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COMMUNICATION

Non-competitive fluorescence polarization immunoassay for detection of H5 avian influenza virus using a portable analyzer

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Abstract

Nowadays, the diagnosis of viral infections is receiving broad attention. We have developed a non-competitive fluorescence polarization immunoassay (NC-FPIA), which is a separation-free immunoassay, for a virus detection. H5 subtype avian influenza virus (H5-AIV) was used as a model virus for the proof of concept. The fluorescein-labeled Fab fragment that binds to H5 hemagglutinin was used for NC-FPIA. The purified H5-AIV which has H5 hemagglutinin was mixed with the fluorescein-labeled Fab fragment. After that, the degree of fluorescence polarization was measured with a portable FPIA analyzer. H5-AIV was successfully detected with an incubation time of 15 min. In addition, the portable FPIA analyzer enables performance of on-site NC-FPIA with a sample volume of 20 μL or less. This is the first research of detecting a virus particle by FPIA. This NC-FPIA can be applied to rapid on-site diagnosis of various viruses.

Keywords Fluorescence polarization immunoassay · Avian influenza · Virus detection · Rapid diagnosis

Introduction

The outbreak of infectious diseases caused by viruses has a great impact not only on the health of humans and animals but also on social and economic aspects. For example, the emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in 2019 which causes COVID-19 has resulted in many deaths worldwide and has had a great economic impact [1, 2]. Also, epidemics of serious animal infectious diseases such as avian influenza and African swine fever may cause catastrophic damage to livestock industries [3, 4]. Fast initial response for a suspected infection is essential to minimize the damage caused by such viral infections [5]. Therefore, a rapid and highly sensitive method for the diagnosis of viral infections is critically required. The detection of viral nucleic acid by a polymerase chain reaction (PCR) is used as a gold standard method for the diagnosis of viral infections [6–8]. PCR testing has good sensitivity and

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specificity and is suitable for definitive diagnosis. However, PCR testing requires complicated procedures and long analysis times, conditions which are not suitable for an initial diagnosis. On the other hand, simple analytical methods for detecting antigens and antibodies by lateral flow immunoassay (LFIA) have been developed [9–12]. Typical LFIA devices with visual observation do not have high accuracy and sensitivity. In order to obtain high sensitivity with LFIA devices, expensive materials such as metal nanoparticles and quantum dots, and read-out equipment are often required for higher sensitivity. However, these methods compromise the simplicity and inexpensiveness of LFIA. To construct an appropriate detection system for the initial diagnosis of a viral infectious disease, we have developed a novel virus detection method using fluorescence polarization immunoassay (FPIA) to enable rapid on-site detection of the virus.

FPIA is one example of a homogeneous immunoassay. The difference in rotational movement due to the antibody binding to the fluorescence-labeled antigen is measured as the change in the degree of fluorescence polarization (P) [13]. Since FPIA does not require bound-free separation, it has the advantage that the assay procedure is rapid and simple compared to heterogeneous immunoassays like enzyme-linked immunosorbent assay (ELISA). Conventional FPIA is a competitive immunoassay, which uses a fluorescence-labeled target (tracer) and an antibody as reagents. Most applications of conventional competitive FPIA have been for quantifying small molecules such as drugs and mycotoxins [13]. Using the advantages of FPIA, we have developed a portable FPIA analyzer that can measure multiple samples simultaneously on site with a small amount of sample [14–16]. In addition, recently, we have demonstrated non-competitive FPIA (NC-FPIA) using a nanobody [17] or a Fab fragment [18] for proteins to expand the applications of FPIA. On the other hand, the detection of virus particles by FPIA has not been reported so far. Shokri et al. [19] reported the competitive fluorescence anisotropy immunoassay for the capsid protein of citrus tristeza virus. However, the pretreatments of sample and long incubation time were needed in their method. If NC-FPIA using our portable FPIA analyzer can be applied to a virus particle detection, it will greatly contribute to speeding up the diagnosis of viral infectious diseases. NC-FPIA requires adding only a fluorescence-labeled antibody fragment and short incubation time. Furthermore, unlike ELISA, FPIA has the advantage of being easy to apply to various animal species because FPIA does not require a suitable secondary antibody for each animal species.

