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# Immunochromatographic strip assay development for avian influenza antibody detection

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## Abstract

To detect antibody on pen-side is a rapid way to know the avian influenza (AI) infectious status in a chicken flock. The purpose of this study was to develop an immunochromatographic strip (ICS) assay to detect the antibody against the AI virus (AIV) for field applications. The ICS was constructed by fixing an AIV strain A/chicken/Taiwan/2838V/2000 (H6N1) onto a nitrocellulose membrane as the antigen at the test line and goat anti-rabbit IgG antibody at the control line. The colloidal gold conjugated with rabbit anti-chicken IgG was used as the tracer. The present ICS was used to detect antibodies against avian influenza virus in 326 chicken serum samples from the field. Compared with HI, this ICS could detect antibodies against H5 and H6 AIVs. The hemagglutination inhibition (HI) test was used as the standard to evaluate the ICS accuracy. The results showed that the sensitivity and specificity of this ICS reached 95.2% (159/167) and 94.3% (150/159), respectively. The Kappa value of the HI and ICS was 0.896 ( $P < 0.001$ ). In conclusion, this ICS could be used as a rapid test to detect antibodies against AIVs in the field.

Key Words: avian influenza virus, antibody, colloidal gold, immunochromatographic strip

Abbreviations: AI = avian influenza; AIV = avian influenza virus; HA = hemagglutinin; HI = hemagglutination inhibition; HP = highly pathogenic; ICS = immunochromatographic strip; IEP = isoelectric point; LP = low-pathogenic; NP = nucleoprotein; PBS = phosphate buffer saline; SPF: specific-pathogen-free

## Introduction

Avian influenza (AI) is a highly contagious disease caused by the avian influenza virus (AIV).

AIVs are divided into low-pathogenic (LP) AIV and highly pathogenic (HP) AIV according to its pathogenicity index for chickens<sup>16)</sup>. Although HPAIV causes death of chickens, some remaining

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birds or chickens infection with LPAIV show antibody conversion<sup>1)</sup> and the antibody lasts much longer than viruses after infection<sup>5,7,16)</sup>. Thus, to detect antibody is easier to know the AIV infection status in the field.

It is well known that the hemagglutination inhibition (HI) test is a gold standard serological assay for the detection of AI antibodies, but it is laborious, requiring a continuous source of appropriate erythrocytes, and trained personnel to perform the procedures<sup>16)</sup>. Another method commonly used is the enzyme-linked immunosorbent assay, which takes 3–5 hours to obtain the data. Most importantly, farmers like to know the AI infection condition in the field immediately. Thus, a rapid and simple antibody detection method is needed for routine field testing on poultry farms.

The Immunochromatographic strip (ICS) test is a lateral flow immunological technique<sup>13)</sup>. This technology has several advantages over traditional immunoassays, such as simplicity of procedure, rapid operation, immediate results, low cost, no requirements for skilled technicians and expensive equipment. It is suitable for the pen-site detection of antibodies<sup>11)</sup>. Here we report an ICS coated with inactivated AIV for the rapid diagnosis of AI antibodies.

