FULL PAPER *Immunology*

Detection of antibodies against avian influenza virus by protein microarray using nucleoprotein expressed in insect cells

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ABSTRACT. Avian influenza (AI) is an infectious disease caused by avian influenza viruses (AIVs) which belong to the influenza virus A group. AI causes tremendous economic losses in poultry industry and pose great threatens to human health. Active serologic surveillance is necessary to prevent and control the spread of AI. In this study, a protein microarray using nucleoprotein (NP) of H5N1 AIV expressed in insect cells was developed to detect antibodies against AIV NP protein. The protein microarray was used to test Newcastle disease virus (NDV), infectious bursal disease virus (IBDV), AIV positive and negative sera. The results indicated that the protein microarray could hybridize specifically with antibodies against AIV with strong signals and without cross-hybridization. Moreover, 76 field serum samples were detected by microarray, enzyme-linked immunosorbent assay (ELISA) and hemagglutination inhibition test (HI). The positive rate was 92.1% (70/76), 93.4% (71/76) and 89.4% (68/76) by protein microarray, ELISA and HI test, respectively. Compared with ELISA, the microarray showed 100% (20/20) agreement ratio in chicken and 98.2% (55/56) in ornamental bird. In conclusion, this method provides an alternative serological diagnosis for influenza antibody screening and will provide a basis for the development of protein microarrays that can be used to respectively detect antibodies of different AIV subtypes and other pathogens.
KEY WORDS: avian influenza virus, eukaryotic expression, nucleoprotein, protein microarray

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The influenza viruses are members of the family *Orthomyxoviridae* whose genome comprises eight single-stranded RNA segments of negative polarity. According to antigenic differences in their nucleoprotein (NP) and matrix protein (M1), influenza viruses are classified into three genera or types: A, B and C. All avian influenza viruses (AIVs) belong to type A, and the large group is further characterized into differential subtypes based on specific hemagglutinin (HA) and neuraminidase (NA). Currently, 16 hemagglutinin (H1 to H16) and 9 neuraminidase (N1 to N9) subtypes have been isolated in AIV [10, 28]. Wild waterfowl and shorebirds are recognized as the natural reservoir of influenza virus, and all subtypes of influenza virus could be identified from birds [23, 27]. AIV poses a significant threat to the poultry industry worldwide. Moreover, AIV has the potential to cross species barriers to trigger human pandemics [8, 11], such as human infections with H7N9 that occurred in Shanghai, Zhejiang and other provinces in China in 2013. Therefore, active serologic surveillance is necessary to prevent and control the spread of AIV.

The hemagglutination inhibition (HI), neuraminidase inhibition (NI) test and agar gel precipitation (AGP) are frequently applied to detect antibodies against AIV [5, 17, 19, 20, 22]. The HI and NI assays are relatively inexpensive and utilized as standard procedure in most labs. However, the HI and NI assays are laborious and rely on having well matched control reference reagents. The AGP test is time-consuming and requires large quantities of both antigens and antibodies to form the precipitation lines. Consequently, various enzyme-linked immunosorbent assay (ELISA) was developed for the detection of antibodies to influenza virus, which is more sensitivity relative to the HI, NI and AGP test [24, 30]. As a result of technology development, microarray technology was applied in disease diagnosis, which allows the simultaneous analysis of thousands of parameters within a single experiment. Currently, protein microarray has shown great potential for disease diagnosis [13, 14] and serology detection [2, 21, 26]. Traditional protein microarray requires expensive equipments, considerable skills and high costs. Thus, this method is rarely applied in veterinary clinics and still in the initial stages of research. In previous report, our laboratory developed a protein chip combining with colloidal gold immunological amplification and a silver staining method to detect antibodies against four avian viruses [26]. This method can scan visually color change

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without expensive equipments.

In this study, we developed a protein microarray method to detect antibodies against type A influenza virus by using NP protein expressed in insect cells. The protein microarray is specific, sensitive and provides a viable alternative for screening assay of antibodies against AIV.

