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Rapid detection of anti-H5 avian influenza virus antibody by fluorescence polarization immunoassay using a portable fluorescence polarization analyzer

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ABSTRACT

A rapid, facile and selective detection of anti-H5 subtype avian influenza virus (AIV) antibody in serum by fluorescence polarization immunoassay (FPIA) was achieved. A fragment of recombinant H5 subtype AIV hemagglutinin was produced and labeled with fluorescein to use it as a labeled antigen in FPIA. This labeled antigen was mixed with anti-AIV sera (H1–H16 subtypes) and FP of the mixture was measured using a portable FP analyzer on a microdevice. It was found that FP increased in proportion to the concentration of anti-H5 AIV antibody (serum) and was significantly higher than FP obtained with the other sera. The selective detection of anti-H5 subtype AIV antibody was confirmed. The required volume of original sample was 2 μL and analysis time was within 20 min. This detection system realizes an efficient on-site diagnosis and surveillance of AIV.

1. Introduction

Avian influenza is a poultry disease caused by influenza A virus infection. Influenza A virus has many subtypes based on the type of virus surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Wild aquatic birds are the primary natural host of influenza A virus, and 16 HAs (H1–H16) and 9 NAs (N1–N9) have been found so far in such birds [1,2]. Avian influenza virus (AIV) usually infects intestinal cells of aquatic birds and does not show pathogenicity; however, it can infect poultry and cause pathogenicity [3,4]. The virus that causes a high mortality when poultry were infected is designated as a highly pathogenic avian influenza virus (HPAIV). To date, some H5 and H7 subtype AIVs have been reported to be HPAIVs [5–8]. H5N1 subtype HPAIV, which appeared after 1996, has been reported not only in poultry but also in humans. The mortality rate for humans infected with H5N1 is reported to exceed 50% [9]. If these HPAIVs have acquired human-to-human infectivity, the deaths of many persons world-wide due to a pandemic become a concern.

Rapid initial response for a suspected infection and continuous surveillance are indispensable to prevent poultry epidemics of HPAIV and further human threats [10,11]. When a diagnosis is performed at the site of a disease outbreak, a rapid, simple and accurate antigen detection method is required. On the other hand, in the process from a disease occurrence to its cleanup and under a usual surveillance system, antibody detection to check for previous infections also plays an important role. Even in poultry that do not currently have the virus, the history of past infection can be determined by detecting antibodies. This is essential for investigating the routes of transmission of infectious diseases. As a recent example, antibody tests for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) have received broad attention to prevent the spread of the novel coronavirus disease (COVID-19) [12,13]. Although some antibody tests, such as the agar gel

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immunodiffusion test, hemagglutination inhibition test and enzyme-linked immunosorbent assay are used for the detection of anti-AIV antibody [14–18], these methods require complicated operations and a long reaction time. Therefore, it is impractical to use these methods outside the laboratory. If the method is developed that can rapidly and easily provide antibody test results outside the laboratory, field applications become possible and the surveillance system using the antibody test can be significantly strengthened.

Fluorescence polarization immunoassay (FPIA) is widely used in various fields and it has the degree of polarization (P) as a parameter [19, 20]. Advantages of FPIA compared to other immunoassays are the simplicity and rapidity of its procedures and the short measurement time. FPIA does not need a washing step and the reaction is completed by simply mixing the reagents together. In this study, we developed a system that selectively detects anti-H5 AIV serum by measuring P value of the mixture of an anti-AIV serum and fluorescein-labeled H5 subtype HA glycoprotein using a newly designed microdevice based on our previously developed microdevice with a portable FP analyzer [21–24] (Fig. 1(a)). Besides being portable, our FP analyzer is low cost and has high throughput, and these features make it possible to conduct FPIA outside the laboratory. All procedures from sampling to obtaining measurement results can be completed on-site, and that contributes to a significant increase in efficiency of diagnosis and surveillance.