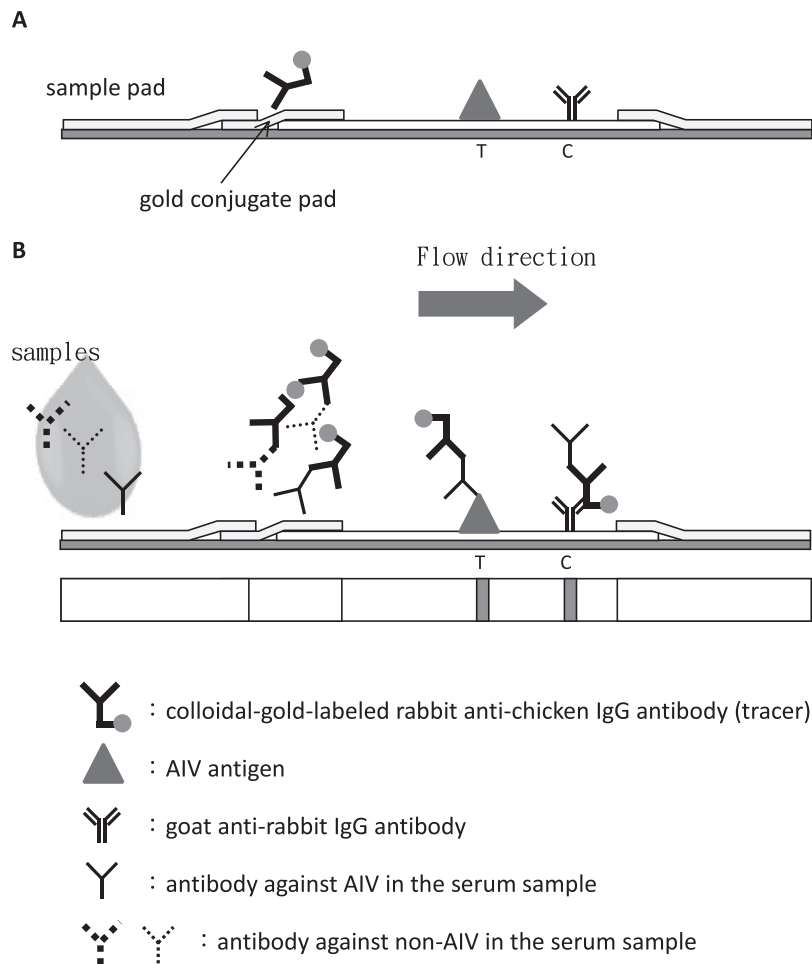
## Materials and Methods

*Virus preparation:* A/chicken/Taiwan/2838V/2000 (H6N1)<sup>17)</sup> was propagated in the allantoic cavity of specific-pathogen-free (SPF) chicken embryos (Animal Health Research Institute, Danshui, Taiwan) and inactivated with 1% of 0.1 M 2-bromoethylamine hydrobromide (Sigma, St Louis, MO) at 37°C for overnight. Cell debris was centrifuged out at 4,000 rpm × 30 mins at 4°C. The dead virus confirmed by egg inoculation was centrifuged at 70,000 g × 2 hours and purified in gradient sucrose at 50,000 rpm for 2 hours. The virus band was taken in TEN buffer (10 mM Tris base, 1 mM EDTA, 0.1 M NaCl) and centrifuged

again. After centrifugation, the pellet was dissolved in 500 µL TEN buffer for coating onto the strip. For the HI test, two virus strains, A/duck/Yunlin/2004 (H5N2) and A/chicken/Taiwan/2838V/2000 (H6N1) were used according to the method described<sup>1)</sup>.

*Isoelectric focusing:* The isoelectric point (IEP) of the rabbit anti-chicken IgG (H+L) (AffiniPure, Jackson ImmunoResearch Lab Inc, Uppsala, Sweden) was determined by isoelectric focusing gel in an automatic flatbed electrophoresis system (PhasGel IEF 3-9, GE Healthcare Life Science, Uppsala, Sweden) according to the manufacturer's procedures. The proteins were separated in the gradient gel zone according to their charge.

*Preparation of colloidal gold-labeled IgG:* The colloidal gold (40 nm) (Rega Biotechnology Inc, Taipei, Taiwan) was adjusted to pH 6, 7, and 8 with 10% HCl and 0.2 M K<sub>2</sub>CO<sub>3</sub>. Two hundred µL of rabbit anti-chicken IgG at 0, 5, 10, 20, 25, 30, 35, 40 µg/200 µL was added into 1 mL of colloidal gold. Two hundred µL of 10% (w/v) NaCl was added into each mixture. After 5 mins of standing, triplicates of 200 µL of each mixture were measured at 580 nm in a spectrophotometer (SpectraMax M5/M5e microplate reader, Molecular Devices Inc, Sunnyvale, CA). A coagulation curve was obtained to evaluate the minimal quantity of IgG required to stabilize gold against the coagulating effects of NaCl and to determine the effect of the quantity of IgG added, the pH of colloidal gold, and the desorption of IgG from gold. After determination, 2.5 mL of bovine albumin (Sigma, St Louis, MO) was added for another 30 mins to block the excess colloidal gold reactivity. The mixture was centrifuged at 7,000 rpm for 45 mins at 4°C. Filtered borax buffer (2 mM borax buffer containing 0.1% PEG-2000, pH 9) was used to wash the precipitate for 2 times. Finally 1 mL of borax buffer was added to become colloidal gold-labeled rabbit antibody solution.



**Fig. 1. Schematic diagram of Immunochromatographic strip procedures for anti-AIV antibody detection.** A) The gold-labeled rabbit anti-chicken IgG antibody is put in the gold conjugate pad. Concentrated AIV is immobilized at the test line (T) and goat anti-rabbit IgG antibody is immobilized at the control line (C). B) The chicken antibodies in the serum samples are bound by tracer in the sample pad and flow to the test line, at which the specific anti-AIV antibody is captured by immobilized AIV, producing a color line. The residual tracers are captured by goat anti-rabbit IgG antibody, producing a colored line in the control region. The strip showing two lines is positive to anti-AIV antibody.