MATERIALS AND METHODS

Virus: AIV A/goose/Guangdong/1/96 (H5N1) and A/chicken/Shanxi/2/06 (H5N1) (CK/SX/06) were maintained in the National Avian Influenza Reference Laboratory, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

Serum samples: Negative reference sera were obtained from specific pathogen free (SPF) chickens. Positive sera for AIV were obtained from vaccinated SPF chickens with H1-H15 subtype inactivated virus (A/duck/Alberta/35/76 (H1N1), A/ duck/Germany/1215/73 (H2N3), A/duck/Ukraine/1/63 (H3N8), A/duck/Czech/1/56 (H4N6), A/turkey/England/N28/73 (H5N2), A/turkey/Canada/63 (H6N8), A/african starling/England/983/79 (H7N1), A/turkey/Ontario/6118/ 68 (H8N4), A/turkey/Wisconsin/1/66 (H9N2), A/turkey/England/384/79 (H10N4), A/duck/England/1/56 (H11N6), A/duck/Alberta/60/76 (H12N5), A/gull/Maryland/704/77 (H13N6), A/mallard/Gurjev/263/82 (H14N5) and A/duck/Australia/341/83 (H15N8)). Negative reference sera and positive sera for H1-H15 subtype AIV were maintained in the National Avian Influenza Reference Laboratory, Harbin Veterinary Research Institute. Positive sera for Newcastle disease virus (NDV) and infectious bursal disease virus (IBDV) were obtained from the avian disease research groups at the Harbin Veterinary Research Institute. All serum samples including negative reference sera and positive for differential virus were obtained from 6- to 10-week-old SPF egg-laying chickens. Seventy-six field serum samples from egg-laying or ornamental birds were used in HI, ELISA and microarray analyses. Among these samples, 20 samples were from submitted one-year-old sick chickens in poultry farms in Harbin, and 56 samples were collected from immunized ornamental birds with H5 subtype inactivated vaccine in Harbin Zoo.

Cloning of NP gene: Viral RNA of A/goose/Guangdong/1/96 (H5N1) was extracted with TRIzol LS Reagent (Life Technology, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. The first-strand cDNA synthesis was performed using the Uni12 primer (5′-AGCAAAAGCAGG-3′) by M-MLV Reverse Transcriptase kit (Life Technology). The forward primer (5′-CATGGATCCTCAGGGCACCAAACGA-3′) and reverse primer (5′-TTTTCTCGAGTTGTCATATTCCTCTGC-3′) were used for PCR amplification. These primers provide a *BamHI* site at the start of the amplified gene and an *XhoI* site at the end. The PCR products were purified by a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, U.S.A.), digested with *BamHI* and *XhoI* (NEB, Ipswich, MA, U.S.A.) and cloned into the pFastBacHTa expression vector (Life Technology). A recombinant plasmid pFastBacHTa-NP, which contained the NP gene, was extracted, and the sequences were verified by PCR and sequencing analysis.

Expression of NP protein: Sf21 cells were cultured in Grace's insect media, which contained 10% fetal bovine serum (Biochrom AG, Berlin, Germany). The bacmid DNA was introduced into Sf21 insect cells by a transfection kit (Life Technology) according to the manufacturer's protocol. Transfected cells were harvested at 72 hr after infection and were used to infect fresh insect cells.

Immunofluorescence assay: Sf21 cells were seeded in 6-well plates and infected with NP recombinant baculovirus. At 48 hr post-infection, the cells were fixed with 4% paraformaldehyde for 30 min and incubated with 0.5% TritonX-100 for 15 min at room temperature. After washing in phosphate buffered saline (PBS, pH 7.4), plates were blocked for 1 hr at 37°C with 5% non-fat milk in PBS and rinsed before incubation. 1:200 diluted sera against AIV were incubated for 1 hr at 37°C. After washing three times in PBST (Tween-20 dissolved in PBS to a final concentration of 0.05%) at room temperature, 1:2,000 dilution of goat anti-chicken fluorescein isothiocyanate (FITC) labeled secondary antibody (Sigma, St. Louis, MO, U.S.A.) was added and incubated at 37°C for 1 hr. The results were visualized under a fluorescence microscope.

