiencies, would be useful for obtaining in-house monoclonal anti-*S. aureus* antibodies with low cost, high yield, and high purity and for applying them for detecting *S. aureus*.

3.4. Sandwich ELISA for detecting *S. aureus*

I performed not only indirect ELISA but also sandwich ELISA to compare those responses and find the most appropriate detecting system. The important step for sandwich ELISA is selecting the best pair of capturing and detecting antibodies. Therefore, at first, I screened the pairs from the combination of polyclonal, monoclonal, and three kinds of recombinant antibodies. I seeded each antibody to the plate and blocked the empty space of plate. Afterward, I added 10^1-10^7 CFU of MRSA or MSSA, then washed the plate. Subsequently, I added a capturing antibody, followed by the HRP-conjugated antibody, which binds to the capturing antibody (Figure 4A). As a result, almost all the pairs, except the polyclonal antibody used as a detecting antibody, showed an antigen-dependent S/B ratio (Figure 4B, 4C).

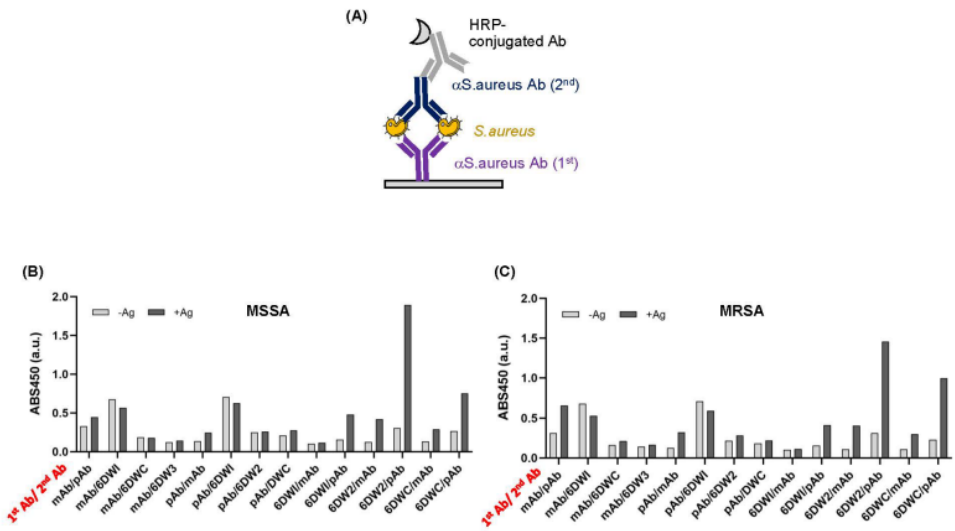

Figure 4. (A) Schematic representation of the sandwich ELISA. (B) ELISA signals of each pair with 10^8 CFU of MSSA, (C) ELISA signals of each pair with 10^8 CFU of MRSA. PBS was added instead of MSSA or MRSA as a negative control.

Among them, the pairs of 6DWC–monoclonal antibody and polyclonal antibody–monoclonal antibody for MSSA, and 6DW2–monoclonal antibody and polyclonal antibody– monoclonal antibody for MRSA showed a higher S/B ratio than others (with the order of capturing antibody–detecting antibody). Therefore, I moved to the next step using these selected pairs for obtaining titration curves. I seeded several concentrations of MSSA or MRSA on the plate and performed sandwich ELISA. As a result, the signals were increased with an antigen-concentration dependent manner, and the EC50 and LOD values were calculated (Figure 5, Table 2).

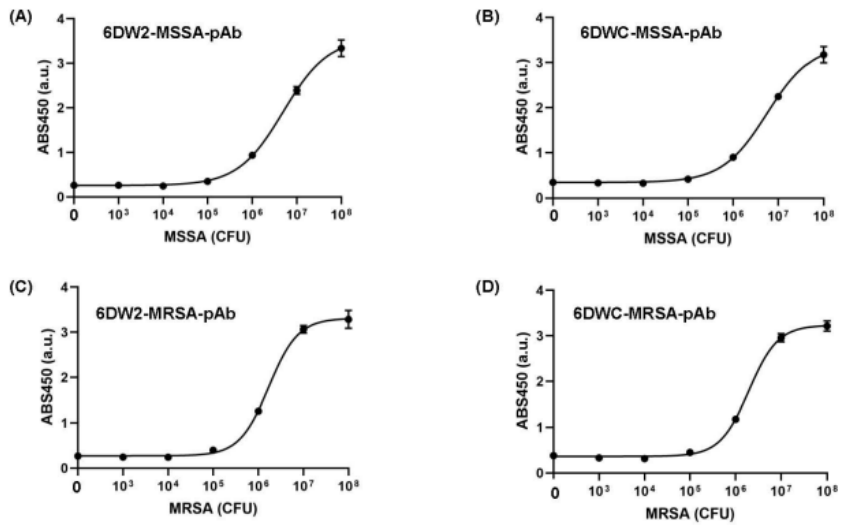

Figure 5. (A) Titration curve for detecting MSSA via sandwich ELISA using 6DW2 as a capture antibody and polyclonal antibody as a detecting antibody, (b) Titration curve for detecting MSSA via sandwich ELISA using 6DWC as a capture antibody and polyclonal antibody as a detecting antibody, (c) Titration curve for detecting MSSA via sandwich ELISA using 6DW2 as a capture antibody and monoclonal antibody as a detecting antibody, (d) Titration curve for detecting MRSA via sandwich ELISA using 6DWC antibody as a capture antibody and polyclonal antibody as a detecting antibody . Error bars represent ±1 SD (n =3).

Table 2. EC50 and LOD values of anti-*S. aureus* antibodies that were determined from the titration curves of sandwich ELISA.

Antigen	Capturing antibody	Detecting antibody	EC50 (CFU)	LOD (CFU)
MSSA	Recombinant Ab (6DW2)	Polyclonal Ab	$5.2 \pm 1.74 \times 10^6$	6.2×10^4
MSSA	Recombinant Ab (6DWC)	Polyclonal Ab	$1.7 \pm 0.27 \times 10^6$	9.3×10^4
MRSA	Recombinant Ab (6DW2)	Polyclonal Ab	$5.8 \pm 1.91 \times 10^6$	4.8×10^4
MRSA	Recombinant Ab (6DWC)	Polyclonal Ab	$1.9 \pm 0.10 \times 10^6$	n.d

Although the LOD values of sandwich ELISA systems were higher than those of indirect ELISA, the sandwich ELISA system can also be used for the practical system because the LOD covers the diagnostic criteria of the concentration of pathogens. As the ELISA method described herein needs below 24 h as an assay time, the total procedure does not require over two days even pre-incubation for several hours is performed prior to the ELISA method to increase the concentration of pathogens. This merit regarding the rapid procedure time is compared to the traditional methods, including PCR-based method, which has low selectivity even though the LOD is 10^3 CFU/mL [47]. Souhir Boujday et al. developed an immunosensor for detecting MSSA that uses gold-coated sensor chip and presented 10^5 CFU/mL as its detectable concentration [49]. There is a possibility that if the antibody developed in this study were immobilized on the gold-coated sensor chip, the sensitivity can be able to be increased, and the performance of optical sensing can be improved. The ELISA-based method offers a similar or better detectable range and LOD than another sensing material-based MSSA detecting methods in the use of Ab-quantum dot, (GO/PDMS)/Paper and Ab/NH2-MIL53.