*Immunochromatographic strip assembly and procedures:* The strip was consisted of five elements (Fig. 1A): the sample pad, the gold conjugate pad, the analytical nitrocellulose membrane, the absorbent pad, and the backing plate (Rega Biotechnol Inc, Taipei, Taiwan). The sample pad and conjugate pad were treated with 20 mM phosphate buffer saline (PBS) containing 1% bovine serum albumin, 0.5% Tween-20, 0.05% sodium azide, 5% sucrose (pH 7.4) for 30 mins and dried at 37°C. The ICS procedure was made according to the schematic diagram in Fig. 1B. Checkboard tests were conducted to obtain the

optimal test conditions. Different volumes (1, 2, 3, 4  $\mu$ L) of the tracer containing gold-labeled rabbit anti-chicken IgG antibody were absorbed in the conjugate pad. Different concentrations of A/chicken/Taiwan/2838V/2000 (H6N1) (6, 8, 10  $\mu$ g/mL) at the test spot and goat anti-rabbit antibody (0.5  $\mu$ L of 62.5, 125, 250, 500  $\mu$ g/mL) (AffiniPure, Jackwon immunoResearch Lab Inc, West Grove, PA) at the control spot were immobilized on the nitrocellulose membrane. The distance between the test spot and the control spot was 5 mm.

A standard chicken anti-H6N1, A/chicken/Taiwan/2838V/2000 antiserum was used to set up

the optimal condition by checkerboard titration. The antiserum against H6N1 AIV was made in nine SPF chickens (Danshui, Taiwan) and reared in isolators kept in separate rooms (the use of chickens were approved by the Institutional Animal Care and Use Committee, National Taiwan University). Each seven-week-old chicken was inoculated intranasally and intramuscularly with  $10^8$  EID<sub>50</sub> of A/chicken/Taiwan/2838V/2000 (H6N1). The chickens were re-inoculated with the same virus by the same routes two weeks after. Blood was obtained three weeks after the last inoculation, and the pooled serum was heat-inactivated for the following tests. After checkerboard testing, a test spot was formed by dragging a pipette tip containing concentrated virus (6 µg/mL) onto the nitrocellulose membrane. A control spot was formed by dragging goat anti-rabbit IgG antibody (250 µg/mL). Mixture of 180 µL of tracer and 660 µL borax buffer was added to the dried conjugate pad. Those dried pads and membrane were assembled as a test strip for antibody detection. The serum samples were diluted at 1 : 30 ratio with PBS and the reading time was fixed at 15 mins at room temperature for the following tests. Alternately, the test and control lines were printed by a high speed printer (Agismart RP-100, Rega Biotechnol Inc, Taipei, Taiwan) and dried in air for further uses.

*Detection limit and analytic specificity of ICS:* A standard anti-H5 serum from an H5N2-infected broiler breeder farm and a standard anti-H6 serum from another H6N1-infected chicken farm were used to determine the detection limit of the ICS. Those farms were not vaccinated with any AI vaccines because vaccination is not allowed in Taiwan. They were confirmed by serological and virological detections before onsets. These sera were collected from AIV-infected farms using virus isolation four weeks after onset. The sera were diluted with PBS at 1 : 30 ratio initially and then two-fold serial dilutions with PBS for to determine the detection limit.

Several anti-avian pathogen antibodies were

tested using this ICS to evaluate the analytic specificity. Those sera included anti-infectious bronchitis virus, anti-Newcastle virus, anti-avian leucosis subgroup A, anti-avian leucosis subgroup J, and anti-reticuloendotheliosis virus (Charles River Lab, Wilmngton, MA).

*Serum samples:* Three hundred and twenty-six chicken serum samples were collected from broiler breeder farms in Central and Northern Taiwan (Table 1). The chickens non-infected or infected with H5 or H6 were confirmed using virus isolation. Besides 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<sup>1,2</sup>. Those sera were measured by HI tests with H5 (A/duck/Yunlin/2004, H5N2) and H6 (A/chicken/Taiwan/2838V/2000, H6N1) AIVs separately. The serum samples were considered positive if there was inhibition at a serum dilution of 1/16 or more against either AIV. Serum samples were diluted at 1 : 30 ratio for ICS test. A single colored spot or line appearing in the control region indicated the absence of anti-AIV antibody. The concurrent presence of colored spots or lines in both control and test regions indicated the presence of anti-AIV antibody in the sample. The sensitivity and specificity of the ICS were calculated based on HI tests. The agreement between the ICS and HI was evaluated with Kappa statistic<sup>14</sup>.