Purification of NP protein: For an abundant expression of the NP protein, Sf21 cells were cultured in shaking flasks with a stirring speed of 150 rpm at 27°C and infected with a twice amplified virus stock. After 72 hr, the infected cells were harvested, centrifuged at 1,000×g for 10 min and washed three times with PBS. The cells were lysed with lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton×100, 1% sodium deoxycholate, 0.1% SDS and 1 mM PMSF). This procedure was performed in an ice bath and gently shaken for 1 hr. The lysate was centrifuged at 10,000×g for 30 min at 4°C. The supernatant, which contained recombinant NP protein, was purified using a HisTrap HP column (GE Healthcare, Uppsala, Sweden) according to the manufacturer's protocol. SDS-PAGE and western blot with AIV positive serum and Alexa Fluor 680 donkey anti-chicken antibody (Life Technologies) were performed to analyze purified NP protein. The concentration of purified NP was determined using a Pierce BCA Protein Assay Kit (Pierce, Rockford, IL, U.S.A.).

Preparation of protein microarray: The purified NP protein was diluted to 2 mg/m*l*, 1 mg/m*l*, 0.5 mg/m*l*, 0.25 mg/m*l*, 0.125 mg/m*l* and 0.0625 mg/m*l* in printing buffer (1% (w/v) bovine serum albumin (BSA) in PBS and adjusted to pH to 7.4 with HCl). SPF chicken serum was chosen as the positive control, and printing buffer was used as the negative control. Samples were contact-printed onto aldehyde-coated slides (Bai'ao Biotechnology Co., Shanghai, China) using a robotic microarray printing system equipped with SMP6 pins (TeleChem International Inc., Sunnyvale, CA, U.S.A.) in rows of six replicates (Fig. 1), and six identical arrays were contained in each slide . During the printing process, 50–60% relative humidity was maintained so that the action of antigen and the shape of spots were maintained. The slides were fixed for 2 hr at room temperature and stored at

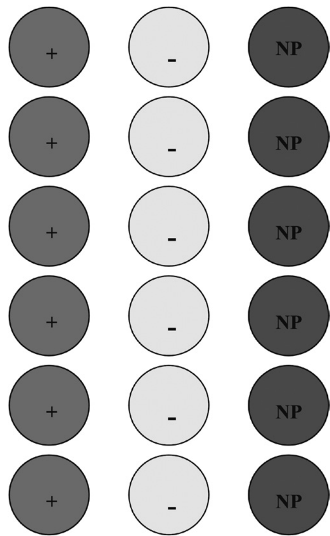

Fig. 1. Layout of NP protein microarray. NP antigen was contact-printed onto aldehyde-coated slides in rows of six replicates. SPF chicken serum and printing buffer were used as the positive control and negative control, respectively. +: positive control spots; –: negative control spots; NP: NP antigen spots.

4°C until further processing.

Sensitivity: The NP antigens were diluted with printing buffer to 2 mg/m*l* to prepare protein microarray. Slides were blocked with 1% BSA for 1 hr at 37°C and rinsed three times with PBST. Subsequently, 30 μl of anti-AIV serum diluted at a two-fold serial dilution (1:200–1:6,400) in antibody dilution buffer (PBS, 1% BSA) was delivered on the array and incubated for 1 hr at 37°C. After washing the slides as described above, 30 μl of colloidal gold-labeled goat anti-chicken IgY secondary antibody (Biosynthesis Biotechnology, Beijing, China) was added on the array. The microarrays were incubated for 1 hr at 37°C. Following, the slides were then rinsed, dried and incubated with the silver enhancing solution for 10 min. The Image J and SPSS software (V.13.0) were used to detect and analyze the signal intensity of each spot [26].