3.5. FLISA

I conducted FLISA using three-fluorescent dye-labeled antibodies against *S. aureus* (made by Mr. Hanool Yun, Department of Biological and Chemical Engineering, Hongik University. Details can be found from his Master's thesis, 2023 Feb) under the same conditions as conventional ELISA: immobilization for 16 h at 4°C, blocking for 2 h, and antibody reaction for 1 h. Using an HRP-conjugated secondary antibody (2 h) and enzyme reaction (0.5 h) was not necessary because the FLISA uses fluorescence rather than enzymatic reaction, the 2.5 h of assay time was reduced at this point, resulting in a total working time of 3.5 h (Figure 6).

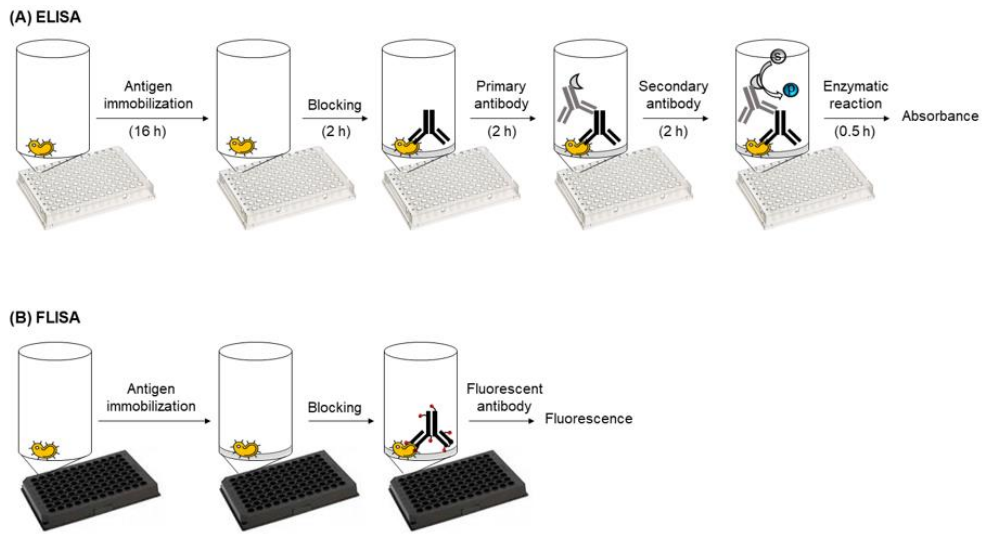

Figure 6. Schematic representations of conventional ELISA (A) and FLISA developed in this study (B) to detect *S. aureus*.

The signals of each fluorescent antibody increased in an antigen concentration-dependent manner. Surprisingly, the maximum intensity of 6DW2 was 43.4-fold, and this value was higher than those of mAb (2.3-fold) and pAb (35.2-fold) (Figure 7, Table 3). I confirmed that the antigen dose-dependent signal did not originate from the antigen, free dye, or non-conjugated antibody. The LOD values for mAb, pAb, and 6DW2 against *WKZ-1*, an MSSA strain, were 9.3 x 10^4 CFU, 8.3 x 10^5 CFU, and 7.4 x 10^5 CFU, respectively. The EC50 values for mAb, pAb, and 6DW2 against *WKZ-1* were 2.2 ± 0.80 x 10^5 CFU, 2.5 ± 0.38 x 10^6 CFU, and 2.0 ± 0.13 x 10^6 CFU, respectively. When I immobilized *WKZ-2*, an MRSA strain, and performed the same procedure, the maximum intensities of mAb, pAb, and 6DW2 were 2.6-fold, 32.6-fold, and 38.6-fold, respectively (Figure 8, Table 3). The LOD values for mAb, pAb, and 6DW2 against MRSA were 9.1 x 10^4 CFU, 6.5 x 10^5 CFU, and 6.3 x 10^5 CFU, respectively. The EC50 values for mAb, pAb, and 6DW2 against MRSA were 1.2 ± 0.03 x 10^5 CFU, 1.7 ± 0.05 x 10^6 CFU, and 1.7 ± 0.02 x 10^6 CFU, respectively. The maximum fold increase, LOD, and EC50 values of 6DW2 against both *WKZ-1* and *WKZ-2* were superior to those of the commercial pAb. In contrast, the maximum

fold increase of 6DW2 against both *WKZ-1* and *WKZ-2* was higher than that of the commercial mAb, but the sensitivity and EC50 values of mAb were higher than those of 6DW2. Commercial mAb is IgM, which is an isotype of immunoglobulin that is composed of pentameric or hexameric macroimmunoglobulins. When IgM was used in my experiment using an indirect ELISA, the response was better than that of pAb and 6DW2 [42]. Although the exact reason for this phenomenon is not clear, and the sensitivity of the commercial mAb was slightly lower than that of the recombinant antibody, I decided to use a recombinant antibody for the next experiment because of the significantly lower price of the recombinant antibody than that of the commercial antibody.

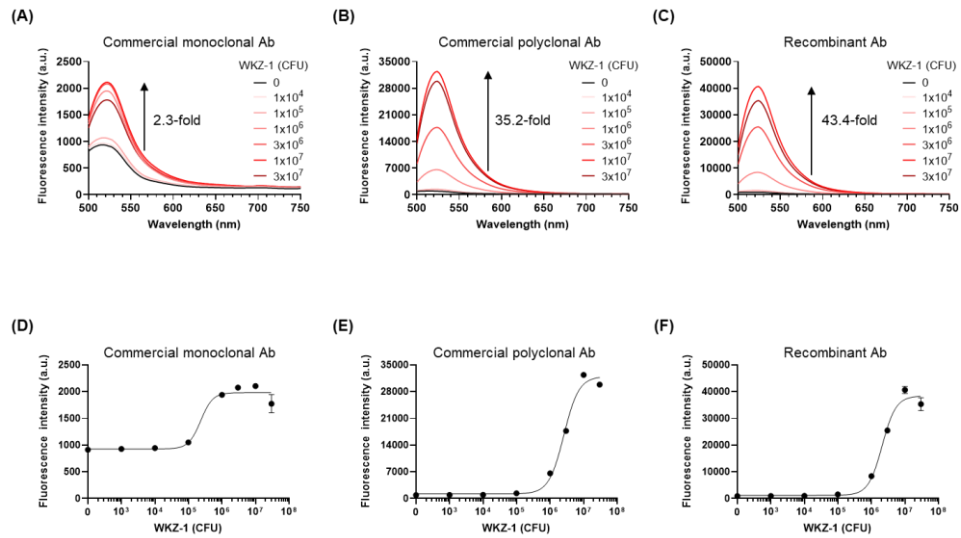

Figure 7. Average fluorescence spectra of FLISA using commercial mAb (A), commercial pAb (B) or recombinant Ab (C) in the presence of *WKZ-1* at the indicated concentrations (n=3). Antigen concentration-dependent fluorescent response of commercial mAb (D), commercial pAb (E) or recombinant Ab (F) against *WKZ-1*. The maximum fluorescence intensity of each sample was plotted. Error bars represent ±1 SD (n = 3).

