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Immunochromatographic Strip Using Baculovirus Expression Nucleoprotein to Detect Avian Influenza Antibody

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Abstract

As a part of routine surveillance, detecting avian influenza (AI) antibody has a longer time than detecting AI virus (AIV) itself because the former lasts longer than the latter. In order to detect AI antibody in the field, we developed a membrane-based immunochromatograpic strip (ICS). The nucleoprotein (NP) gene of an H5N2 AIV was cloned and expressed in a baculovirus expression system. The expression NP was applied in an enzyme-linked immunosorbent assay (NP-ELISA) and compared with the hemagglutination inhibition (HI) test. The sensitivity and the specificity of the NP-ELISA based on HI reached 100% and 96.6%, respectively. In addition, the NP was applied in ICS to detect anti-AIV antibodies. The sensitivity and the specificity of NP-ICS based on HI were 96.5% and 89.1%, respectively. The present NP-ICS detects antibodies at least against H5 and H6 AIVs and provides a quick and convenient method for AIV antibody detection in the field.

Keywords: Avian influenza antibody, Baculovirus expression system, Chickens, Immunochromatography, Nucleoprotein

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Introduction

The avian influenza virus (AIV) is a member of the family *Orthomyxoviridae*, genus *Influenzavirus A*. Type A influenza virus is subtyped according to the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). Up to the present, 16 HA subtypes and 9 NA subtypes have been found in birds (Fouchier *et al.,* 2005; Rohm *et al.,* 1996; Webster *et al.,* 1992). In Taiwan low pathogenic AIV (LPAIV) strain H6N1 was first isolated from meat ducks in 1972 (Lu *et al.,* 1985). The LPAIV H5N2 was reported in 2004. Infection with LPAIV usually results in an egg production drop with a few mortalities. Since then, AIV surveillance has been performed in Taiwan. In addition to the antigen, antibody detection is important and commonly used for AI surveillance, with methods including the agar gel immunodiffusion test, the hemagglutination inhibition (HI) test and enzyme-linked immunosorbent assay (ELISA). However, most of these tests are time-consuming and require well-equipped laboratories with well-trained medical technicians. For these reasons, they are not suitable for clinical practice in the field. In contrast, the immunochromatographic strip (ICS) is a membrane-based strip, using gold-labeled antibody as the tracer which can be interpreted with a naked eye (Posthuma-Trumpie *et al.,* 2009) in the field.

Although the ICS for AIV antigen detection is widely used (Manzoor *et al.,* 2008; Miyagawa *et al.,* 2011; Miyoshi-Akiyama *et al.,* 2009; Tsuda *et al.,* 2007), there have been few applications for antibody detection (Peng *et al.,* 2007). The antibody surveillance of diseases in chickens has a longer window than antigen detection because antibodies last much longer than AIV antigen after infection(Chen *et al.,* 2010). The presence of antibodies indicates virus infection because vaccination is not permitted in Taiwan. The local veterinarians like to know the AI infectious status of their chickens. To develop a rapid antibody detection method for use in the field is important for AI surveillance. The present study develops a rapid, easy-to-operate method to detect AIV antibodies on poultry farms.

Materials and Methods

Viruses

Two AIVs, A/duck/Yunlin/3233/2004 (H5N2) and A/chicken/Taiwan/2838V/2000 (H6N1), were propagated in specific-pathogen-free (SPF) chicken eggs (Animal Health Research Institute, Taiwan). The cell debris in the allantoic fluid was centrifuged ouf at 3,000 xg for 30 min

and the supernatant was then concentrated by centrifugation at 70,000 xg for 2 hours at 4℃. The pellet was dissolved in a small amount of TEN buffer (10 mM a space Tris base, 1 mM EDTA, 0.1 M NaCl). The virus particle was purified in 20-50% sucrose gradient by centrifugation at 50,000 rpm (268,000 xg) for 2 hours at 4℃ (Rotor MLS-50, Optima Ultracentrifuge, Beckman). The virus band was collected and stored in a -20℃ freezer for western blotting.

