A fluorescence polarization immunoassay for the rapid detection of antibody against influenza A virus in chicken and goat sera

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Abstract. Highly pathogenic influenza A viruses (IAVs) cause substantial damage to the poultry industry. A simple and quick testing method is required for strict control of this infectious agent. The fluorescence polarization immunoassay (FPIA) is a rapid test based on antigen–antibody binding, which can detect antigen-specific antibody in the infected animal samples within a few minutes. FPIA is a one-step reaction assay that does not require a secondary antibody and complicated steps. We evaluated the usefulness of FPIA for the detection of anti-IAV antibodies, including those against internal proteins and H5 subtype HA, in sera. In the FPIA using fluorescent peptides of internal NP and M1 proteins, millipolarization units (MPUs), which increase depending on the amount of antibody, were higher in antibody-positive sera than in antibody-negative sera. Moreover, in FPIA using fluorescent recombinant H5 subtype HA proteins, anti-H5 serum gave the highest MPUs among the antisera raised in goats against individual H1–H5 subtype IAVs. Our results support the utility of FPIA for the detection of anti-IAV antibodies, especially the anti-H5 antibody.

Key words: fluorescence polarization immunoassay; influenza A virus; rapid testing method.

Introduction

Transboundary animal diseases (TADs) are highly contagious animal diseases that spread rapidly around the world. The livestock industry suffers critical economic damage with the spread of TADs across a nation, and eradication of pathogens has become a difficult task (<http://www.fao.org/emergencies/emergency-types/transboundary-animal-diseases/en/>). To prevent the spread and lessen the damage of TADs, rapid testing methods are needed for the identification of infected animals.

Fluorescence polarization immunoassay (FPIA) is an affinity-based immunoassay, whose basic principle was first devised in 1926.¹² In FPIA, the fluorescence-labeled antigen (Ag) is used as a tracer for the detection of antibody (Ab), which specifically binds to a tracer Ag. A tracer rotates in a solution. The rotation rate of the tracer is high when the tracer Ag is mixed with a sample such as Ab-negative serum. However, the rotation rate of the tracer mixed with an Ab-positive sample decreases because its molecular size becomes larger by the binding of Ab and Ag. The tracer is excited by a plane-polarized light beam, and the FP value is measured. The FP value is inversely correlated with the rotation rate, thereby indicating the absence or presence of Ab in the sample.⁹ FPIA is a one-step assay; it does not require a secondary Ab and washing procedure. It enables a simple and rapid (within a few minutes) detection of pathogen-specific Ab in the tested sample. Moreover, Ag can be

detected by competitive FPIA in which Ag-specific Ab and tracer are added to a sample.^{7,13}

Highly pathogenic avian influenza (HPAI) is one of the TADs of domestic poultry caused by the HPAI virus (HPAIV; *Orthomyxoviridae*, *Alphainfluenzavirus*, *Influenza A virus*). Avian influenza A virus (IAV) spreads globally as a result of the international trade of poultry and the global movement of migration of wild aquatic birds, which are the natural hosts of avian IAV.⁶ Avian IAVs are classified into many subtypes by the antigenicity of hemagglutinin (HA; H1–H16) and neuraminidase (N1–N9) surface glycoproteins. It is widely known that low pathogenicity AIV (LPAIV) of H5 and H7 subtypes can convert into HPAIV strains.¹ The HPAIV strains, such as the H5N1 viral subtypes, may cause severe disease with high mortality in the poultry industry and may cause illness and death in humans who live or work closely

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with the birds (https://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/). Considering these conditions, the monitoring of H5 and H7 subtype avian IAVs is essential. The development of a rapid testing method for the detection of virus and antiviral Ab in infected animals is required to strictly control the spread of these virus subtypes.

We evaluated the utility of FPIA for the detection of anti-H5 avian IAV Ab in sera. For strict HPAI control, the dual testing of Abs against all subtypes and H5- or H7-specific subtypes is needed. Additionally, when avian IAV infections occur, timely detection of H5 or H7 subtype viral Ags is crucial. We describe herein the development of a simple and rapid FPIA for the detection of Abs against avian IAVs.