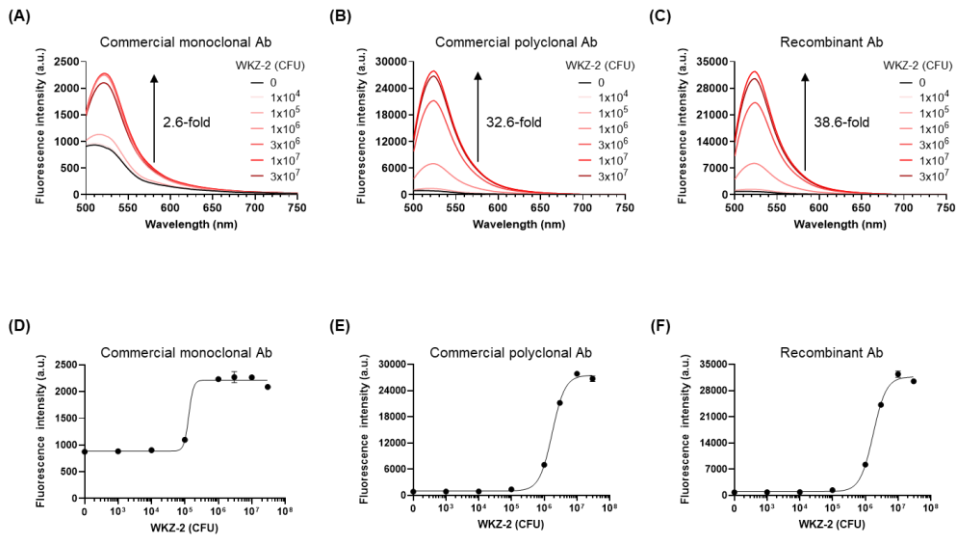

Figure 8. Average fluorescence spectra of FLISA using commercial mAb (A), commercial pAb (B) or recombinant Ab (C) in the presence of *WKZ-2* at the indicated concentrations (n=3). Antigen concentration-dependent fluorescent response of commercial mAb (D), commercial pAb (E) or recombinant Ab (F) against *WKZ-2*. The maximum fluorescence intensity of each sample was plotted. Error bars represent ±1 SD (n = 3).

Table 3. LOD, EC50, and fold increase of antibodies that were determined from the titration curves of FLISA.

Antibody	Immobilization	WKZ-1			WKZ-2		
		LOD (CFU)	EC50 (CFU)	Fold increase	LOD (CFU)	EC50 (CFU)	Fold increase
Recombinant Ab	16 h, 4 °C	7.4×10^5	$2.0 \pm 0.13 \times 10^6$	43.4	6.3×10^5.	$1.7 \pm 0.02 \times 10^6$	38.6
Commercial mAb	16 h, 4 °C	9.3×10^4	$2.2 \pm 0.80 \times 10^5$	2.3	9.1×10^4	$1.2 \pm 0.03 \times 10^5$	2.6
Commercial pAb	16 h, 4 °C	8.3×10^5	$2.5 \pm 0.38 \times 10^6$	35.2	6.5×10^5	$1.7 \pm 0.05 \times 10^6$	32.6
Recombinant Ab	2 h, 30 °C	5.5×10^6	$1.2 \pm 0.06 \times 10^7$	27.8	3.1×10^6	$1.0 \pm 0.06 \times 10^7$	24.8

Although the assay time was decreased from 20.5 h to 19 h by eliminating the secondary antibody reaction and enzymatic reaction for FLISA, the entire assay time was still long. To further reduce the assay time, I adjusted each step of FLISA. First, I performed a conventional ELISA with different antigen immobilization times and temperatures. I immobilized cells under several conditions, 4°C, 30°C, and 37°C for 1, 2, 3, or 16 h in the presence or absence of antigen. When the antigen was immobilized at 30°C or 37°C, the signal was higher than the signal that was immobilized at 4°C. Indeed, the signal in the presence of antigen was improved alone with the immobilization time, and saturated at 16 h, 2 h, and 3 h for 4°C, 30°C, and 37°C, respectively. Overall, when immobilization was conducted at 30°C for 2 h, the signal was almost the same as that at 4°C for 16 h, which was the maximum response, and I determined that immobilization at 30°C for 2 h was the best time-reduced condition. Next, the cells were blocked for several reaction times. As a result, the titer in the present of antigen was decreased when I reduced the blocking time from 2 h to 1.5 h, 1 h, 0.5 h, or 0 h. Therefore, I determined the best blocking time to 2 h. Finally, I optimized the concentration of fluorescent antibody. When I

added 0.1 μg, 0.3 μg, 0.5 μg, 0.7 μg, and 1 μg of fluorescent antibody, the titer overall titer was increased in the large amount of antibody. However, when I added 2, 3, or 5 μg of antibody, there were no distinguishing titers at the high concentration of antigen. This may be because the high concentration of the fluorescent antibody caused fluorescence quenching. It has been reported that a Homo-dimer can be formed between the same dyes when the distance between the dyes is small. Based on these results, I decided to per-form an antibody-antigen binding reaction using 1 μg of fluorescent antibody. Using the optimized FLISA working conditions (5 h of assay time), I performed FLISA using fluorescent 6DW2 against *WKZ-1* or *WKZ-2* (Figure 9).

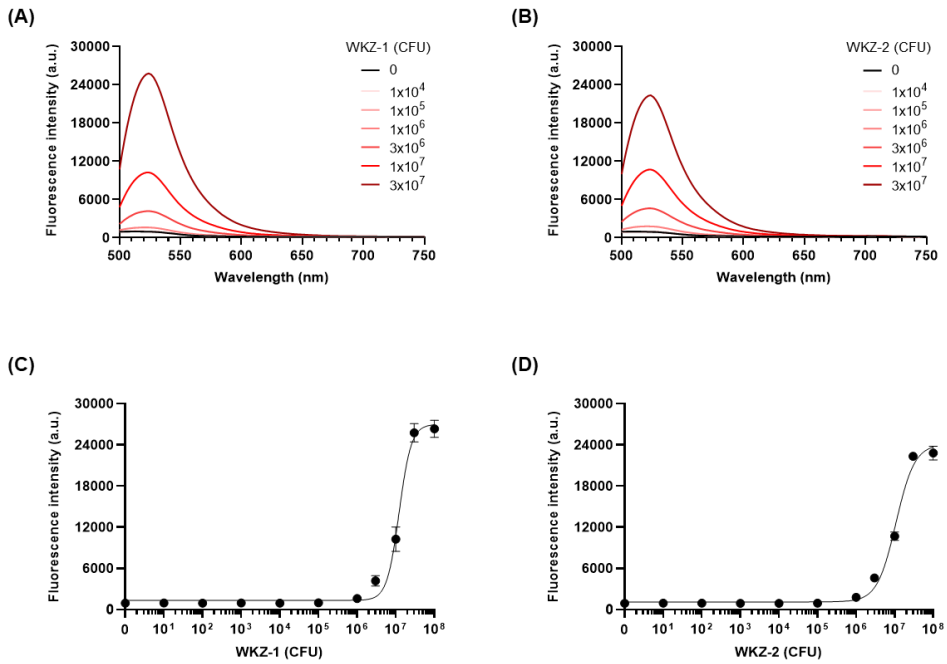

Figure 9. Average fluorescence spectra of Rapid FLISA using recombinant antibody in the presence of *WKZ-1* (A) or *WKZ-2* (B) at the indicated concentrations (n=3). Antigen concentration-dependent fluorescent response against *WKZ-1* (C) or *WKZ-2* (D). The maximum fluorescence intensity of each sample was plotted. Error bars represent ±1 SD (n = 3).