Specificity: To determine the specificity of the microarray, protein microarrays were hybridized with positive sera of H1-H15 AIV subtypes, NDV, IBDV and AI negative sera that were obtained from SPF chickens. Meanwhile, 76 field serum samples (diluted 1:200) were tested with the protein microarray.

Comparison of protein microarray, ELISA and HI: To compare the protein microarray with ELISA and HI, positive sera of H1-H15 AIV subtypes and 76 field serum samples were also analyzed by ELISA and HI. The microtiter plates were coated with NP antigen (6.25 μg/well) overnight at 4°C, blocked with a solution of 5% of skimmed milk and incubated with serum samples. After washing three times with PBST, bound antibodies were detected using horseradish peroxidase-conjugated rabbit anti-chicken IgY (Sigma). Following this incubation, all unbound materials were removed, and the wells were washed prior to addition of a TMB substrate solution (Cell chip biotechnology, Beijing, China). Finally, the reaction was stopped by sulfuric acid and read by microplate reader at an absorbance of 450 nm (Bio-Rad, Hercules, CA, U.S.A.). Meanwhile, HI antibody assays were performed by following the World Organization of Animal Health manual (OIE, 2004). Four hemagglutination units (HAU) of CK/SX/06 antigen and H1-H15 antigen were used for detection of field serum samples and H1-H15 immune sera, respectively.

RESULTS

Cloning, expression and purification of NP: PCR and double enzyme digestion were performed to verify the recombinant plasmid. As expected, an approximately 1,500 bp product was obtained (Data not shown). The result of sequencing showed the predicted amino acid sequence of NP was consistent with the previously reported sequences

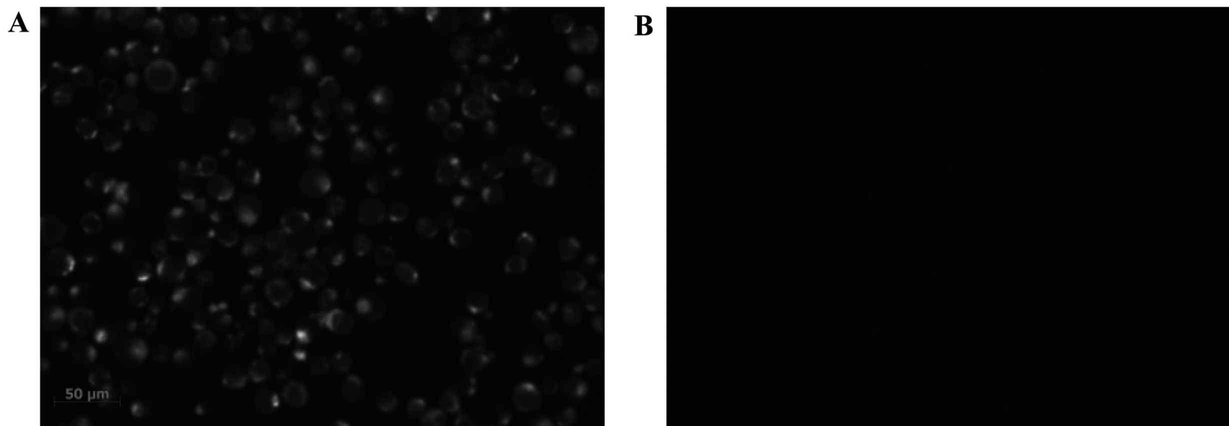

Fig. 2. The immunofluorescent analysis of NP protein. Indirect immunofluorescent staining of Sf21 cells was performed with positive serum for AIV, incubated with a FITC-conjugated goat anti-chicken second antibody and then observed with a fluorescence microscope. A: Recombinant baculovirus infected Sf21 cell; B: Uninfected Sf21 cell.