Serum samples

The chicken serum samples were collected from chicken farms in the field around Taiwan. The chickens both non-infected and infected with H5 or H6, were confirmed using virus isolation. In addition to negative virus detection at the time of sampling, the non-infected farms were confirmed by HI test 1-2 months afterwards. Almost all chickens had seroconversion three weeks after infection (Chen *et al.,* 2008; Chen *et al.,* 2010). Those sera were measured with a commercial AI antibody detection kit (Idexx, Westbook, ME) and the positive sera were further tested using HI tests with H5 (A/duck/Yunlin/3233/2004, H5N2) and H6 (A/chicken/Taiwan/2838V/2000, H6N1) AIVs separately. For convenient sampling, 78 positive and 88 negative sera were used for NP-ELISA and 86 positive and 64 negative sera were used for the NP-ICS test. All sera used in this study were stored in in a freezer at -20℃ before testing. Negative sera were from non-infected chickens.

Preparation and purification of the expression NP

NP gene cloning, expression and purification in the Bacmid system were performed according the method previous reported (Chen *et al.,* 2016). In brief, the full length of the NP gene from A/duck/Yunlin/3233/2004 was amplified using RT-PCR to obtain a fragment of 1,497 bp which was then cloned into pFastBac HT C vector (Invitrogen, Carlsbad, CA). The vector was then transformed into DH10Bac cells for homologous recombination with Bacmid to form Bacmid-NP. The Bacmid-NP was transfected into *S. frugiperda* cells and the recombinant NP was expressed. The expression NP was analyzed using SDS-PAGE. AI positive serum and mouse anti-NP monoclonal antibody (3IN5, HyTest Ltd, Turku, Finland) were used for western blotting to evaluate the expression protein.

The expression NP appeared in the supernatant after 7 days post infection. The supernatant was harvested and purified using ultra-centrifugation (4℃ , 70,000 xg for 2 hours, Avanti^{TM} J-25.5 Centrifuge, Beckman). The pellet containing virus was then re-suspended in TEN buffer (10 mM Tris base, 1 mM EDTA, 0.1 M NaCl).

Indirect ELISA coated with the expression NP (NP-ELISA)

The purified expression NP was coated on ELISA plate (96 Micro Well Plate, Nunc, Denmark) with coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) at 4 ℃ for 16 hours. After washing three times with 300 μL/well PBST (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.05% Tween 20, pH 7.4), the plate was incubated at 37℃ for 1 hour with 100 μL/well blocking buffer (10% skim milk in PBST). The serum samples were diluted in blocking buffer and added to 100 μL/well at 37℃ for 30 minutes. After washing the plate, 100 μL/well rabbit anti-chicken HRP-conjugated antibody (1:2,500 dilution, Jackson Immuno Research Labs, West Grove, PA) was added and incubated at 37℃ for 30 minutes. The plate was then washed 5 times and 100 μL/well tetramethylbenzidine substrate (Kirkegaard& Perry Lab, Gaithersburg, MD) added in the dark for 10 minutes and the reaction was stopped with 100 μL/well stop solution (Kirkegaard & Perry Lab, Gaithersburg, MD). The optical density (OD) of each well was read at 450 nm wavelength in an ELISA reader (Multiskan FC, Thermo). The sample to positive (S/P) ratio was calculated using (sample OD-negative OD) / (positive OD-negative OD).

Assembly of the immunochromatographic strip

Twenty mL of the colloidal gold (40 nm, Rega Biotechnology Inc., Taiwan) was labeled with 220 μg of rabbit anti-chicken IgY (Jackson Immuno Research Labs, West Grove, PA), and finally concentrated in 1 mL of borax buffer (2 mM borax buffer containing 0.1% PEG-20000, pH 9.0).

The NP-ICS was composed of sample pad, conjugated gold pad, nitrocellulose membrane, and absorbance pad. The sample pad and conjugated gold pad were treated with PBS buffer (with 1% BSA, 0.5% Tween 20, 0.05% sodium azide, and 5% sucrose, pH 7.4) for 30 minutes and then air-dried at 37℃ before use. The conjugated gold pad was then received drops of 4 μL of rabbit anti-chicken IgY-labeled colloidal gold. The nitrocellulose membrane was received drops of 0.5 μL of 160 μg/mL purified NP as the test line, 0.5 μL of 250 μg/mL goat anti-rabbit IgG (Jackson Immuno Research Labs, West Grove, PA) as the control line. A pipet was used to drop spots instead of lines.

During the operational procedure, the serum was diluted at 1:30 in PBS buffer and transferred to the sample pad. After 20 minutes, if two red-purple lines developed, one at the test line and one at the control line, then the sample was considered to be positive. If a red-purple line developed only at the control line, it was considered negative (Fig. 1).

