Rapid, Sensitive, and Selective Detection of H5 Hemagglutinin from Avian Influenza Virus Using an Immunowall Device

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ABSTRACT: Avian influenza virus (AIV) infection, caused by influenza virus type A, is an infectious, acute respiratory disease of birds related to influenza outbreaks worldwide. The highly pathogenic AIV subtype H5N1 has crossed species barriers to infect mammals, including humans, with fatal outcomes and has received attention as a potential pandemic threat. A rapid and timely detection in poultry is vitally important to prevent virus spread. Despite their great sensitivity, conventional detection methods such as real-time reverse transcription-polymerase chain reaction and the agar gel precipitation test are time-consuming and labor-intensive and require special training. In this work, an immunowall device was evaluated as an easier and faster way for detecting AIV H5-hemagglutinin (AIV H5-HA). For detection, fluorescence-labeled or enzyme-labeled antibody was employed as a labeling antibody in a sandwich immunoassay. Both were shown in this paper to be easier and faster assays for detection compared with the conventional enzyme-linked immunosorbent assay (ELISA) kit. In addition, high selectivity was achieved for AIV H5-HA detection after the evaluation of other different HA virus subtypes. The limit of detection was 0.23 ng/mL for the enzyme-labeled antibody. This value was equivalent to that of the immunowall device with the enzyme-labeled antibody offered a rapid, sensitive, selective, and simple immunoassay system for future H5 AIV real sample detection.

INTRODUCTION

Influenza, also known as “the flu”, is a contagious, acute respiratory illness caused by influenza viruses. There are three main types of influenza viruses: A, B, and C. Among them, type A influenza viruses infect a wide variety of avian species and mammals, and type A is the only type associated with influenza pandemics, making it the most significant for public health worldwide.1 Influenza virus type A is further divided into subtypes based on the combinations of different virus surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). So far, 16 HAs (H1−H16) and 9 NAs (N1−N9) have been identified. The HA subtypes govern the ability of the virus to bind to and enter cells, where the epidemiologically most important multiplication of the virus then occurs.2−4

Avian influenza virus (AIV) infection is an infectious disease of birds that is caused by influenza virus type A. Wild waterfowl, gulls, and shorebirds are known to be the natural reservoirs of this virus. Other bird species are also susceptible to being infected with a wide range of symptoms: from those of a mild illness to a highly contagious and rapidly fatal disease.5−6 On the basis of their virulence, influenza A viruses are categorized into two groups: low pathogenic AIV (LPAIV) and highly pathogenic AIV. To date, highly pathogenic forms have been limited to those of the H5 and H7 subtypes. When two different viruses infect the same cell, some of their gene segments are easily exchanged because of the organization of the influenza virus into eight gene segments.8 Thereby, LPAIVs can begin to evolve rapidly and can mutate into a
highly lethal version of the same initially mild strain that can kill chickens within 48 h, with the mortality approaching 100%.6−8 Avian H5N1 influenza in particular has shown a fast mutation and propensity for acquiring genes from viruses and infecting other animal species.9−11 Globally, between 1997 and 2015, 907 human cases of H5N1 influenza infection with a 53.3% mortality rate have been reported in 16 countries.12 The increasing incidence of H5N1 influenza in birds also increases the opportunities for direct infection of humans and, subsequently, the risk of transmission from person to person. Quick and timely on-site detection is vitally important to subsequently, the risk of transmission from person to person.

Laboratory diagnosis of AIV infection is usually based on established “gold standard” viral culture and genetic detections using real-time reverse transcription-polymerase chain reaction (RT-PCR). Despite their great sensitivity, these methods are time-consuming and labor-intensive, requiring a week or more for completion, and they need specially trained workers and facilities.8,13−22 Other popular methods include antibody-based immunoassays such as conventional enzyme-linked immunosorbent assay (ELISA).23−28

Currently, on-site disposable detection systems are thought to be the best option to prevent infection spread. They offer the advantages of not requiring sample transport and avoiding exposure to the virus sources and subsequent equipment decontamination.29 We previously reported a microdevice for immunoassay using a three-dimensional photopolymer structure (immunowall) fabricated in a microchannel, called the immunowall device. The immunowall device provides a fast, easy, and highly sensitive detection of disease biomarkers.30−32 In the immunowall device, a biotinylated capture antibody is immobilized on the wall structure surface through biotin−streptavidin (SA) interaction. Introducing an analyte and secondary and labeled antibodies produces a sandwich complex anchored on the sides of the wall. To improve the sensitivity, an enzyme label was used, and the signal was amplified by the enzymatic reaction with a fluorogenic substrate.32

In this work, we carried out the detection of H5-HA from AIV using the immunowall device. A comparison between fluorescence-labeled and enzyme-labeled antibodies was made to achieve a highly sensitive detection. The performance was contrasted with that of a conventional ELISA kit. Also, the selectivity was evaluated for different HA proteins: H1, H3, H5, and H7.