## Results

*Binding of colloidal gold with IgG:* The IEP of rabbit anti-chicken IgG antibody (H+L) was determined to be about 6.55 by isoelectric focusing, similar to previous results<sup>6</sup>. The pH of adsorption, the IEP of the IgG population, and the quantity of the rabbit IgG added were important in the production of protein-gold complexes. The coagulation curves showed that gold auto-coagulated less when the amount of the IgG

**Table 1. Comparison of anti-AIV antibodies in chicken sera using HI and ICS**

Case no.	AIV infection	Week age	H5 HI	H6 HI	ICS
3695	Yes <sup>a</sup>	73	11/11 <sup>b</sup>	11/11	10/11
3799	Yes	45	0/20	20/20	16/20
3800	No	60	0/20	0/20	1/20
3801	Yes	47	3/20	20/20	19/20
3802	Yes	62	0/20	10/20	2/20
3803	No	25	0/10	0/10	0/10
3804	No	24	0/10	0/10	3/10
3806	No	13	0/10	0/10	0/10
3807	No	30	0/10	0/10	0/10
3808	No	32	0/10	0/10	0/10
3809	Yes	53	0/10	10/10	8/10
3817	Yes	58	0/6	6/6	6/6
3818	No	29	0/9	0/9	0/9
3819	Yes	49	20/20	0/20	20/20
3820	Yes	53	20/20	1/20	20/20
3830	Yes	36	1/10	10/10	10/10
3831	Yes	36	2/10	10/10	10/10
3832	Yes	39	0/10	10/10	10/10
3833	Yes	39	0/10	10/10	10/10
3834	Yes	56	20/20	0/20	20/20
A4905	No	32	0/10	0/10	0/10
A4908	No	32	0/10	0/10	1/10
A4909	No	31	0/10	0/10	1/10
A4911	No	19	0/10	0/10	0/10
A4913	No	18	0/10	0/10	1/10
A4915	No	18	0/10	0/10	0/10

a. The chickens non-infected or infected with H5 or H6 were confirmed by virus isolation.

b. Number positive/number tested.

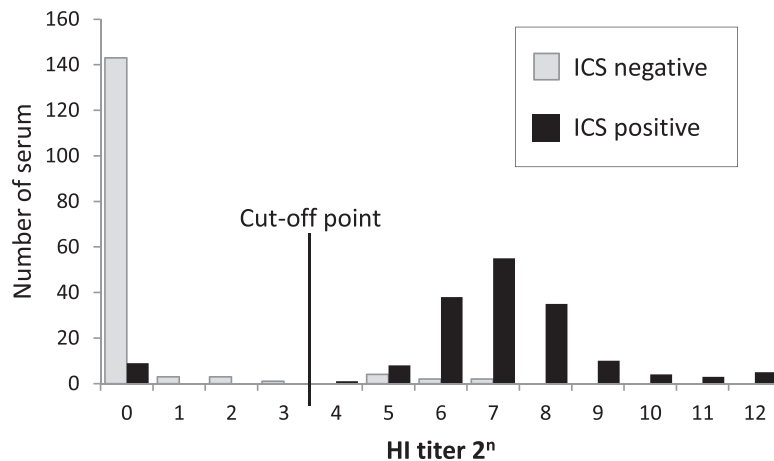
increased to 10 µg/200 µL or more. The coagulation curves decreased from 5 to 10 µg/200 µL and then reached stable afterwards. To minimize the amount required of IgG to stabilize colloidal gold, 10 µg of IgG at pH 8 was select to stabilize 1 mL of colloidal gold for the labeling.

*Detection limit and analytic specificity of ICS:*

One anti-H5 positive chicken serum (anti-H5 HI: 2<sup>8</sup>, anti-H6 HI: 2<sup>2</sup>) and one anti-H6 positive chicken serum (anti-H5 HI: 2<sup>2</sup>, anti-H6 HI: 2<sup>8</sup>) were serially diluted with PBS after initial 1 : 30 dilution. Each diluted sample was tested by ICS. The result showed that the detection limit of the

present ICS was measured by testing the positive serum at 1 : 60 dilution (1 : 2 dilution after the initial 1 : 30 dilution). Thus, the detection limit of this ICS was about 4 HI titer. The sera against other avian virus pathogens were negative by this ICS. So this ICS was specific to AIV antibody detection.