As a result, 27.8-fold response, 1.2 ± 0.06 x 10^7 of EC50, 5.5 x 10^6 of LOD was obtained in the presence of *WKZ-1*, and 24.8-fold response, 1.0 ± 0.06 x 10^7 of EC50, 3.1 x 10^6 of LOD was obtained in the presence of *WKZ-2*. As the sensitivity in case of the 5 h assay time were only one-digit lower than the FLISA assay with 18.5 h, indicating the assay time was decreased from 18.5 h to 5 h but the response was mostly maintained. When I conducted FLISA against other cells, such as *BL21*, *KCTC1682*, *Bacillus subtilis*, and *Shigella flexneri*, no dose-dependent response was observed, indicating that the FLISA signals were *S. aureus*-specific.

3.6. FLISA for detecting clinical MRSA

Next, I applied the FLISA system to identify clinical samples isolated from MRSA-infected patients. First, I confirmed whether the 6DW2 antibody bound to clinically isolated MRSA strains via indirect ELISA. I cultured the MRSA strains from ten patients in LB medium and collected the cells. I added PBS to 10^6 CFU of cells and immobilized them in each well of a 96-well plate. After blocking, I added 6DW2 followed by HRP-conjugated anti-mouse IgG antibody as a secondary antibody. All strains

showed high titers. Notably, I used commercially available anti-*S. aureus* pAb instead of 6DW2; the titers were lower than those of 6DW2, indicating the usefulness of 6DW2 over commercial pAb in clinical studies.

Prior to conducting FLISA using clinical samples, I preincubated the cells to determine the time required to reach the LOD of the FLISA system. I inoculated a single colony from a clinical strain-streaked plate and incubated the culture. I obtained fractions from the cultivate every 1 h and kept the sample at 4°C before finishing the collection of the last fraction (8 h culture). I immobilized each fraction on a 96-well black immunoplate at 30°C for 2 h and conducted FLISA in the above established manner (Figure 10). The averaged LOD values from each ten samples was 315 ± 47 min, which was corresponded to 5.2 ± 0.8 h. Although 5.2 h of preincubation of cells is required to apply this FLISA system, such prerequisite introduces much faster additional time than traditional incubation time of approximately 16 to 24 h until the bacteria have grown sufficiently to diagnosis [50, 51].

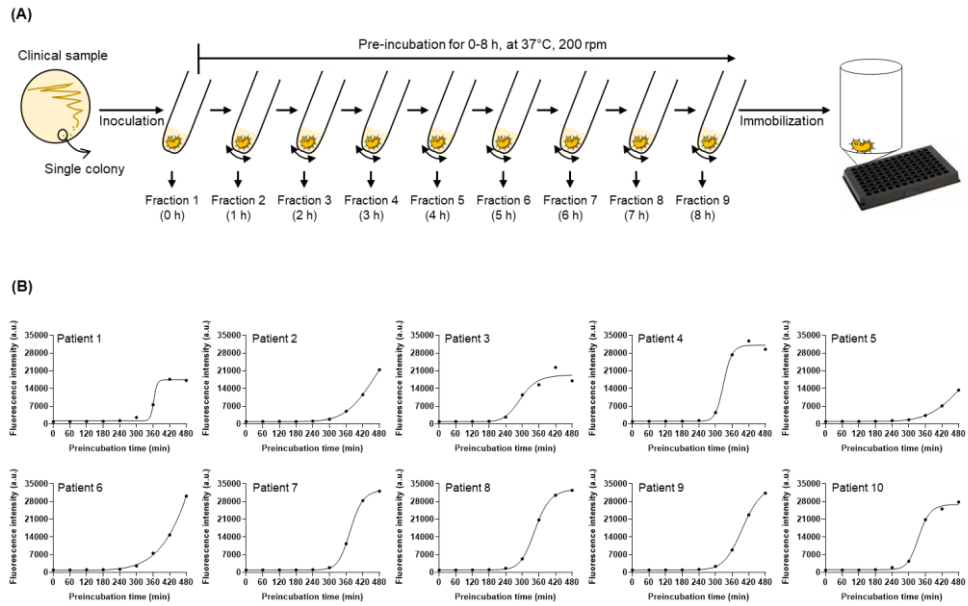

Figure 10. (A) Schematic representation of FLISA using clinical MRSA strains isolated from patients. (B) Fluorescent responses of preincubated clinical samples.

3.7. Comparison of methods for detecting clinical MRSA

As the LOD of FLISA using 6DW2 was one-digit higher than that of ELISA using 6DW2, I conducted preincubation followed by ELISA for the diagnosis of the clinical sample to confirm whether the LOD values affected the pre-incubation time. I prepared nine fractions with different incubation times (from 0 to 8 h) and immobilized each fraction in a 96-well plate for 16 h at 4°C. Next, I conducted ELISA with 2 h of blocking, 1 h of primary antibody reaction, 1 h of secondary antibody reaction, and 0.5 h of enzymatic reaction. As a result, the average minimum preincubation time for detection was 270 ± 53 min (4.5 ± 0.8 h), which was approximately 45 min faster than FLISA. However, the assay time of conventional ELISA requires 20.5 h, the total time, including the preincubation and assay time, was 25.0 ± 0.8 h, which was approximately 15 h longer than FLISA.

The conventional ELISA established in our previous study included 16 h of immobilization. I assumed that the sensitivity of ELISA would be maintained when the immobilization condition was changed to 2 h at 30°C, resulting in a reduced whole working time as the ELISA titers in the

presence of 10^6 CFU of *WKZ-2* were similar. First, I conducted "Rapid ELISA" by immobilizing serially diluted *WKZ-1* or *WKZ-2* for 2 h at 30°C instead of 16 h at 4°C and additionally optimized the time for primary or secondary antibody reaction and the amount of primary or secondary antibody. When I conducted Rapid ELISA with the optimized condition (immobilizing preincubated samples for 2 h at 30°C instead of 16 h at 4°C, reacting 1 ug of primary antibody for 1 h and reacting 0.2 ug of secondary antibody for 1 h) the LOD value for *WKZ-1* and *WKZ-2* was 3.7 x 10^5 and 4.2 x 10^5, respectively, and the EC50 value for *WKZ-1* and *WKZ-2* was 1.47 ± 0.22 x 10^6 and 1.78 ± 0.22 x 10^6, respectively, which were similar to the conventional ELISA. When I conducted Rapid ELISA using clinical samples, the average preincubation time was 263 ± 46 min (4.4 ± 0.8 h) and the assay required 5 h 40 min.

Overall, FLISA requires 10.3 ± 0.8 h for diagnosis MRSA clinical sample, which is faster than ELISA (25 ± 0.8 h) and similar to Rapid ELISA (10.1 ± 0.8 h), indicating the usefulness of the FLISA as a speedy detection system (Table 4). Even though the total required time of Rapid ELISA was slightly faster than that of FLISA, the assay steps of FLISA are much more

conventional and faster than Rapid ELISA because the secondary antibody reaction and enzymatic reaction steps were eliminated for FLISA. Although the preincubation time of FLISA was approximately 1 h slower than that of Rapid ELISA, there was no need for human handling for incubation. Therefore, the total working times of FLISA and Rapid ELISA were similar; however, the convenience of the diagnosis of FLISA was superior to that of Rapid ELISA.