In this paper, we applied NC-FPIA using the Fab fragment to virus detection. The H5 subtype avian influenza virus (H5-AIV) was used as a model virus for the proof of concept. AIV is a bird-adapted influenza A virus and this virus is characterized by two membrane proteins, hemagglutinin (HA) and neuraminidase (NA) [20]. There are several highly pathogenic strains of H5-AIV, and human infections have been reported. Therefore, rapid initial response is required when an H5-AIV infection is suspected [21]. For the detection of H5-AIV, we prepared a fluorescein-labeled Fab fragment (F-Fab) and used it for NC-FPIA. After the F-Fab was reacted with H5-HA on the H5-AIV surface, the P value was measured with a portable FPIA analyzer. As far as we know, this is the first report of virus detection by FPIA.

Materials and methods

Materials

H5N1 hemagglutinin polyclonal antibody (anti-H5-HA rabbit IgG polyclonal antibody) was purchased from Bioss Antibodies (USA). Albumin from bovine serum (BSA) was purchased from FUJIFILM Wako Pure Chemical Corporation (Japan). Fluorescein labeling kit-NH_2 was purchased from Dojindo Molecular Technologies, Inc. (Japan). The Micro BCA Protein Assay Kit and phosphate-buffered saline (PBS) were purchased from Thermo Fisher Scientific, Inc. (USA). Polydimethylsiloxane (PDMS; SILPOT 184 W/C) was purchased from Dow Corning Toray Co., Ltd. (Japan). The PDMS included black silicon rubber to decrease the background signal of fluorescence. SU-8 3050 and the SU-8 developer were purchased from Nippon Kayaku Co., Ltd. (Japan). Rosetta-gami™ 2 (DE3) pLysS Competent Cells and expression vector pET-32b (+) were purchased from Novagen (USA). SuperScript® III Reverse Transcriptase was purchased from Invitrogen (USA). Isogen-LS RNA extraction reagent was purchased from Nippon Gene (Japan). Isopropyl-β-d-thiogalactopyranoside was purchased from Wako Pure Chemical Industries (Japan). TaKaRa Ex Taq was purchased from TaKaRa (Japan). Nickel–nitrilotriacetic acid (Ni–NTA) agarose was purchased from Qiagen (Germany).

Preparation of antigen

Recombinant HA of H5-AIV (H5-rHA), which is derived from H5N3 subtype AIV (A/whistling swan/Shimane/499/1983), was produced using the bacterial expression system as we previously described [16]. The purification of H5N3 subtype AIV (A/duck/Hong Kong/820/1980) and H1N1 subtype influenza A virus (A/Puerto Rico/8/34) was performed as previously described [22]. Shimane strain and Hong Kong strain were kindly provided by Dr. Toshihiro Ito (Tottori University, Japan) and Dr. Yoshihiro Sakoda (Hokkaido University, Japan), respectively. A/Puerto Rico/8/34 strain was purchased from ATCC (Manassas, VA: Catalog No. VR-95™). The concentration of H5-rHA and purified H5N3 and H1N1 viruses was measured by the Micro BCA Protein Assay Kit.

Preparation of F-Fab

Anti-H5-HA Fab fragment was purchased from Hokudo Co., Ltd. (Japan). Fab fragment was produced by the papain digest of commercial anti-H5-HA rabbit IgG polyclonal antibody (Bioss antibodies). Labeling of fluorescein on the amino groups of the Fab fragment was conducted following the procedures described in the manufacturer's instructions of the fluorescein labeling kit-NH_2. Unreacted fluorescent molecules were separated using a modified polyethersulfone membrane (Nanosep 3 K Omega, Pall Corporation, USA). Concentration of Fab fragment and fluorescein was determined based on absorbance at 280 nm and 500 nm acquired with a fluorometer (NanoDrop One, Thermo Fisher Scientific, Inc.). The labeling rate (fluorescein concentration (M)/Fab fragment concentration (M)) was 1.45.

PDMS microdevice fabrication

The PDMS microdevice was designed and fabricated by the standard soft lithography technique, in accordance with the literature [16]. In short, the mold was fabricated from negative photoresist SU-8 3050 and a silicon wafer (Sumco Co., Japan). The mixture of PDMS prepolymer with black silicon rubber and the cross-linking agent was poured onto the mold. After 24 h of curing at room temperature, the PDMS was removed from the master mold by peeling off and cutting.

Assay procedure

NC-FPIA was demonstrated using a portable FPIA analyzer with a microfluidic device (Fig. S1) [15, 16]. Antigen (purified virus or H5-rHA) in PBS, 80 ng/mL F-Fab, and 1% BSA in PBS were added to a microtube. The mixing volume ratio was antigen:F-Fab:1% BSA-PBS = 8:1:1. BSA was used as a blocking agent and reduced the variation by non-specific adsorption (Fig. S2). The mixture was incubated at room temperature for 15 min. Then, 20 μL of the mixture was injected into the microfluidic device and the P value was measured with our portable FPIA analyzer. All data points are means ± standard deviations ($n = 3$).