2. Experimental

2.1. Chemicals

Phosphate-buffered saline (PBS; pH 7.4) solution and Micro BCA Protein Assay Kit were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA). The Sylgard 184 silicone elastomer kit for polydimethylsiloxane (PDMS) microdevice fabrication was purchased from Dow Corning Toray Co., Ltd. (Tokyo, Japan). The PDMS included black silicon rubber to decrease the background signal of fluorescence. A negative photoresist (SU-8 3050) for PDMS microdevice fabrication was purchased from Nippon Kayaku Co., Ltd. (Tokyo, Japan). Isopropyl-β-d-thiogalactopyranoside was used to induce the expression of the partial HA gene. The protein extracted from the bacteria was purified using nickel–nitrilotriacetic acid (Ni–NTA) agarose. The recombinant protein obtained was concentrated using an ultrafiltration membrane, and then dialyzed against PBS. The recombinant protein was identified by Western blotting using anti-H5 mouse serum, and its concentration was measured by the Micro BCA Protein Assay Kit.

2.2. Fluorescein-labeled antigen

As an antigen for the FPIA, recombinant H5 subtype AIV HA (H5-rHA) was produced using the bacterial expression system as we previously described [25] with slight modifications. Briefly, viral RNA was extracted from H5N3 subtype AIV (A/whistling swan/Shimane/499/1983) [26]. The viral RNA was extracted from the virus with Isogen-LS, and then reverse transcribed into cDNA by using SuperScript III Reverse Transcriptase. Amplification of the partial HA gene (aa 284–425), PCR was performed using the TaKaRa Ex Taq and the following primers: 5'-GGCCATGGAACTGGAGTATGGTAACTGT-3' and 5'-GGCCATGCGATTCTATTAAATT-3', which include restriction enzyme site sequences (underlined). The PCR cycle included an initial cycle at 94 °C for 1 min, then 30 cycles at 94 °C for 20 s, 58 °C for 30 s, and 72°C for 2 min, and a final extension at 72°C for 7 min. The PCR product was ligated into the expression vector pET-32b. The expression vector prepared was used to transform the Rosetta-gamiTM 2 (DE3) pLysS, and isopropyl-β-d-thiogalactopyranoside was used to induce the expression of the partial HA gene. The protein extracted from the bacteria was purified using nickel–nitrilotriacetic acid (Ni–NTA) agarose. The recombinant protein obtained was concentrated using an ultrafiltration membrane, and then dialyzed against PBS. The recombinant protein was identified by Western blotting using anti-H5 mouse serum, and its concentration was measured by the Micro BCA Protein Assay Kit. The resultant H5-rHA was labeled using the Lightning-Link Fluorescein Conjugation Kit.

2.3. Anti-AIV sera

The goat antisera against H1–H15 subtypes and the chicken anti-serum against H16 subtype AIV were kindly provided by Dr. Yoshio Sakoda (Hokkaido University, Sapporo, Japan). The immunogen of each serum is shown in Table 1.

2.4. Microfluidic device

The basic PDMS microfluidic device was fabricated using the standard soft lithography technique, as described in our previous work [24]. Briefly, the mold was fabricated from negative photoresist SU-8 3050 and a silicon wafer (Sumco Co., Tokyo, Japan). The negative photoresist SU-8 3050 was spin-coated onto a silicon wafer. The PDMS prepolymer with black silicon rubber was poured onto the mold and cured. The cured PDMS was pasted on a glass slide. Figs. 1(b) and (c) show a photograph and a schematic drawing of the microfluidic device. We designed the microfluidic device for this experiment based on the basic device. The microdevice had nine channels arranged in the field of view of the portable FP analyzer, and nine samples could be measured at one time. The width of the channel was 200 μm and the depth is 900 μm. The depth of the microfluidic device was significantly different from our previous device. By employing a channel having a high aspect ratio, it was possible to increase the fluorescence intensity per unit area and reduce specific surface area. These features contributed to highly sensitive measurement (Fig. S1). The numbers in Fig. 1(c) denote inlets and outlets, and the solution was injected into the inlet with a pipet (Eppendorf, Hamburg, Germany).
2.5. Portable FP analyzer

We used our previously developed portable FP analyzer for FP measurement [24]. This analyzer made the FP measurement based on the principle of synchronization between the orientation of the liquid crystal molecules and the sampling frequency of a CMOS [21–23]. Thus, P value could be obtained as a two-dimensional image. An image of a microdevice captured by the CMOS was processed to produce a P image according to the formula in the literature [21] using home-built image processing software. The portable FP analyzer had dimensions of 65 cm (W 35 cm × D 15 cm × H 15 cm) and a weight of 5.5 kg.