Nevertheless, in the case of FCM, the most rapid and accurate detection was possible among other assays including FLISA, with a clinical strain preincubation time of 2.8 ± 0.5 h and a total assay time of 3.8 ± 0.5 h for detection.

Table 4. Entire assay time of each detection method

		ELISA	Rapid ELISA	FLISA	FCM
Clinical sample treatment	Preincubation	4.5 ± 0.8 h (270 ± 53 min)	4.4 ± 0.8 h (263 ± 46 min)	5.3 ± 0.8 h (315 ± 47 min)	2.8 ± 0.5 h (172 ± 35 min)
Assay time	Pathogen immobilization	16 h (4°C)	2 h (30°C)	2 h (30°C)	-
	Blocking	2 h	2 h	2 h	-
	Primary Ab	1 h	10 min	1 h	1 h
	Secondary Ab	1 h	1 h	-	-
	Enzyme reaction	0.5 h	0.5 h	-	-
	Total	25 ± 0.8 h	10.1 ± 0.8 h	10.3 ± 0.8 h	3.8 h ± 0.5 h

3.8. Flow cytometry analysis

The fluorescent antibody was reacted with *S. aureus* or *P. aeruginosa* cells and analyzed using FCM with a laser capable of exciting Alexa647 dye. To acquire a sufficient cell population to obtain a stable event parameter of the instrument, I had to adjust the minimum total cell number to 3×10^6 CFU (Figure 11). Therefore, I incubated fluorescent antibodies with various concentrations of the pathogen, ranging from 1×10^4 CFU to 3×10^6 CFU, and adjusted the total cell number by adding non-pathogenic bacteria, *BL21*, up to 3×10^6 CFU. After mixing, I eliminated the free dye and unbound fluorescent antibodies by removing the supernatant after centrifugation and adding PBS and then conducted FCM analysis. The histogram of the sample in the presence of 3×10^6 CFU of *BL21*, which was used as a negative control, was on the left side, and the histograms of the sample in the presence of pathogens were shifted to the right (Figure 12A, 12B). Clear differences in the fluorescent patterns were observed among individual samples in the presence of different numbers of cells that were distinguishable from the fluorescent patterns of 3×10^6 CFU of *BL21* cells. I gated the peak range in the

presence of 3 × 10^6 CFU of the pathogen, which is the maximum signal, and determined the positive signal ratio of the samples by calculating the ratio between the fluorescence intensity of the *BL21* only sample in the gated range and the fluorescence intensity of the pathogen-including sample in the gated range.

The fluorescent dye-labeled antibody against *S. aureus* showed a dose-dependent binding efficiency to both *WKZ-1* and *WKZ-2* (Figure 12C, 12D). The LOD values of the recombinant antibody against *WKZ-1* and *WKZ-2* were 5.17 × 10^3 CFU and 2.73 × 10^3 CFU, respectively, which were lower than the value in the use of a commercial antibody, 1.59 × 10^4 CFU and 9.65 × 10^3 CFU for *WKZ-1* and *WKZ-2*, respectively (Table 5). When I used these recombinant and commercial antibodies for indirect ELISA in our study, the LOD values of the recombinant antibody against *WKZ-1* and *WKZ-2* were 1.5 × 10^3 CFU and 3.6 × 10^2 CFU, respectively, which were lower than the values for the commercial antibody, 1.0 × 10^4 CFU and 7.5 × 10^3 CFU, respectively. The ELISA results corresponded to the results of the FCM analysis, indicating the usefulness of the recombinant antibody for more sensitive detection of *S. aureus* than can be performed

by a commercial antibody. Notably, a sample could be analyzed within 5 min of assay time without the preincubation of cells by using the FCM method described in this study. Thirty minutes of hands-on time for reacting fluorescent antibody and cells followed by 5 min for washing were only necessary for preparing the samples. The LOD values of FCM were estimated as 10^3-10^4 CFU order, which are similar to those of ELISA and considerably lower than those of bacterial detection methods. Because the assay time for FCM is much faster than that of other conventional methods such as ELISA, which requires 1-2 d to obtain results, a few hours of preincubation for cultivating the cells to reach a cell number over the LOD value can be additionally performed before the FCM analysis.

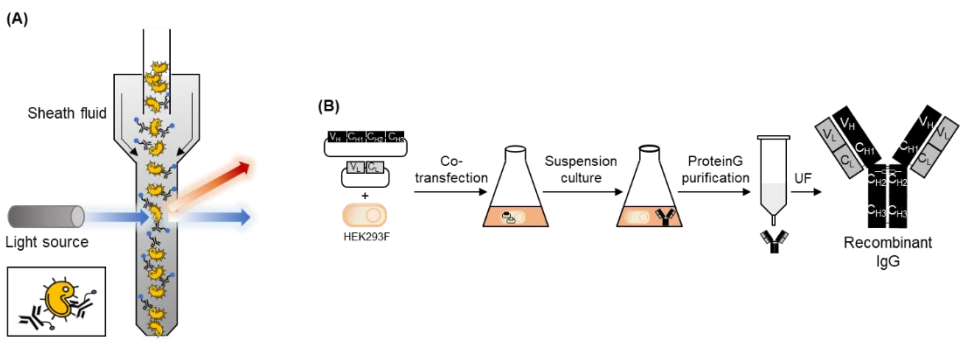

Figure 11. (A) Schematic image of flow cytometry analysis using a fluorescent antibody to detect pathogens. (B) Schematic representation of an entire step for producing a recombinant full-sized antibody using HEK293F cells. UF indicates ultra-filtration.

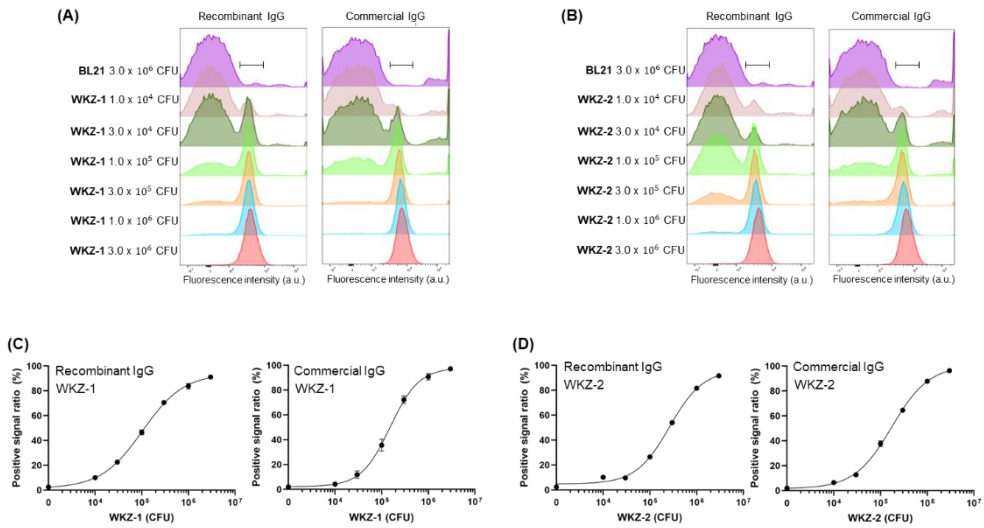

Figure 12. (A) Overlaying histograms obtained by flow cytometry analysis after incubation of fluorescent recombinant or commercial antibody with different numbers of *WKZ-1* and *BL21*. The total cell numbers of each assay were adjusted to 3 x 10^6 CFU by adding *BL21* cells to the different numbers of pathogen cells (B) Overlaying histograms obtained by flow cytometry analysis after incubation of fluorescent recombinant or commercial antibody with different numbers of *WKZ-2* and *BL21*. The total cell numbers of each assay were adjusted to 3 x 10^6 CFU by adding *BL21* cells to the different numbers of pathogen cells (C, D) Positive signal ratio of the mean of fluorescence intensity of range-gated in each histogram. Error bars represent ±1 SD (n = 3).