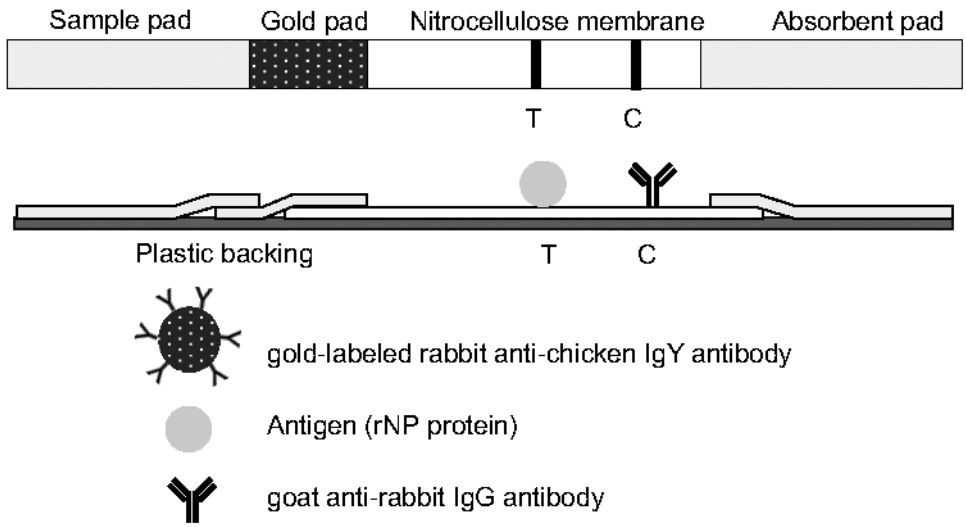

Fig. 1 The immnuochromatographic strip is assembled with sample pad, gold pad, nitrocellulose membrane, and absorbent pad. All pads are adhered to a plastic backing. The gold pad receives drops of 4 μL of colloidal gold-labeled rabbit anti-chicken IgY. Nitrocellulose membrane is dropped with 0.5 μL of 160 μg/mL purified NP on the test lines, and 0.5 μL of 250 μg/mL goat anti-rabbit IgG is dropped on the control line. Positive result of NP-ICS shows 2 lines, both on the test line and the control line. A negative result shows only one line on the control.

Results

Expression NP

The full length of the NP gene was 1,497 bp and translated to 498 amino acids. The expected expression NP's size was about 56 kDa. Both the AI positive serum and mouse anti-NP monoclonal antibody could detect the protein on western blotting (Fig. 2). After infection with the Bacmid-NP, the Sf9 cells began to express NP within 48 hours, reached peak at 96 hours after infection, and then appeared in the supernatant mostly at 168 hours.

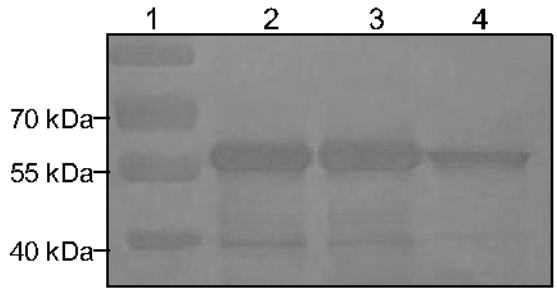

Fig. 2　The baculovirus expression NP was purified and analyzed by western blotting. Mouse anti-NP monoclonal antibody was used to detect recombinant NP. The expected band appears at about 56 KDa. (Lane 1: Page Ruler prestained protein ladder, Lane 2&3: purified expression NP, Lane 4: purified AIV strain A/duck/Yunlin/3233/2004).