Results and discussion

We constructed a NC-FPIA with a portable FPIA analyzer for virus detection. Unlike conventional competitive FPIA, only fluorescence-labeled Fab is used as the reagent. F-Fab was added to a virus sample and the P value was measured after incubation (Fig. 1). If we apply conventional competitive FPIA to a virus, antigens that competitively react with the antibody are needed as a tracer. The smaller the molecular weight of the tracer is, the greater the difference in the P value before and after binding to the antibody is, resulting in high sensitivity. Therefore, it is desirable to use only a part of the virus like a membrane protein as a tracer. However, it is difficult to react a small tracer and a virus to the antibody competitively because the diffusion coefficient in the solution

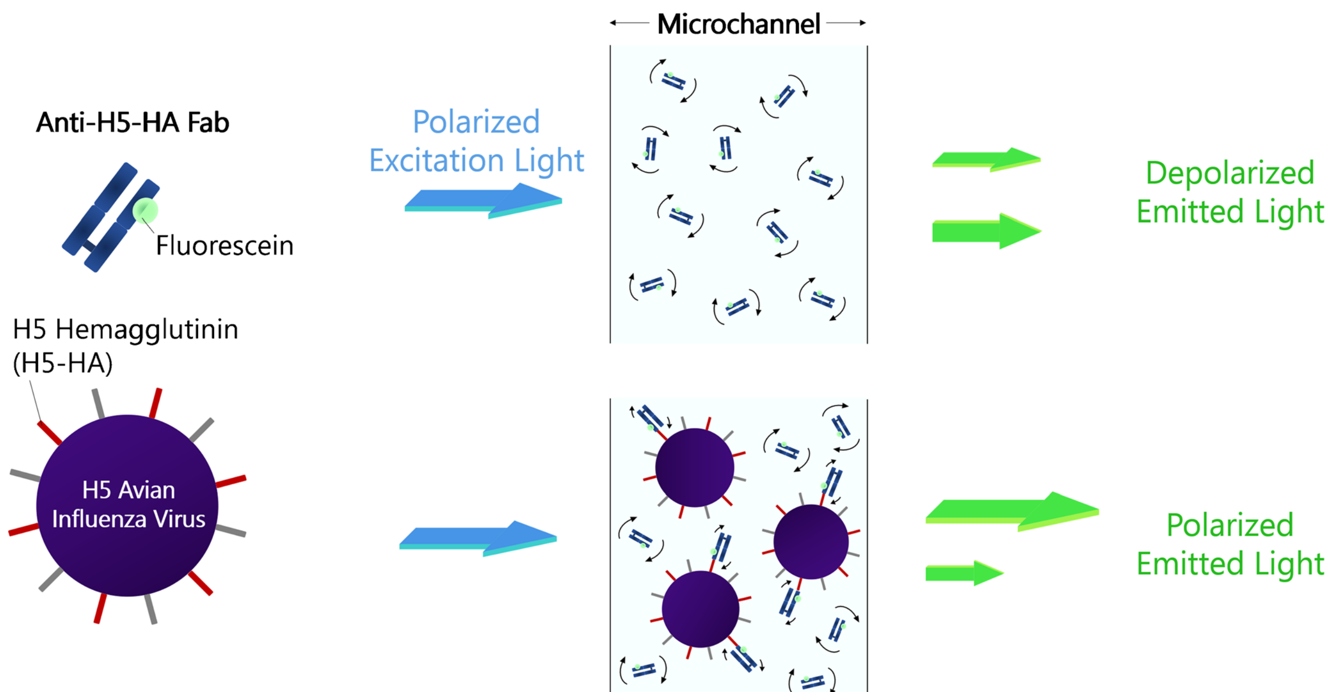

Fig. 1 Schematic illustration of the NC-FPIA using F-Fab for H5-AIV quantification

greatly differs between the small tracer and the virus particle. In our proposed NC-FPIA, only F-Fab was used as a reagent. When a F-Fab is bound to a virus, the *P* value becomes high due to the much slower rotational motion of the virus-Fab complex. High detection sensitivity can be achieved by using a Fab fragment (~50 kDa) instead of the commonly used IgG antibody (~150 kDa) [18].