Table 5. EC50 and LOD values of Abs that were determined from the titration curves of FCM. n.d. means not detected.

Strain	Antibody	EC50 (CFU)				LOD (CFU)
		1st trial	2nd trial	3rd trial	Average	
WKZ-1	Commercial Ab	1.45×10^5	1.51×10^5	1.60×10^5	$1.52 \pm 0.08 \times 10^5$	1.59×10^4
	rAb (HEK293)	1.01×10^5	1.06×10^5	1.10×10^5	$1.06 \pm 0.05 \times 10^5$	5.17×10^3
WKZ-2	Commercial Ab	1.79×10^5	1.71×10^5	1.77×10^5	$1.76 \pm 0.04 \times 10^5$	9.65×10^3
	rAb (HEK293)	2.73×10^5	2.55×10^5	2.66×10^5	$2.65 \pm 0.09 \times 10^5$	2.73×10^3
UCBPP PA14	Commercial Ab	6.30×10^5	4.43×10^5	4.61×10^5	$5.12 \pm 1.03 \times 10^5$	6.61×10^4
	rAb (HEK293)	2.06×10^6	n.d.	n.d.	n.d.	n.d.
	rAb (*E. coli*)	2.26×10^6	9.73×10^5	1.65×10^6	$1.63 \pm 0.64 \times 10^6$	3.16×10^5
ATCC 27853	Commercial Ab	1.49×10^6	1.40×10^6	n.d.	n.d.	1.41×10^5
	rAb (HEK239)	1.97×10^6	n.d.	n.d.	n.d.	n.d.
	rAb (*E. coli*)	4.81×10^6	4.07×10^6	7.28×10^5	$3.20 \pm 2.18 \times 10^6$	1.37×10^5
ATCC BAA-2108	Commercial Ab	8.69×10^5	2.17×10^6	1.25×10^6	$1.43 \pm 0.67 \times 10^6$	5.16×10^4
	rAb (HEK293)	4.25×10^6	n.d.	2.24×10^6	n.d.	8.48×10^4
	rAb (*E. coli*)	n.d.	5.13×10^6	5.13×10^6	n.d.	n.d.

Next, I prepared a fluorescent dye-labeled antibody against *P. aeruginosa* and mixed it with various numbers of three types of *P. aeruginosa* strains and *BL21* up to 3 × 10^6 CFU to adjust the entire cell number to be equal to 3 × 10^6 CFU in the individual assay. As a result, the signal increased in a pathogen cell-number-dependent manner (Figure 13). The LOD value of the recombinant antibody and commercial antibody against *ATCC BAA-2108* was 8.48 x 10^4 CFU and 5.16 x 10^4 CFU, respectively, indicating that the recombinant antibody can detect the pathogen slightly more sensitively than the commercial antibody can. The exact LOD value of the recombinant antibody against *UCBPP PA14* and *ATCC 27853* could not be calculated, because the signal on the titration curve was not fully saturated; thus, the equation of the curve was not estimated. However, when I compared the curve patterns and EC50 values of recombinant antibody and commercial antibody, I estimated that the LOD value for detecting the *UCBPP PA14* of the recombinant antibody might be higher than that of the commercial antibody, and the value for detecting the *ATCC 27853* of recombinant antibody might be similar to that of the commercial antibody. The lowest LOD value of the ELISA assay

using a commercial antibody for *UCBPP PA14*, *ATCC 27853*, and *ATCC BAA-2108* was 10^5 CFU order, 10^1 CFU order, and 10^4 CFU order, respectively, and the LOD values using a recombinant antibody for ATCC27853 and *ATCC BAA-2108* was 10^4 CFU order (the LOD of PA14 was not calculated) [52]. These results demonstrated that the FCM analysis described herein can detect *P. aeruginosa*, and fluorescent recombinant or commercial antibodies can be used for the assay. The fact that a recombinant antibody can be produced in a more cost-effective manner than the price of a commercial antibody can compensate for the higher LOD value against *ATCC 27853* of the recombinant antibody than the commercial antibody.

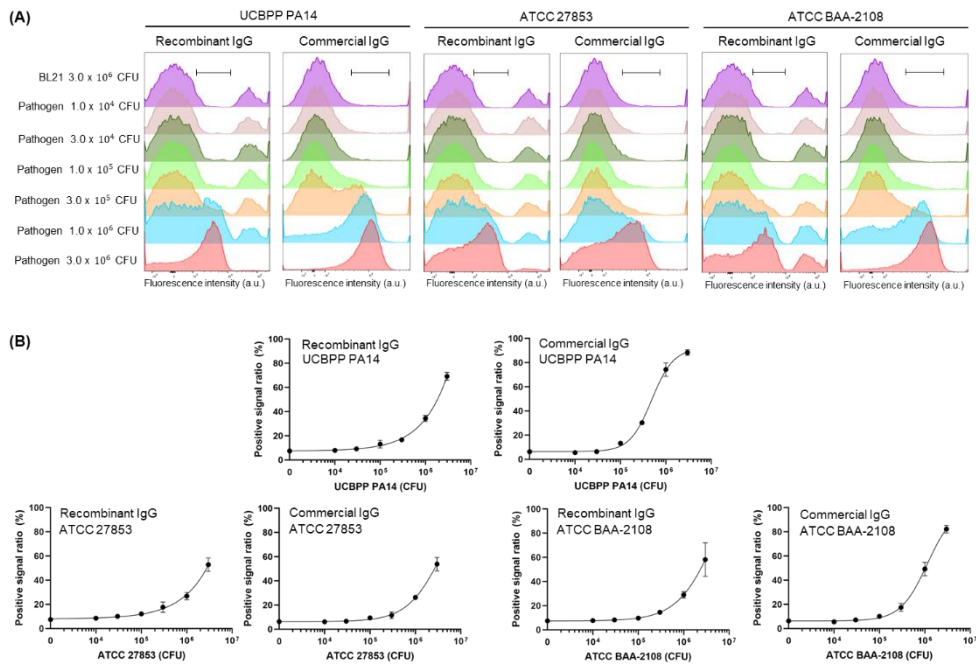

Figure 13. (A) Overlaying histograms obtained by flow cytometry analysis after incubating fluorescent recombinant or commercial antibody with different numbers of *P. aeruginosa* and *BL21*. The total cell numbers of each assay were adjusted to 3 x 10^6 CFU by adding *BL21* cells to the different numbers of pathogen cells (B) Positive signal ratio of the mean of fluorescence intensity of range-gated in each histogram. Error bars represent ±1 SD (n = 3).