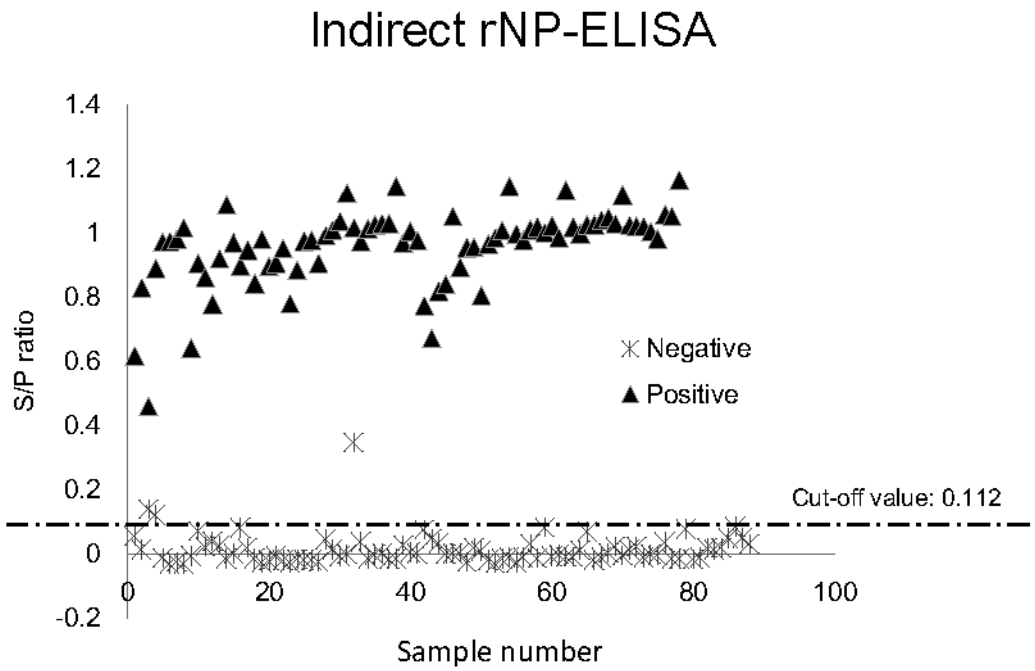

Fig. 3　S/P ratio values of 78 positive and 88 negative sera using indirect ELISA coating with expression NP.

Indirect NP-ELISA

After checkerboard titration, 100 ng/well of expression NP was coated onto the ELISA plate and the serum was diluted using 200X for the ELISA test. Seventy-eight positive and 82 negative as well as 8 SPF chicken sera were used for cut-off point determination. The mean S/P ratio of negative serum was 0.0126 with standard deviation of 0.0497. Thus the cut-off point was set to 0.112 (mean S/P ratio plus 2 standard deviations) (Fig. 3). Seventy-eight positive and 88 negative chicken serum samples confirmed by both a commercial ELISA (Idexx) and HI tests with H6N1 and H5N2 AIVs were used to evaluate the present NP-ELISA. The results showed that the sensitivity and specificity of the NP-ELISA were 100% (78/78) and 96.6% (85/88). The agreement between NP-ELISA and HI test was 98.2%.

NP-ICS assay

Eighty-six positive and 64 negative chicken sera confirmed using a commercial ELISA (Idexx) and HI test with H6N1 and H5N2 were used to evaluate the accuracy of the present NP-ICS assay. The results showed that the sensitivity and the specificity of the NP-ICS were 96.5% (83/86) and 89.1% (57/64), respectively. The agreement between the NP-ICS and HI test was 93.3%. The detection limit of the strip was obtained using 2-fold serial dilution, and the highest dilution was $1:2^6$, corresponding HI titer of 2^2-2^3 because the original titers of those sera were 2^8-2^9. In order to confirm that the NP-ICS would not have a cross reaction with other disease antibodies, the strip was tested with hyperimmune sera against different avian viruses, including infectious bronchitis virus (IBV), Newcastle disease virus (NDV), avian leucosis virus subgroup A (ALV-A), avian leucosis virus subgroup B (ALV-B), avian leucosis virus subgroup J (ALV-J), and reticuloendotheliosis virus (REV) (Charles River Lab, North Franklin, CT). The results showed that all hyperimmune sera were negative except the AI H5 and H6 subtype sera (Fig. 4).

Discussion

In Taiwan, the AIV was first reported in 1972 (Lu *et al.,* 1985). Since then, surveillance has been performed for AI control, which includes RT-PCR for antigen detection and serological test for antibody. Currently, ELISA is used to screen anti-AIV antibody and then HI for subtyping. These required substantial amounts of equipment and technician. Therefore, a rapid and simple diagnostic kit was designed in this study. This rapid test could be used by local veterinarians in the field.