Materials and methods

Virus and serum samples

H5N3(Shimane) and H5N3(Hong Kong) avian IAV strains are LPAIVs (Suppl. Table 1) that belong to the Am-nonGsGD (American lineage) and EA-nonGsGD (Eurasian lineage) clades, respectively.² The H5N3(Shimane) strain was kindly provided by Dr. Toshihiro Ito (Tottori University, Tottori, Japan). The HA gene sequence of the H5N3(Shimane) strain (Suppl. Fig. 1) was obtained using next-generation sequencing, which was kindly performed by Drs. Tetsuya Mizutani and Tsutomu Omatsu (Tokyo University of Agriculture and Technology, Tokyo, Japan).⁸ The goat antisera against IAVs of H1–H15 subtypes (Suppl. Table 1) were kindly provided by Dr. Yoshihiro Sakoda (Hokkaido University, Sapporo, Japan). Control (unimmunized) specific pathogen-free chicken serum, H4N6(Shiga)-immunized chicken serum (14 d after immunization), and H9N2(Yokohama)-immunized chicken serum (28 d after immunization) were kindly provided by the National Institute of Animal Health (Tsukuba, Japan). H5N3(Shimane)-immunized mouse serum (2–4 wk after immunization) was obtained in our laboratory.

Peptides and recombinant proteins

The NP and M1 peptides and recombinant HA proteins were prepared as Ags for the measurement in ELISA and FPIA. NP_{380–393} (ELRSRYWAIRTRSG) and M1_{61–72} (GFVFTLTVPSEK) peptides were purchased from AnaSpec. These NP and M1 peptides are epitopes that are restricted to major histocompatibility complex class I^{11,14} and class II,⁴ respectively. Because a high-molecular-weight protein, such as full-length HA, is not suitable as a tracer in FPIA, partial recombinant HA proteins were prepared. The H5N3(Shimane)-derived recombinant partial HA proteins, which were named H5(Shimane)-HA1, -HA2, and -HA3 (Suppl. Fig. 2), were prepared as follows. Viral RNA was isolated (ISOGEN-LS; Nippon Gene), and reverse transcription was performed

(SuperScript III reverse transcriptase; Invitrogen). With the use of the prepared cDNA, the HA1, HA2, and HA3 regions were amplified by PCR (Ex Taq, TaKaRa; Suppl. Table 2). The PCR product was inserted into the pET-32b expression vector (Novagen); the vector was then introduced into the Rosetta-gami2 Competent Cell (Novagen). Vector-derived protein was produced in the presence of 0.4 mM of isopropyl- β -D-thiogalactopyranoside (Wako), and the protein was purified using Ni-NTA agarose (Qiagen). The fraction containing the target protein with a size of ~30 kDa, which is the molecular weight suitable for use as a tracer in FPIA, was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting using H5N3(Shimane)-immunized mouse serum as the first Ab. The concentration of the recombinant protein prepared was measured (Micro BCA protein assay kit; Thermo Fisher Scientific).

ELISA

In ELISA, with the use of NP and M1 peptides as Ags, 2 μ g/mL of peptide solution was prepared. The carbonate buffer (pH 9.6) was used as a solvent, and 50 μ L of peptide solution was added into each well to coat the 96-well plate (Immobilizer; Nunc). This plate was held at 4°C for 18 h. Blocking took 1 h at 25°C with PBS containing 1% alkali-soluble casein (Wako), and then sera from 21 controls (unimmunized) and 5 H4N6(Shiga)-immunized and 2 H9N2(Yokohama)-immunized chickens were diluted 100–12,800 times and added to the Ag-coated plate. A total of 0.3 μ g/mL of horseradish peroxidase (HRP)-conjugated anti-chicken IgG Ab (Rockland) was used as the secondary Ab. After the addition of TMB substrate reagent (BD Biosciences), absorbance was measured at 450 nm. Using H5(Shimane)-HA1–HA3 as Ags, 2.5 μ g/mL of the protein solution was used to coat the 96-well plate. Goat antisera raised against H1–H15 subtypes of avian IAV were diluted 400 times in the blocking buffer. HRP-conjugated anti-goat IgG Ab (Jackson ImmunoResearch Laboratories) was used as the secondary Ab in this case.