**EXPERIMENTAL SECTION**

**Materials and Reagents.** An azide-unit pendant water-soluble photopolymer (AWP, 6%) was purchased from Toyo Gosei Co., Ltd. (Tokyo, Japan). SA was purchased from ProSpec-Tany TechnoGene Ltd. (East Brunswick, NJ). Bovine serum albumin (BSA), skim milk powder, hydrochloric acid, and magnesium chloride hexahydrate were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Trizma base and Tween 20 were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO). Phosphate-buffered saline (PBS; pH 7.4) solution and 9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-y1) phosphate (DDAO phosphate) diammonium salt were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA). Mouse anti-H5N1 HA monoclonal antibody, recombinant H5N1 HA, recombinant H1N1 HA, recombinant H3N2 HA, recombinant H7N9 HA, and rabbit anti-avian influenza A HA polyclonal antibody were purchased from Sino Biological, Inc. (Beijing, China). DyLight 650-labeled goat antirabbit polyclonal antibody and alkaline phosphatase-labeled goat antirabbit polyclonal antibody were purchased from Abcam (Cambridge, MA). The biotin labeling kit-NH$_3^+$ was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan).

**Fabrication of the Immunowall Device.** Figure 1 shows a photograph and schematic representation of a microchip and the evaluated immunowall device. The microchip was fabricated by Sumitomo Bakelite Co., Ltd. (Tokyo, Japan) from a cyclic olefin copolymer and had 40 microchannels. The immunowall had a width of 40 μm and was fabricated inside a microchannel using a mixture of a water-soluble photopolymer (AWP) and SA. The AWP/SA ratio was 5:5 for the fluorescence-labeled antibody and 7:3 for the enzyme-labeled antibody.

![Figure 1](image-url)
Assay Procedure. The volume of each solution injected into a microchannel was 0.25 μL. First, a concentration of 50 μg/mL of biotinylated mouse anti-H5N1 HA monoclonal antibody (capture antibody) in PBS containing 0.5% of BSA (0.5% BSA–PBS) was injected into a microchannel and incubated for 1 h. Next, a standard of recombinant HS-HA protein in PBS was added with an incubation time of 15 min. Then, 1% of skim milk in PBS (blocking solution) was added and incubated for 5 min (this step is only required when the alkaline phosphatase-labeled antibody is employed). This blocking process avoided the nonspecific adsorption of the detection and labeling antibody on the immunowall surface. Subsequently, anti-AIV H5-HA antibody (detection antibody) (50 μg/mL) in 0.5% BSA–PBS was injected and incubated for 30 s. Finally, DyLight 650-labeled antibody or alkaline phosphatase-labeled antibody (labeling antibody) (50 μg/mL) in 0.5% BSA–PBS was injected and incubated for 30 s. After each incubation at room temperature and humidity conditions, the solution was removed by a vacuum pump, and the immunowall was washed five times with a washing buffer. The final washing buffer injection was not removed from the microchannel.

For the experiments with the alkaline phosphatase-labeled antibody, an extra step was required: the immunowall device was placed on a fluorescence microscope stage, and 30 μM of the fluorogenic substrate (DDAO phosphate) in Tris-HCl buffer (pH 8.0, 1 μM Mg2+) was injected into the microchannel. The enzymatic reaction was carried out in the dark at room temperature for 30 min.

Image Capture and Analysis. The immunowall device was placed on the stage of an inverted fluorescence microscope (BZ-9000; Keyence; Tokyo, Japan) with a filter cube Cy5 filter (OP-87766; excitation 620/60 nm, emission 700/75 nm; Keyence; Tokyo, Japan). Fluorescence images were taken at 10, 20, and 30 min for alkaline phosphatase-labeled antibody assay (CCD camera exposure time was 0.5 s) and without any time interval for DyLight 650-labeled antibody assay (CCD camera exposure time was 7 s). For each standard, the assay was performed in triplicate.

The fluorescence intensity of the captured images was measured through ImageJ software. Figure 2 shows typical fluorescence images. The detection area, 20 pixels × 1000 pixels or 100 pixels × 1000 pixels, was adjusted manually depending on the assay (red dotted line in Figure 2).