2.6. Measurement procedure

Anti-AIV serum diluted in a prescribed ratio and 640 ng/mL fluorescein-labeled H5-rHA were mixed at a volume ratio of 1:1. The mixture was incubated at room temperature for 15 min. Then, 20 μL of the mixture was injected into the microfluidic device and P value was measured with our portable FP analyzer. The FP analyzer acquired P images in the detection area. The P value measured was determined by reading out the luminance on the P image in the detection area of the microdevice. As shown in Fig. 1 (c), nine channels were placed within the detection area. Therefore, nine samples could be measured simultaneously.

3. Results and discussion

In the FPIA measurement, P value is expressed by the following equation:

\[ P = \frac{I_V - I_H}{I_V + I_H} \]

where \( I_V \) and \( I_H \) are the fluorescence intensities of the vertical and horizontal components of the emitted light, respectively. A free fluorescein-labeled antigen emits random fluorescence when excited by polarized light and it gives a low P value. When a fluorescence-labeled antigen is bound to an antibody, P value is high due to the much slower Brownian motion of the antibody-H5-rHA complex [19,20]. Fig. 2 shows the schematic illustration of FPIA in this work. When anti-H5 antibody in serum binds to a fluorescein-labeled H5-rHA, P value becomes high. Therefore, anti-H5 AIV serum containing a large amount of anti-H5 HA antibodies shows a higher P value than that of the antibody-negative serum. In this work, we used the fragment of H5-rHA (not whole H5-rHA) as an antigen to improve the sensitivity. Using a smaller fluorescein-labeled antigen reduces P value of the blank sample. Fig. 3 shows an example of a P image obtained using our portable FP analyzer. Samples used were a mixture of anti-H5 serum and fluorescein-labeled H5-rHA and a mixture of anti-H14 serum and fluorescein-labeled H5-rHA. Blank indicates only fluorescein-labeled H5-rHA. P value of each sample was calculated by analyzing the luminance value of each channel of this image by home-built image processing software.

First, we confirmed whether the prepared fluorescein-labeled H5-rHA binds with antibodies in anti-H5 AIV serum. Fig. 4 shows P value as a function of incubation time after mixing the fluorescein-labeled H5-rHA and anti-H5 serum. The solution mixture was incubated in a microtube for a certain time before being injected into a microdevice, and P value was measured with our portable FP analyzer. Time 0 means the fluorescein-labeled H5-rHA solution without anti-H5 serum. As the incubation time increased, P value increased and became an almost constant value at 5 min. Therefore, the change of P value due to the changing rotational motion of the fragment of recombinant H5 subtype AIV HA labeled by fluorescein could be detected by our analyzer. In the next experiments, the incubation period was set at 15 min at which time the reaction of the fluorescein-labeled H5-rHA and a serum was sufficiently progressed. Then, Fig. 5 shows the measured P value results after mixing fluorescein-labeled H5-rHA and anti-H5 AIV serum diluted with PBS. P value increased as the serum dilution rate decreased (increased concentration). The more antibodies the serum had, the greater the number of antigen-antibody complexes that were formed in the excess amount of fluorescein-labeled H5-rHA. Increasing P value with increasing concentration of serum meant that antigen-antibody complexes successfully formed. Therefore, we concluded that this fluorescein-labeled H5-rHA could be used for detection of anti-H5 antibody in serum. The limit of detection was about 20-fold dilution. Our microdevice required 20 μL of solution per one channel. Since serum and fluorescein-labeled H5-rHA were mixed at a volume ratio of 1 : 1, the required serum volume for an analysis was 10 μL. When a serum with 5-fold dilution was used for the analysis, the required volume of the original serum was 2 μL. It would be sufficiently small for application to on-site diagnosis and surveillance. Therefore, 5-fold dilution of anti-serum was used in the next experiment.