We prepared a calibration curve for the purified H5N3 AIV (Fig. 2). The *P* value increased with increasing virus concentration, and the detection limit was 2.8 μg/mL. The difference of the *P* value between the maximum value in this calibration curve and blank was 13.0 mP. We clarified that the Fab-based NC-FPIA can detect viruses as well as protein targets with equivalent sensitivity [17]. It is possible to change the measurable concentration range by changing of the F-Fab concentration or using a Fab fragment with different affinity to H5-HA. Then, we evaluated the selectivity of F-Fab for H5-AIV. Figure 3 shows the results of the selectivity test for H5-AIV using the purified H1N1 virus as a negative control. The *P* value after reaction between F-Fab and H5N3 AIV was significantly higher than that of blank and H1N1 virus. In addition, it was confirmed that F-Fab had the high affinity for H5-rHA (Fig. S3). These results indicated that F-Fab had high selectively to H5-HA. To achieve the detection of H5-AIV with much higher sensitivity and selectively, using the Fab fragment which has a higher selectivity and affinity for H5-HA is ideal. However, the present results are promising in that Fab-based NC-FPIA has a greater possibility for achieving the rapid on-site diagnosis of viral infectious diseases.

From the above results, we demonstrated that AIV can be detected by NC-FPIA with an incubation time of 15 min by using a Fab fragment that selectively binds to a protein on the virus surface. Also, since a portable FPIA device is used for measurement, the sample volume required for one assay is 20 μL or less. By preparing a suitable fluorescently labeled Fab fragment, this method can be applied to various viral infectious diseases like COVID-19.

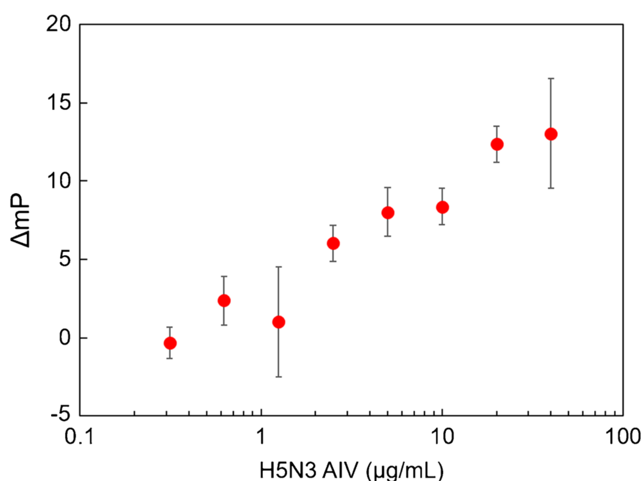

Fig. 2 Calibration curve for the purified H5N3 AIV in PBS. F-Fab concentration: 80 ng/mL. $\Delta mP = 1000 \times (P_{sample} - P_{blank})$

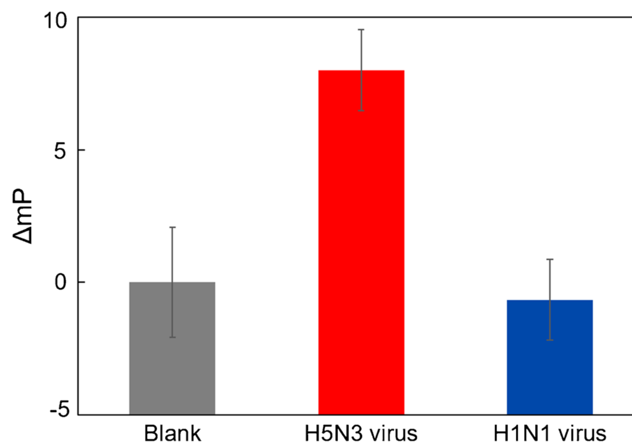

Fig. 3 Selectivity of the F-Fab for NC-FPIA of H5-virus. H1N1 virus was used as a negative control. Concentrations of H5N3 virus and H1N1 virus were each 5 μg/mL. $\Delta mP = 1000 \times (P_{sample} - P_{blank})$

Conclusions

In this paper, we demonstrated that H5-AIV can be detected by NC-FPIA using Fab fragment. The reaction is completed by simply mixing the F-Fab with the sample, and the portable FPIA device enables rapid virus detection on site. Detection with high sensitivity and selectively by this method can be achieved by using a Fab fragment which has a high affinity and selectively. The use of smaller antibody fragments such as a nanobody and a single-chain fragment variable is also considered to be effective in improving sensitivity. In the future, we plan to optimize the assay conditions in detail and evaluate the performance with field samples. Our research group has also demonstrated the possibility of antibody testing using a portable FPIA analyzer [16]. Rapid on-site antigen and antibody tests for viral infectious diseases on the same platform will be implemented in the future.

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Declarations

Conflict of interest The authors declare no conflicts of interest.

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