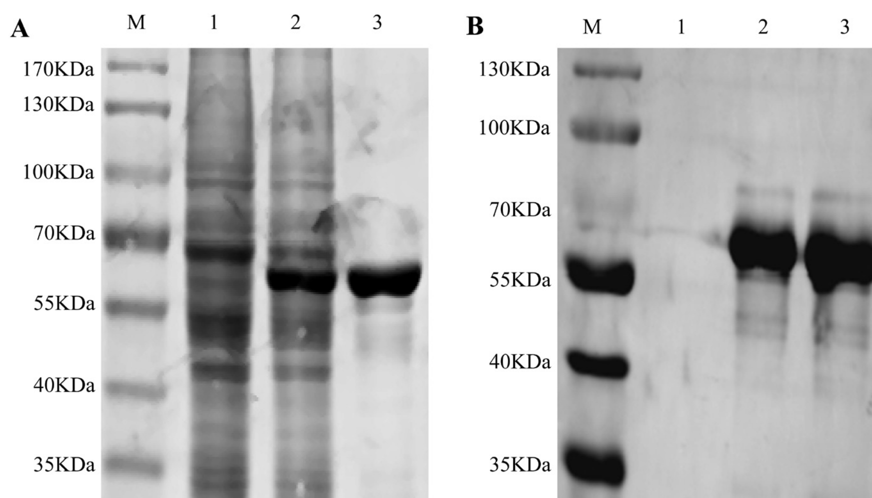

Fig. 3. SDS–PAGE and western blot analysis of recombinant protein. (A) SDS-PAGE analyses of the expression of NP protein. M, protein molecular marker; lane 1, uninfected Sf21 cells; lane 2, infected Sf21 cells; lane 3, purified NP; (B) Western blotting analysis of NP protein. Lane 1, uninfected Sf21 cells; lane 2, infected Sf21 cells; lane 3, purified NP.

(GenBank ID: AAD51925.1). Subsequently, immunofluorescence staining was employed to analyze the expression of NP, and strong fluorescence signals were detected in the infected cells (Fig. 2). SDS PAGE analysis confirmed that a protein of approximately 60 kDa was expressed, but was not detected in uninfected cells (Fig. 3A). In addition, we performed a western blot analysis using an anti-AIV chicken polyclonal antibody. The result in Fig. 3B shows that a specific band was presented. A high concentration of the NP antigen was obtained in the final step.

Antigen concentration of the protein microarray: When the antigen was spotted onto the slides at 0.125 mg/m*l*, 0.25 mg/m*l*, 0.5 mg/m*l*, 1 mg/m*l* and 2 mg/m*l*, respectively, the signal intensity rose in relation to the antigen concentration on the slides. In Fig. 4, when the antigen concentration was 0.5 mg/m*l*-2 mg/m*l*, the signal intensity did not show a significant change. To ensure high signal intensity, 2 mg/m*l* was selected as the antigen concentration in this experiment.

Sensitivity and specificity test: Positive sera (diluted 1:200–1:6,400) for AIV were hybridized with 2 mg/m*l* NP antigen. The signal intensities changed according to the sera dilution (Fig. 5). When the serum was diluted 1:6,400, the positive signals were still obtained by a common microscope. To evaluate the specificity of the NP protein microarray, positive sera of AIV, NDV and IBDV and SPF chickens negative sera were also hybridized with the protein microarrays. Scanning image of microarrays showed the spots of a positive control present strong signals, and a negative control does not present any signal. Parts of scanning image were displayed in Fig. 6. The NP antigen reacted specifically with all positive sera for AIV and showed no cross-reactivity with positive sera for NDV, IBDV and negative control sera. The results indicate that this protein microarray can be used to test specific antibodies against AIV.