RESULTS AND DISCUSSION

H5-HA Detection with Fluorescence-Labeled Antibody. The conventional immunowall method30 was employed to prepare a calibration curve for the H5-HA standard in PBS with the DyLight 650-labeled antibody for a concentration range between 1 and 100 ng/mL (Figure 3). In this case, the fluorescence-labeled antibody was immobilized on both sides of the immunowall, where fluorescence was shown (Figure 2a). The limit of detection (LOD), 10.7 ng/mL, was determined considering the average of the blank signal plus 3 standard deviations. Thus, the detection range of H5-HA was from 10.7 to 100 ng/mL, with a total incubation time of 16 min. This detection method is known to be faster but not as sensitive as the conventional one. Therefore, the signal amplification with the enzyme-labeled antibody was tested in the next experiment to achieve higher sensitivity.

H5-HA Detection with Enzyme-Labeled Antibody. In this analysis mode, fluorescent molecules were generated by an enzymatic reaction in the presence of an excess amount of substrate (DDAO phosphate). The diffusion of the fluorescent enzymatic product (DDAO) into the immunowall induced fluorescence in the entire wall area (Figure 2b). Our previous research has suggested that DDAO molecules could be accumulated inside the wall and could efficiently magnify the fluorescence intensity.32 First, a standard solution of H5-HA in PBS (20 ng/mL) was used as a model to probe the fluorescence intensity variation during 30 min. It was found that the fluorescence intensity considerably increased as a function of time (Figure 4a), and the value of the signal/blank ratio (S/B) because of the DDAO accumulation inside the immunowall was slightly improved.

Different solutions of the H5-HA standard in PBS (concentration range 0.1–100 ng/mL) were measured with the alkaline phosphatase-labeled antibody and the DDAO phosphate substrate. The resulting calibration curves are shown in Figure 4b. Although the fluorescence intensity increased over time, the behavior of the calibration curves was the same. The LODs, calculated by considering the
The immunowall device with an enzyme-labeled antibody is a good option for future rapid and sensitive detection using a very low volume of sample. **Selectivity Experiments.** The selectivity experiments were carried out with four types of HA proteins H1, H3, H5, and H7 in the immunowall device with fluorescence-labeled and enzyme-labeled antibodies. Solutions of each standard at 100 ng/mL in PBS (fluorescence-labeled antibody) and 20 ng/mL in PBS (enzyme-labeled antibody) were tested and compared against a blank signal. The normalized values of fluorescence intensity were calculated considering the average of the H5-HA signal as 1. The results are shown in Figure 5.

Among the different HA protein subtypes, H5-HA was the only one that presented a significantly different signal to the blank. From these results, it was concluded that the immunowall device with an enzyme-labeled antibody is a promising option for future AIV H5-HA virus detection.

**CONCLUSIONS**

In this work, AIV H5-HA detection using the immunowall device was optimized. Fluorescence-labeled antibody and enzyme-labeled antibody were used as labeling antibodies. Alkaline phosphatase-labeled antibody with DDAO phosphate as the fluorogenic substrate showed an increase in sensitivity because of enzymatic amplification. The LOD of the H5-HA solution in PBS was 0.23 ng/mL (almost 50 times lower than that of the fluorescence-labeled antibody), and the detection range was 0.23–100 ng/mL. Only 31 min were required as incubation time of 31 min.

Table 1 summarizes the results for both the immunowall device analysis mode and the conventional microtiter plate method. The conventional ELISA kit offered the lowest value of LOD (0.08 ng/mL) but had the longest analysis time (260 min). Immunowall devices had the advantages of requiring a very small volume of sample (only 0.25 µL), a shorter assay time, and the fluorogenic substrate showed an increase in sensitivity because of enzymatic amplification. The LOD of the H5-HA solution in PBS was 0.23 ng/mL (almost 50 times lower than that of the fluorescence-labeled antibody), and the detection range was 0.23–100 ng/mL. Only 31 min were required as analysis time with a small volume of sample (0.25 µL). Also, the high selectivity of the optimized method was proved against other subtypes of AIV HAs. The immunowall device with the enzyme-labeled antibody offers a rapid, sensitive, selective, and simple immunoassay system for H5 AIV detection.

Future tasks involve testing to detect H5 AIV from real samples of chickens and other kinds of poultry with the immunowall device. As a short-term goal, the development of a miniaturized detection system with the immunowall device for on-site detection will be pursued.
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