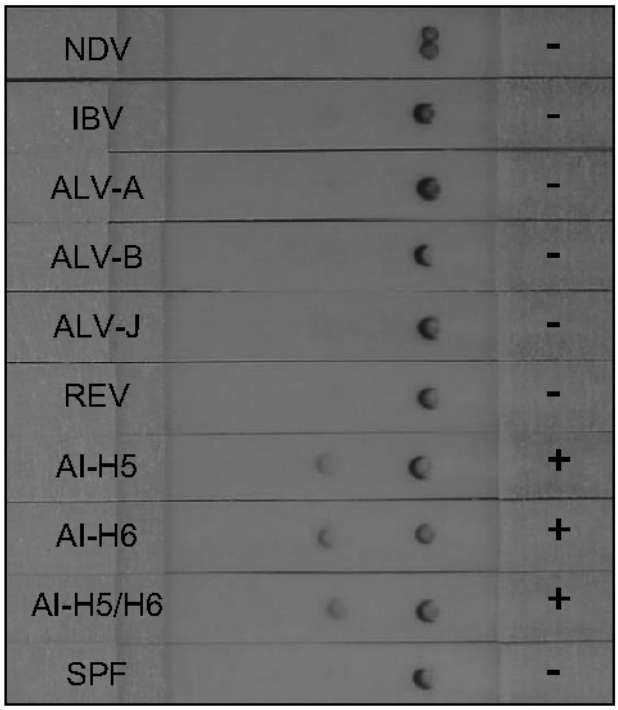

Fig. 4　Specificity of the NP-ICS. ICS is negative to antisera against NDV, IBV, ALV-A, ALV-B, ALV-J, and REV but is positive to sera against H5 and H6 AIVs. SPF serum is used as the negative control.

The NP is a highly conserved gene in AIV, and only 0-10 amino acids have changed in the past 50 years (Gorman *et al.,* 1990). The NP gene used in this study, A/duck/Yunlin/3233/2004, was performed with different AIV subtypes using blastn, and the similarity was 96% compared with other NP genes. The conservative feature is useful for the diagnosis of all subtypes of AIV. In this study, we used baculovirus as vector to infect insect cell and produced the expression NP. This system has been proved effective by many researchers. According to the western blotting, the NP could be detected by NP monoclonal antibody and AI positive serum at approximately 56 kDa from supernatant and purified NP.

The expression NP was proved to have good immunogenicity using indirect ELISA and western blot.The sensitivity and the specificity of the NP-ICS were slightly lower than the indirect NP-ELISA (Table 1). This is because the reaction only takes 20 minutes and lacks washing steps, but on the other hand, it has the advantages of being time saving and easy to perform. The

specificity to AIV was also tested using several common diseases, including IB, ND, ALV-A, ALV-B, ALV-J, and REV. No cross reaction was found on NP-ICS. Due to the feature of the NP-ICS, it can be developed as a non-instrument rapid diagnosis kit to apply to the field for quick surveillance.

Table 1 Comparison of the NP-ELISA and NP-ICS with hemagglutination inhibition test.

The present tests		Hemagglutination inhibition test	
		+	—
NP-ELISA	+	78	3
	—	0	85
NP-ICS	+	83	7
	—	3	57

In conclusion, we developed a rapid diagnostic kit for detecting antibodies against at least AI H5 and H6. This NP-ICS kit does not contain viruses, which can eliminate the concerns with virus dissemination when operating at farms. For AI surveillance, quick screening in the field is important and this quick and easily operated method can be used at pen-sites.

Conflict of interest

The authors have no conflicts of interest to declare.

Acknowledgments

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應用桿狀病毒表現核蛋白質以免疫色層分析試片檢測家禽流行性感冒抗體

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摘 要

因禽流感抗體遠比病毒存在雞隻體內時間久，故檢測抗體較檢測病毒較易檢測出陽性雞群，為監測中最常使用的方法之一，因此本研究目的在於建立免疫色層分析試片檢測禽流感抗體。選殖 H5N2 禽流感病毒之核蛋白質基因，於桿狀病毒系統表現禽流感病毒之核蛋白質，作為抗原進行酵素連結免疫吸附法檢測雞隻禽流感抗體，依血球凝集抑制抗體為金標準，以此重組蛋白質建立之間接型 ELISA 測試，結果此 ELISA 敏感度為 100%，特異度為 96.6%。此外此重組核蛋白質應用於免疫色層分析試片的敏感度為 93.2%，特異度為 87.5%。結果顯示本禽流感檢測方法可用於檢測至少 H5 及 H6 亞型之 AI 抗體，可以應用於現場快速檢測禽流感抗體。

關鍵詞：禽流感抗體、桿狀病毒表現系統、雞、免疫色層分析、核蛋白質

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