Detection of serum samples: Antisera to different AIV subtypes from vaccinated SPF chickens and the field serum samples from 20 chickens and 56 ornamental birds were tested by protein microarray, ELISA and HI test. Antisera to H1-H15 AIV subtypes from chickens showed strong positive signals, which were consistent with ELISA and HI test (Table 1). Six of 76 field serum samples showed negative signals by protein microarray test, and five samples showed negative signals by ELISA. However, eight of 76 field serum samples showed negative signals by HI test (HI titer (Log2)≥2 is considered to be positive). The positive rate was 92.1% (70/76, positive number/total number), 93.4% (71/76) and 89.4% (68/76) by protein microarray, ELISA and HI test, respectively. Compared with ELISA, the coincidence rate of microarray was 100% (20/20) in chicken and 98.2% (55/56) in ornamental bird (Tables 2 and 3). Compared with HI, the coincidence rate of microarray was 100% (20/20) in chicken and 96.4% (54/56) in ornamental bird (Tables 2 and 3). The data showed that this protein microarray was specific and sensitive for AIV antibody detection compared with the HI test.

DISCUSSION

Microarray technology allows the simultaneous analysis of thousands of parameters within a single experiment. This method plays an important role in basic research, disease diagnostics, drug discovery and other research fields [3, 4, 7]. Here, we developed a type of protein microarray method that was used to detect sera antibodies against the type A influenza virus, and compared the results tested protein microarray, ELISA and HI, respectively. The coincidence rate was 98.7% (75/76) and 97.4% (74/76) with ELISA and HI. When positive sera of AIV were diluted 1:6,400, the positive signals were still obtained by a common microscope in protein microarray test. Those results demonstrate that this protein microarray is reliable, sensitive and specific without

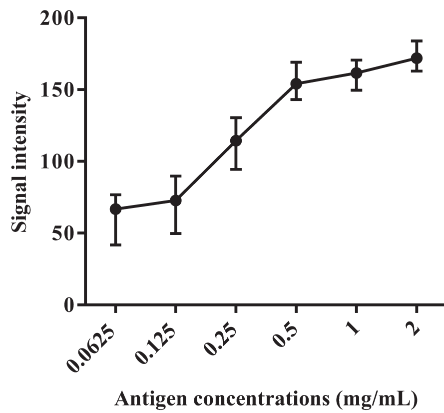

Fig. 4. The effect of antigen concentration on signal intensity. The purified antigens were immobilized onto aldehyde-modified slides at the concentration of 0.125 mg/m*l*, 0.25 mg/m*l*, 0.5 mg/m*l*, 1 mg/m*l* and 2 mg/m*l* with printing buffer. The microarray was then hybridized with positive serum (diluted 1:200) for avian influenza, washed and reacted with the colloidal gold-labeled antibody. The signal intensity rose in relation to the antigen concentration on the slides. When the antigen was coated at concentrations of 0.5, 1 and 2 mg/m*l*, the signal intensity did not change clearly according to the concentration of antigen used.

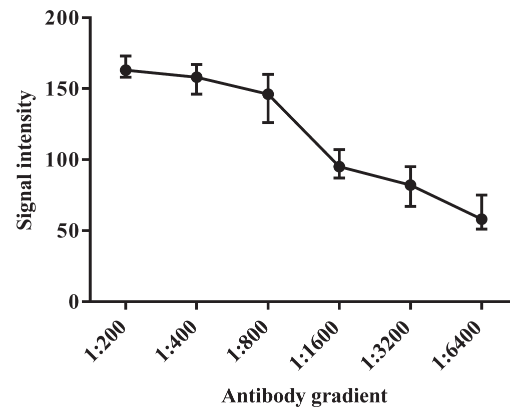

Fig. 5. The signal intensity using different serum dilutions. The purified NP antigen was printed onto slides at 2 mg/m*l* and hybridized with two-fold serial dilutions (1:200 to 1:6,400) of positive sera against AIV for 1 hr at 37°C. The signal intensities changed according to the sera dilution. Positive signals were still obtained by a common microscope when the serum was diluted 1:6,400.