FPIA

NP and M1 peptides, as well as H5(Shimane)-HA1–HA3 proteins, were labeled (Lightning-Link fluorescein conjugation kit, Innova Biosciences; Alexa fluor 488 protein labeling kit, Invitrogen, respectively). In FPIA, using fluoro-NP and -M1 peptides as the tracers, 50 μ L of fluoropeptide diluted in PBS (pH 7.4) was added to the black 96-well polystyrene plate (Nunc). The concentration of diluted fluoropeptides was 4.0–1,000 ng/mL. Sera from 21 controls (unimmunized) and 5 H4N6(Shiga)-immunized and 2 H9N2(Yokohama)-immunized chickens were diluted in PBS. The dilution rate of the sera was 100–200 times. PBS (50 μ L) or the diluted chicken sera were added to the black 96-well plate and mixed with the tracers. The final concentration of fluoropeptides

Figure 1. Detection of anti-avian IAV Ab in chicken serum by fluorescence polarization immunoassay (FPIA) using fluoro-NP and -M1 peptides (peps). **A.** ELISA with NP or M1 pep was conducted on the H9N2(Yokohama)-immunized chicken serum. Control (unimmunized) serum served as a negative control. The concentration of NP pep was 2 µg/mL. The sera were diluted 100–12,800 times. **B.** FPIA with fluoro-NP or -M1 pep was conducted on the H9N2(Yokohama)-immunized chicken serum. PBS and control serum served as the negative controls. Equal volumes of fluoropeptide and serum were mixed. The concentration of fluoro-NP and -M1 peps was 2.0–500 ng/mL in the mixture. The dilution rates of sera mixed with fluoro-NP and -M1 peps were 200 times and 400 times, respectively, in the mixture. The results represent 2 similar experiments. **C.** ELISA with NP pep was conducted on 21 controls and 5 H4N6(Shiga)-immunized and 2 H9N2(Yokohama)-immunized chicken sera. The concentration of NP pep was 2 µg/mL. The sera were diluted 100 times. **D.** FPIA with fluoro-NP pep was conducted on control and H4N6(Shiga)-immunized and H9N2(Yokohama)-immunized chicken sera. The concentration of fluoro-NP pep was 50 ng/mL in the mixture. The sera were diluted 200 times in the mixture. A Mann–Whitney U test was performed to determine statistical significance. ** $p < 0.01$.

was 2.0–500 ng/mL in the mixture. The final dilution rate of the sera was 200–400 times in the mixture. In FPIA, using fluoro-H5(Shimane)-HA1–HA3 proteins as the tracers, equal volumes (50 µL) of diluted fluoroprotein and diluted anti-H1–H15 Ab-positive goat sera were mixed. The final concentration of fluoroproteins in the mixture was 313 ng/mL. The final dilution rate of the sera in the mixture was 20 times. After a 5-min reaction at room temperature, FP was measured (GENios Pro microplate reader; Tecan), and millipolarization units (MPUs) were calculated (XFluor4; Tecan). The measurement conditions were as follows: the excitation wavelength was 485 nm, emission wavelength was 535 nm, gain was 70–85, the number of reads was 10, the integration time was 40 µs, the target temperature was 37°C, and the G-factor was 1.1253.

Statistical analysis

Mann–Whitney U tests were performed to evaluate the statistical difference between control (21 unimmunized) and IAV-immunized [5 of H4N6(Shiga)-immunized plus 2 of H9N2(Yokohama)-immunized] chicken serum groups in ELISA and FPIA.

Results

Verification of FPIA with fluoro-NP and -M1 peptides for the detection of anti-IAV Ab in chicken serum

In the ELISA using the NP and M1 peptides as the Ags, the absorbance in the H9N2(Yokohama)-immunized chicken serum was higher than in the control (unimmunized) serum (Fig. 1A). In FPIA with the low (2.0 ng/mL) and high (500 ng/mL) concentrations of fluoro-NP and -M1 peptides as the tracers, there was no difference in the MPUs among PBS, control serum, and immunized serum. However, in the middle peptide concentrations (7.8–125 ng/mL), the MPU for the immunized serum was higher than that for the PBS and control serum (Fig. 1B). In both ELISA and FPIA, the absorbance and MPUs in the IAV-immunized serum group, which contains 5 H4N6(Shiga)-immunized and 2 H9N2(Yokohama)-immunized chicken sera, were statistically higher than those in the control serum group (Figs. 1C, 1D).