*Serum samples:* The anti-AIV antibody in 326 chicken serum samples were measured by HI tests with H5 (A/duck/Yunlin/2004, H5N2) and H6 (A/chicken/Taiwan/2838V/2000, H6N1) AIVs. The serum samples were considered positive showing antibody positive against either H5 or



**Fig. 2. Hemagglutination inhibition titer frequency distribution of Immunochromatographic strip (ICS)-positive and -negative sera. The cut-off point is  $2^4$  HI.**

H6 AIVs. Among 326 samples, 167 of them were positive and 159 were negative to AIV by HI. Among 167 positive serum samples, 159 of them showed positive by ICS and among 159 negative serum samples, 150 of them showed negative by ICS. The HI titer frequency distribution of ICS-positive and ICS-negative sera is shown in Fig. 2. The sensitivity and specificity of the ICS based on HI test were 95.2% (159/167) and 94.3% (150/159), respectively. The kappa value of these two tests was 0.896 with excellent reproducibility between HI and ICS ( $P < 0.001$ ).

## Discussion

Surveillance of AI antibody is suitable for monitoring AIV infection status in chickens<sup>3,4</sup>. The presence of antibodies in chickens indicates virus infection since vaccination is not allowed in most countries, including Taiwan. Most commercially available rapid diagnosis kits and published paper are applied to detect virus antigen<sup>9,10,12,15</sup> but not antibody. It is well known that AIV lasts only 2–3 weeks after infection and disappears when antibody forms. Conversely, antibody lasts for several months and even years<sup>16</sup>. Thus it is easier to monitor AIV infection in poultry farms using antibody detection than to detect AIV. In addition, serology is useful as a

method to retrospectively assess the status of a flock. As we know, no commercially available antibody detection using ICS is provided for the moment. The rapid detection of AI antibody on pen-site in flocks using this ICS is significant for detecting AIV infectious status in the field.

Some chickens infected with LPAIVs showed no any clinical signs. Thus, the farmers like to know if their chickens got infection in the past. The present rapid test kit is necessary for them to understand the AIV infection conditions of their chickens. In addition, they like to know if antibody forms after vaccination if vaccination is permitted in a country.

The whole killed virus was used at the test line in the present ICS, which could catch the anti-nucleoprotein (NP) and/or anti-matrix protein antibodies from different influenza subtypes as the enzyme-linked immunosorbent assay and the agar-gel immunodiffusion test do. They are not subtype-specific but type-specific. Therefore, this ICS might be more useful for anti-AIV antibody detection than previous reports, which caught only anti-H5 antibody or anti-NP antibody because of the difference of capture antigens immobilized at the test line<sup>4,11</sup>.

To show hemagglutination, virus particles should be enough to form a lattice of virus-erythrocyte complex. The serum affects the lattice formation inhibits hemagglutination and

shows HI activity. So the HI is very sensitive for anti-AIV antibody detection. However, HI test has the disadvantages of requiring a long time and trained personnel. Enzyme-linked immunosorbent assay needs a micro-plate reader, which is not always available at field sites. Although the present ICS could not catch the antibody titers lower than 4 HI, these titers are considered to be negative according to OIE standard. Thus, the lower detection limit of the ICS is accepted. Although lower detection limit than HI, this ICS provides a rapid pen-side antibody detection method in a short time without the need for any laboratory equipment. The proposed method is a useful tool for farmers to determine the infectious status of their chickens. Performing HI requires AIVs and some facilities, which are not always available in remote areas and developing countries. Under such circumstances, the present ICS would be a practical adjunct for pen-site diagnosis.

The present ICS might detect more anti-nucleoprotein (NP) antibodies than anti-hemagglutinin (HA) antibodies since the whole AIV contains more NP than HA<sup>8)</sup>. The NP of AIV is a type-specific protein and very conservative antigen. It can induce strong immune responses. Although NP is different from hemagglutinin, both could be used for antibody reactions in infected chickens. So the present ICS could catch anti-H5 antibody in chicken sera using H6 AIV as the capture antigen at the test line.

There were nine HI negative sera showed ICS positive. The reason for this might be the non-specific binding of labeled rabbit anti-chicken IgG antibody with the whole virus at the test line. The interference of compounds present in serum similar in structure could be the reason of such non-specific binding. These nine false positive sera were from 6 different chicken flocks. Such false positive sera might not be an influence because they stand for a small amount in a flock resulting in a low positive rate. Our previous report showed that nearly one hundred percent of chickens in an infected flock have the antibody

two weeks after infection<sup>2)</sup>. Many of them show HI antibody titers higher than 2<sup>8</sup> which can be detected by the present ICS. We have successfully developed an ICS, which is rapid and easy to perform. The present ICS does not require equipment or skilled personnel and has great potential for field monitoring of antibodies against AIVs in the field.

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