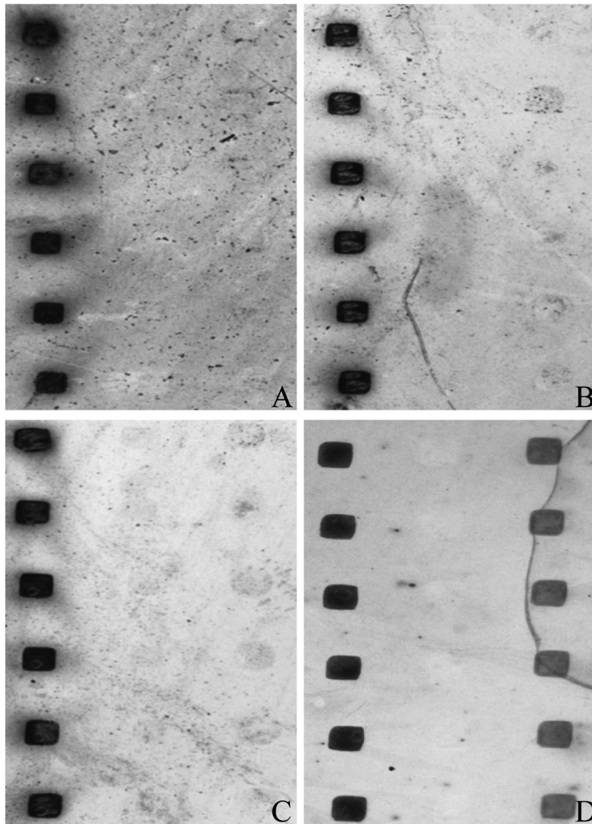

Fig. 6. The specificity detection of the protein microarray. Positive chicken sera of AIV, NDV and IBDV and negative sera from SPF chickens were hybridized with the protein chip. (A) SPF chicken sera; (B) anti-NDV positive sera; (C) anti-IBDV positive sera; (D) anti-AIV positive sera.

cross-reactivity between AIV antisera and negative serum samples.

Generally, microarrays require expensive scanning equipment to obtain results. However, the immune colloidal gold technique can avoid this limitation. This technique, which has high sensitivity and good specificity, has been rapidly developed in recent years [1, 6, 9, 29]. Protein microarray, which is combined with the colloidal gold and silver staining method, has been used to simultaneously detect antibodies against four poultry diseases [26]. This method is inexpensive, easy and visible. In this study, the same method is used to detect antibodies against the type A influenza virus.

Serological methods, such as HI and NI tests, have often been applied to detect antibodies against AIV in most labs [12, 16, 25]. Compared with HI test, there are some advantages to using protein microarrays. One advantage of the protein microarray is the application in DIVA (differentiating infected from vaccinated animals) strategies when recombinant vaccines without the NP protein are used in poultry. Moreover, the use of small volumes of reagents and antibodies is also a characteristic of protein microarrays, which made it economical and useful for those conditions when large quantities of samples were not easy to get. It is well known that wild waterfowl and shorebirds play an important role in disseminating AIV among different regions [15, 18]. However, it was hard to obtain large volumes of sera from these birds. Thus, there are practical limitations to the application of traditional serological tests. Optimistically, protein microarray can provide qualitative information regarding antibodies in wild waterfowl and shorebirds, which is valuable and useful for the surveillance of field situations and for controlling the prevalence of influenza viruses, but the HI assay is still necessary for subsequent subtyping.