Verification of FPIA with fluoro-H5-HA proteins for the specific detection of anti-H5 IAV Ab-positive serum

In both ELISA and FPIA with H5(Shimane)-HA1–HA3 proteins, the absorbance and MPUs in the anti-H5N3(Hong Kong) Ab-positive goat serum were highest among the anti-H1–H15 Ab-positive sera (Figs. 2A, 2B). The aa sequences

Figure 2. Detection of anti-H5 subtype avian IAV Ab by fluorescence polarization immunoassay (FPIA) using fluoro-H5-HA proteins. **A.** ELISA with H5(Shimane)-HA1, -HA2, and -HA3 proteins was conducted on anti-H1–H15 Ab-positive goat sera. The sera were diluted 400 times. **B.** FPIA with fluoro-H5(Shimane)-HA1, -HA2, and -HA3 proteins was conducted on anti-H1–H15 Ab-positive goat sera. The concentration of fluoroprotein was 313 ng/mL in the mixture. The sera were diluted for 20 times in the mixture. The results represent 2 similar experiments.

of HA1, HA2, and HA3 regions were >90% identical between H5N3(Shimane) and H5N3(Hong Kong) strains (Suppl. Fig. 2). These results suggest that, with the use of HA proteins as tracers, FPIA can be utilized to detect Ab against the specific HA subtype virus in sera.

Discussion

We used only one peptide region in a FPIA using fluoro-NP and -M1 peptides as tracers; a longer peptide, which contains several epitopes, should be selected in future studies for the detection of broad avian IAV strains. We found that partial HA recombinant proteins derived from H5N3(Shimane) specifically detected anti-H5(Hong Kong) serum, but their reactivity with antisera from animals immunized with other H5

subtype strains including HPAIV has not been evaluated. The aa sequence of the epitope is different among H5 clades in some cases.^{2,3} The selection of aa region, which is conserved among the broad H5 clades as a tracer, is one of the most vital issues in future studies. Such a highly conserved tracer will contribute to broad detection of H5 subtype avian IAVs with high sensitivity and specificity.

The difference that we found in the MPUs between Ab-positive and -negative serum was lower than that in previous reports using FPIA for the detection of Abs against *Brucella* and equine infectious anemia virus.^{10,15} The low differences in MPU between Ag-positive and -negative sera may result from chicken serum components that often show nonspecific reactions. The identification of factors affecting the MPU and the establishment of suitable sample pre-treatment methods may improve the accuracy of the FPIA. Our study was also limited to an analysis of goat sera and a small number of anti-IAV Ab-positive chicken sera (<10 samples). In previous studies evaluating the utility of FPIA to detect Abs against infectious agents, >70 Ab-positive samples were analyzed to determine sensitivity and specificity.^{5,10,15} Hence it will be essential to analyze many wild bird and poultry sera for the evaluation of the sensitivity and specificity of anti-IAV Ab detection using FPIA.

Acknowledgments

We thank Dr. Toshihiro Ito (Tottori University, Tottori, Japan); Dr. Yoshihiro Sakoda (Hokkaido University, Sapporo, Japan); the National Institute of Animal Health (Tsukuba, Japan); and Drs. Tetsuya Mizutani and Tsutomu Omatsu (Tokyo University of Agriculture and Technology, Tokyo, Japan). We thank Sachiko Matsuda for technical assistance. We thank Editage (<https://www.editage.jp>) and Enago (<https://www.enago.jp>) for English language editing.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

This work was partially supported by JSPS KAKENHI grant 15H05260 from the Japan Society for the Promotion of Science, Japan. This research was partially funded by the Ministry of Agriculture, Forestry and Fisheries of Japan.

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Supplementary material

Supplementary material for this article is available online.

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