3.9 Flow cytometry analysis for detecting clinical MRSA

Preparation of clinical strains was also conducted in the same way as FLISA. Fractions of clinical strains cultured from 0 to 8 h were obtained and used for FCM measurement (Figure 14). As a result, the preincubation time required to detect clinical strains was 2.8 h, and the total assay time including this was very short at 3.8 h.

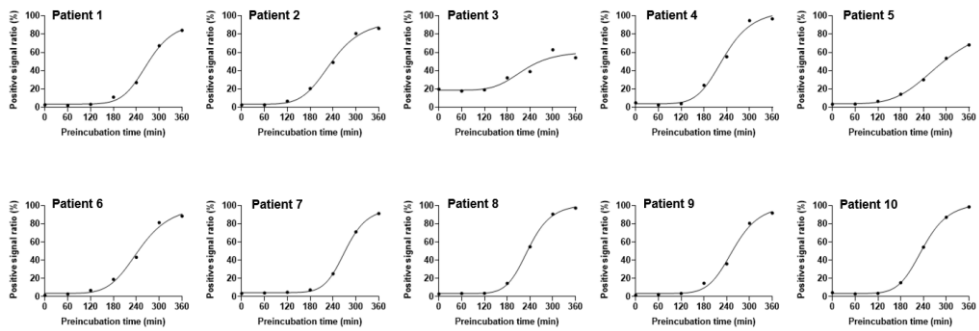

Figure 14. Flow cytometry analysis of preincubated clinical samples.

3.10. Recombinant scFv against *P. aeruginosa*

Although the FCM analysis of the use of recombinant antibody showed the detection of *P. aeruginosa* in a range from 10^4 to 10^5 CFU within 5 min, I conducted further experiments with a single-chain Fv (scFv), the smallest antibody fragment, against *P. aeruginosa* to extend the candidate of the superior antibody. When I used this fluorescent scFv for FCM analysis, I observed that the peak on the histogram was shifted to the right in a pathogen concentration-dependent manner (Figure 15A). The LOD values of scFv against *UCBPP PA14* and *ATCC 27853* were 3.16 x 10^5 CFU and 1.37 x 10^4 CFU, respectively. Although the exact LOD value of scFv against *ATCC BAA-2108* could not be calculated, it was estimated to be 10^4-10^5 CFU from the titration curve (Figure 15B, Table 5). The LOD values of scFv were similar to or slightly higher than those of the commercial antibodies and recombinant IgG. However, the *E. coli*-based scFv-type recombinant antibody is superior to mammalian cell-based IgG-type antibody production, namely, the former's convenient production, and cost-efficiency. Further, *E. coli*-based scFv production requires 5 days, whereas mammalian cell-based IgG production requires 2-3 weeks,

including several days for inoculating and preculturing the cells and 7 days for the formation of antibodies in the cells. Moreover, the media for mammalian cell culture is more expensive and easier to get contaminated than the *E. coli* expressing medium. Therefore, the fluorescent anti-*P. aeruginosa* scFv developed in this study can be used as a probe for FCM analysis, especially when a shorter antibody-producing time and simpler antibody-producing method are required.

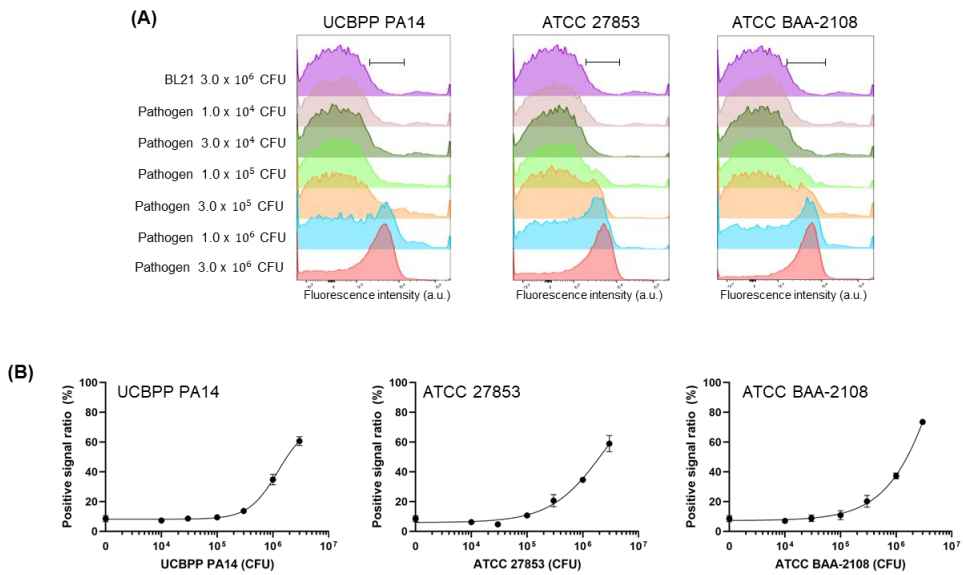

Figure 15. (A) Overlaying histograms obtained by flow cytometry analysis after incubation of fluorescent scFv with different numbers of *P. aeruginosa* strains and *BL21*. The total cell numbers of each assay were adjusted to 3 x 10^6 CFU by adding *BL21* cells to the different numbers of pathogen cells (B) Positive signal ratio of the mean of fluorescence intensity of range-gated in each histogram. Error bars represent ±1 SD (n = 3).

4. CONCLUSION

In this study, I produced recombinant anti-*S. aureus* antibodies using mammalian HEK293F cells with high yield and purity. Then I developed four different detecting methods and compared those assay times and sensitivities. First, I performed indirect ELISA to confirm the binding efficiency of each antibody to MSSA and MRSA, resulting in very high sensitivity with the order of 10^2 CFU. When I performed sandwich ELISA in the use of recombinant antibodies with commercial antibodies, the selected pairs showed LOD values with the order of 10^4 CFU, which is relatively higher than the value from indirect ELISA but low enough to cover the diagnostic standard of the concentration of pathogens. The method for producing these antibodies which showed high *S. aureus*-binding efficiency and sensitivity can be used for generating recombinant anti-*S. aureus* antibody to use it for practical pathogen detecting. The procedure to produce a recombinant antibody described herein needs 10 days, which is comparable to the generation of a monoclonal antibody, which needs at least six months for mouse immunization followed by hybridoma cell culture or at least three months for phage display followed

by bio-panning. Once a recombinant antibody is produced on a large scale, it can be stored at -20°C or -80°C and then used for each ELISA analysis. It is obvious that the traditional culture and colony counting method is inadequate for on-the-spot detection because it takes 2-3 days, including pre-incubation time to amplify pathogens for obtaining enough pathogens. On the other hand, the whole procedure for indirect ELISA using a pre-made antibody described herein needs approximately 20 h, which is less time-consuming than the traditional method. Moreover, in the case of the sandwich ELISA, the 96-well plate can be pre-coated to immobilize the capture antibody that provides the assay time down to 4-5 h, which is distinguishable from the conventional detecting methods as well as indirect ELISA when it is developed as a ready-to use ELISA kit. The ELISA method with the newly developed recombinant antibody does not require any amplification and can directly detect *S. aureus* with the low limit of detection of 10^2- 10^3 CFU order. Afterward, I developed a rapid ELISA to decrease the assay time of the conventional ELISA. By optimizing the assay conditions, I reduced the antigen immobilization time from 16 h to 2 h and the primary antibody reaction time from 1 h to 10 min. This

method has higher selectivity and sensitivity comparable to the conventional methods to detect *S. aureus*. The rapid and sensitive *S. aureus* detecting method using a newly developed recombinant antibody described in this study can be used for efficient detection of *S. aureus* with a wide range of applications including medical and pharmaceutical diagnosis, antibiotic discovery, and food safety.