Next, we verified whether our system could selectively detect anti-H5 AIV serum in anti-H1–H16 AIV sera. Fig. 6 shows the P value obtained with anti-H1–H16 sera. The cutoff value for positive anti-H5 AIV antibody was defined as the average + 3SDs. Anti-H5 serum showed the highest P value among all anti-AIV sera and all other antisera except anti-H5 was below the cut-off value. Therefore, we showed that there was no false positive result with other anti-subtype sera. The incubation time was 15 min (total analysis time was within 20 min), which was significantly shorter than the time needed by conventional ELISA-based devices. These findings showed that our FPIA system selectively detected the anti-H5 AIV serum.

| Table 1  | Immunogens of goat anti-AIV sera. |
|----------|-----------------------------------|
| Serum name | Strain name (subtype) of immunogen AIV | GenBank Accession number |
| H1 | A/swine/Hokkaido/1/1981 (H1N1) | HA gene: AB434392 |
| H2 | A/duck/Hong Kong/278/1978 (H2N9) | HA gene: AB292785 |
| H3 | A/duck/Hokkaido/5/1977 (H3N2) | HA gene: AB277754 |
| H4 | A/duck/Czechoslovakia/1956 (H4N6) | HA gene: GU552381 |
| H5 | A/duck/Hong Kong/320/1980 (H5N3) | HA gene: LC042027 |
| H6 | A/shearwater/Australia/1/1972 (H6N5) | HA gene: AB278600 |
| H7 | A/duck/Hong Kong/301/1978 (H7N2) | HA gene: AB302789 |
| H8 | A/turkey/Ontario/6118/1967 (H8N4) | HA gene: GU053171 |
| H9 | A/duck/Hong Kong/448/1978 (H9N2) | HA gene (partial): AB080224 |
| H10 | A/chicken/Germany/N/1949 (H10N7) | HA gene: GG176136 |
| H11 | A/duck/England/1/1956 (H11N6) | HA gene: GU052203 |
| H12 | A/duck/Alberta/60/1976 (H12N5) | HA gene: CY130078 |
| H13 | A/gull/Maryland/704/1977 (H13N6) | HA gene: CY130086 |
| H14 | A/mallard/Astrakhan/263/1992 (H14N5) | HA gene: CY130094 |
| H15 | A/duck/Australia/341/1983 (H15N8) | HA gene: AB295613 |
| H16 | A/black-headed gull/Sweden/5/1999 (H16N3) | HA gene: AY684891 |
4. Conclusions

We developed the rapid and easy-to-use system for the FPIA detection of anti-H5 AIV serum. Analysis time was within 20 min and required sample volume was only 2 μL. Our portable FP analyzer with the microfluidic device makes it possible to conduct on-site analysis. Further study on blocking of protein adsorption to PDMS devices may lead to improved sensitivity and selectivity of a detection. By designing fluorescence-labeled antigen, this system can be applied to other viruses such as SARS-CoV-2. We may safely say that this system for antibody detection is promising for rapid screening of target viruses in the field.
detection is ideal method for controlling infectious diseases by testing large numbers of samples. In the future, we plan to conduct experiments using real samples acquired in the field.

Author contributions

KN, YT, KS, AH, YY, KI, HO and MT conceived and designed the study. KN and YT contributed to data collection and analysis of data. KN, YT, MM, AI, HT, KS, AH, YY, KI, HO and MT joined discussion and provided constructive suggestions. KN and YT wrote the initial draft of the manuscript. MM, AI, HT, KS, AH, YY, KI, HO and MT critically reviewed and made improvements in the manuscript. All authors approved the final version of the manuscript.

Declaration of Competing Interest

There are no conflicts to declare.

CRediT authorship contribution statement

Keine Nishiyama: Conceptualization, Methodology, Investigation, Writing - original draft. Yohei Takeda: Conceptualization, Methodology, Investigation, Writing - review & editing. Masatoshi Maeki: Methodology, Writing - review & editing. Akihiko Ishida: Methodology, Writing - review & editing. Hirofumi Tani: Methodology, Writing - review & editing. Koji Shigemura: Supervision, Software, Validation, Resources. Akihide Hibara: Supervision, Software, Validation. Yutaka Yonezawa: Conceptualization, Resources, Validation. Kunitoshi Imai: Conceptualization, Resources, Validation. Haruko Ogawa: Conceptualization, Supervision, Project administration, Funding acquisition. Manabu Tokeshi: Conceptualization, Supervision, Project administration, Funding acquisition.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.snb.2020.128160.
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