ELISA is another option for AIV surveillance and/or evaluation of vaccine efficacy. The results indicate that similar detection rate is obtained from ELISA and protein microar-

Table 1. Detection of different subtype AIV immune sera

Tests	Immune sera sample (No.)														
	H 1	H 2	H 3	H 4	H 5	H 6	H 7	H 8	H 9	H 10	H 11	H12	H 13	H 14	H15
Microarray	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ELISA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HI (Log_2)	9	7	8	8	8	8	7	9	8	6	7	8	8	7	9

Table 2. Detection of field sera samples from chicken

Tests	Field sera sample (No.)																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Microarry	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	–	+
ELISA	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	–	+
HI (Log_2)	9	8	8	6	2	0	8	8	7	3	3	2	8	6	7	7	6	8	0	8

Table 3. Detection of sera samples from ornamental birds

No.	Name	Microarry	ELISA	HI	No.	Name	Microary	ELISA	HI
1	*Cygnus cygnus*	+	+	7	29	*Grus vipio*	+	+	2
2	*Cygnus cygnus*	+	+	8	30	*Grus vipio*	+	+	0
3	*Tadorna ferruginea*	+	+	8	31	*Grus grus*	+	+	5
4	*Tadorna ferruginea*	+	+	8	32	*Grus grus*	+	+	5
5	*Syrmaticus reevesii*	+	+	7	33	*Grus grus*	+	+	6
6	*Numida meleagris*	+	+	6	34	*Grus grus*	+	+	7
7	*Numida meleagris*	+	+	2	35	*Grus grus*	+	+	6
8	*Grus monacha*	–	+	1	36	*Grus leucogeranus*	+	+	8
9	*Grus monacha*	+	+	6	37	*Grus leucogeranus*	+	+	8
10	*Grus monacha*	+	+	4	38	*Grus leucogeranus*	+	+	8
11	*Grus monacha*	+	+	6	39	*Grus leucogeranus*	+	+	8
12	*Greater flamingo*	+	+	8	40	*Lophura nycthemera*	+	+	6
13	*Greater flamingo*	+	+	8	41	*Lophura nycthemera*	+	+	8
14	*Greater flamingo*	+	+	6	42	*Anser cygnoides*	+	+	7
15	*Greater flamingo*	+	+	8	43	*Anser cygnoides*	+	+	5
16	*Greater flamingo*	+	+	8	44	*Pavo muticus*	+	+	8
17	*Grus japonensis*	–	–	1	45	*Pavo muticus*	+	+	8
18	*Grus japonensis*	–	–	0	46	*Pavo muticus*	+	+	8
19	*Grus japonensis*	+	+	3	47	*Pavo muticus*	+	+	8
20	*Grus japonensis*	+	+	8	48	*Canadensis*	+	+	6
21	*Grus japonensis*	+	+	4	49	*Canadensis*	+	+	7
22	*Anthropoides virgo*	+	+	6	50	*Cygnus atratus*	+	+	4
23	*Anthropoides virgo*	+	+	8	51	*Cygnus atratus*	+	+	7
24	*Anthropoides virgo*	+	+	7	52	*Ciconia ciconia*	+	+	8
25	*Anas poecilorhyncha*	+	+	8	53	*Ciconia ciconia*	+	+	8
26	*Anas poecilorhyncha*	+	+	7	54	*Phasianus colchicus*	–	–	0
27	*Grus vipio*	+	+	5	55	*Phasianus colchicus*	+	+	6
28	*Grus vipio*	+	+	1	56	*Anas platyrhynchos*	+	+	8

ray analysis (92.1% and 93.4%), but the direction of microarray and ELISA is not the same. Differential antibodies are detected in a separate assay with ELISA array. While parallel antibodies can be detected on one chip without non-specific reactions, thus protein microarray may be more convenient than ELISA. In this study, only one antigen was employed to develop a protein microarray for AIV antibodies detection, which does not achieve high-throughput capability. However, this study verifies the feasibility of the method, and this method has great potential in serological investigations and eradication of AIV from certain flocks. In future studies, we can increase the number of antigens in this microarray to achieve high-throughput capability and attempt to simultaneously detect antibodies of different diseases, which may broaden the range of detection for microarrays and provide a basis for animal disease diagnosis and other studies.

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