Next, I established a novel *S. aureus* detection system using fluorescent antibodies and fluorescence spectrometry. The FLISA system detects both MSSA and MRSA strains within 5 h. Notably, the FLISA system uses a 96-well plate that can detect a maximum of 96 samples simultaneously e, indicating a high-throughput detection platform. The FLISA method is composed of only simple steps, such as immobilization, blocking, adding fluorescent antibodies, washing, and measurement. Based on this method, clinical sample could be diagnosed after 5.3 h of preincubation, resulting in whole process time of 10.3 h. As the details for producing the fluorescent recombinant 6DW2 has been described, the FLISA method using the fluorescent antibody can be widely used for rapid and convenient *S. aureus* detection.

Moreover, I also established an FCM-based method that can detect *S. aureus* and *P. aeruginosa* within 1 h, including 1-2 min of mixing fluorescent antibodies and cells, 30 min of reaction, 5 min of washing, and 5 min of analysis. Based on these assays, the minimum number of pathogens for which it was possible to consider a positive signal was 10^3-10^5 CFU. Because the whole process time for obtaining the assay results is very fast, preincubating the cells before reacting them with fluorescent antibodies can be further performed to increase the concentration of pathogens in the samples over the LOD values. If the bacterial load is less than the LOD for this method, preincubating the cells can increase the number of cells in the sample (i.e. bacteria can be cultured before adding fluorescent antibodies). As the whole process time for this FCM-based method is under 1 h (much faster than other conventional methods such as ELISA that requires approximately 20 h), a pre-incubation step can be added prior to the assay, providing an advantage to this FCM-based rapid detection system. In this study, I focused on evaluating an FCM-based rapid detection method. I thus used commercial or recombinant antibodies that I have used in our study. Modifying the antigen-binding

sites of antibodies through point mutation would allow screening or selection for reagents with increased sensitivity. I applied our FCM-based method to detect *S. aureus* in apple juice, which carries a high risk of pathogen contamination. I generated an artificially contaminated beverage sample by adding serially diluted *WKZ-2* strain to juice. I then added AF647 dye-labeled 6DW2 to the sample. After a 30 min incubation, the sample was centrifuged, washed using PBS, and analyzed using FCM. Direct analysis using FCM without centrifugation and washing save 5 min by eliminating pretreatment. However, I washed the sample before loading to FCM because direct injection of commercial juice to FCM can contaminate equipment. I have demonstrated that the FCM-based method described here can detect *S. aureus* in beverage samples with similar efficacy as detection in PBS. Interestingly, when I centrifuged a mock pathogenic apple juice sample and added fluorescent dye-labeled 6DW2 to the pellet, I observed high background signal, indicating that the order of the pretreatment steps in processing a real sample can considerably affect the outcome.

In addition, the previously developed scFv showed binding efficiency to three *P. aeruginosa* strains, which was confirmed by ELISA and FCM. The use of fluorescent IgGs and scFv combined with FCM analysis developed herein can be widely used in applications for detecting *S. aureus* and *P. aeruginosa* in samples originating from food, beverages, and clinical sources. I expect that FCM-based rapid and convenience pathogen detection using multicolour fluorescent antibody cocktails, which can be generated by conjugating fluorescent dyes with different emission wavelengths to antibodies that specifically bind each target pathogen will be useful in an extensive array of applications.

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국문 초록

황색포도상구균 및 녹농균은 대표적인 식중독 원인균으로 증식전에 신속히 검출할 수 있는 방법의 개발이 필요하다. 본 연구에서는 황색포도상구균과 녹농균 각각을 인식하는 재조합 항체를 동물세포를 사용하여 고수율 및 고순도로 생산하였다.

이 항체를 사용하여 식중독균을 검출 가능한 네가지 분석법 ELISA (Enzyme-linked immunosorbent assay), Rapid ELISA, FLISA (Fluorescent-linked immunosorbent assay), FCM (Flow cytometry)을 개발하였고 분석 시간과 민감도를 비교하였다. 먼저, 항생제 내성 황색포도상구균과 녹농균을 검출하기 위해 간접 ELISA 및 샌드위치 ELISA 를 수행한 결과, 양쪽 검출법에서 높은 결합성과 낮은 검출한계 값을 확보하였다. 간접 ELISA 의 경우 메티실린 내성 황색포도상구균에 대한 EC50 값과 최소 검출 농도는 각각 2.3 ± 0.09 x 10^4 CFU 및 3.6 x 10^2 CFU 였으며, 샌드위치 ELISA 의 경우 각각 5.8 ± 1.91 x 10^6 CFU 및 4.8 x 10^4 CFU 였다. 그 후 기존 ELISA 의 분석 시간을 단축하기 위해 Rapid ELISA 법을 확립하였다. 분석 조건을 최적화하여 항원 고정 시간을 16 시간에서 2 시간으로, 1 차 항체 반응 시간을 1 시간에서 10 분으로 단축하여, ELISA 에서 소요되었던 25 시간의 총 분석시간을 10 시간으로

단축하였다. 그 다음으로 형광 항 황색포도상구균 항체를 이용한 새로운 FLISA 기반 황색포도상구균 검출 방법을 개발하였다. FLISA 시스템을 최적화하여 기존 ELISA 시스템 뿐만 아니라 신속 ELISA 시스템에 비해 작업 시간을 5 시간으로 단축하였다. 그리고 황색포도상구균에 대한 형광 항체를 사용하여 FCM 기반 검출 방법을 확립하였다. 이 방법을 통해 최소 10^3-10^5 CFU 의 황색포도상구균을 1 시간 이내에 검출 가능함을 알 수 있었다. 특히, 임상적으로 분리된 메티실린 항생제 내성 황색포도상구균 샘플을 사용하여 황색포도상구균을 검출하기 위한 ELISA, Rapid ELISA, FLISA 및 FCM 분석법의 효율을 비교 검증하였다. 그 결과, FCM 을 통해 병원체 전처리를 포함하여 임상샘플을 검출을 총 3.8 h ± 0.5 시간 안에 완료할 수 있었고, 이는 ELISA (25 ± 0.8 시간), Rapid ELISA (10.1 ± 0.8 시간), FLISA (10.3 ± 0.8 시간)보다 신속한 검출이 가능함을 제시하였다. 이러한 결과는 본 연구에서 생산한 재조합 항체를 이용한 검출 시스템이 병원균을 정확하고 신속하게 검출하여 의학적 진단, 식품 안전 및 신약 개발에 광범위하게 응용될 수 있음을 시사하였다.

주요어: 재조합항체, 황색포도상구균, 녹농균, ELISA, FLISA, 유세포분석법

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