A Simple and Rapid Strip Test for Detection of Antibodies to Avian Infectious Bronchitis Virus

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ABSTRACT. A simple gold-immunochromatographic assay (GICA) based on indirect reaction format was developed for rapid detection of antibodies against avian infectious bronchitis virus (IBV). The detection time of IBV IgG by the GICA was less than 10 min, whereas the HI test and the enzyme-linked immunosorbent assay (ELISA) require 1–2 hr. Reference sera against newcastle disease virus, infectious bursal disease, avian influenza virus H5 and H9 subtypes were all negatives for anti-IBV antibodies using the GICA. Compared with the HI, the sensitivity of the GICA was 92.11% and specificity was 81.71%. The agreement rate between the 2 tests was 85%. Compared with the ELISA, the sensitivity of the GICA was 92.31% and specificity was 97.06%. The agreement rate between the 2 tests was 95%. The GICA test strip is a reliable and useful tool for the on-site surveillance of anti-IBV antibodies.

KEY WORDS: avian infectious bronchitis virus, gold-immunochromatographic assay, nucleoprotein, recombinant antigen.

Avian infectious bronchitis is an acute and highly contagious respiratory disease in chickens. It has already caused major health concerns in the poultry industry around the world [3]. This disease is caused by coronavirus infectious bronchitis virus (IBV), a member of the family Coronaviridae (order nidovirales) and genus Coronavirus [2, 12]. To date, IBV still causes significant impacts on commercial meat and egg birds [8, 11]. IBV primarily targets the trachea, but also kidney, oviduct, and other epithelial surfaces. In laying birds and broilers, infections with IBV can reduce the egg production. Vaccination is a key strategy for the prophylaxis and control of avian infectious bronchitis in poultry. Attenuated vaccines against infectious bronchitis are administrated to one-day-old chicks through drinking water or by spraying [4]. In many laboratories, hemagglutination inhibition (HI) test is the preferred serological assay for the detection of antibodies to IBV, but it is a laborious intensive test [8, 5]. Another common method is the ELISA, but this test is also time-consuming and requires trained personnel and special equipments. Hence, a rapid and simple test to monitor the antibodies induced by IBV vaccines is urgently needed for both routine field practice and in epidemiological surveillance. Recently, gold-immunochromatographic assay (GICA) is a relatively new technique in which a cellulose membrane is used as the carrier, and a colloidal gold-labeled antigen or antibody is used as the tracer [1, 6]. This technology has several advantages when compared to other traditional immunoassays, such as simplicity, speed, and low cost. In this study, we developed a simple GICA for rapid detection of antibodies against avian infectious bronchitis. The performances of GICA method were compared with the well-characterized HI test and the ELISA.

MATERIALS AND METHODS

Materials: Mouse monoclonal antibodies against the Fc fragment of chicken IgG (McAbFc) and recombinant nucleocapsid protein were prepared in our laboratory [9]. As described previously, nucleocapsid (N) protein was generated by expression of GST-N fusion proteins in E. coli, and the purification was performed using glutathione sepharose 4B (GE Healthcare, Buckinghamshire, UK). Product was determined by SDS-PAGE analysis and western blotting. The rabbit anti-mouse IgG was purchased from Sino-American Biotechnology Co. (Luoyang, China). Hydrogen tetrachloroaurate (III) hydrate (HAuCl₄·xH₂O) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Bovine serum albumin (BSA) was purchased from Roche (Indianapolis, IN, U.S.A.). Nitrocellulose membranes, glass fibers, sample pads, and absorbent pads were purchased from Millipore Corporation (Bedford, MA, U.S.A.). The IDEXX FlockChek Infectious Bronchitis Virus Antibody Test Kit was purchased from Beijing Idexx-Yuanheng Biotechnology Corporation (IDEXX Laboratoires Inc., Westbrook, ME, U.S.A.). The IBV was maintained in our laboratory. Forty-three chicken serum samples including twenty-three positive serum samples (one sample was prepared by M41 strain of IBV inactivated vaccine, nineteen samples from the chickens confirmed cases of clin-
ical) and twenty negative serum samples (Positive sera specific for and the antigens of subtypes H5 and H9 AIV were obtained from Harbin Veterinary Research Institute. Positive sera for newcastle disease virus and infectious bursal disease virus were purchased from the China Institute of Veterinary Drug Control. Sixteen serum samples were obtained from the wings of 4-week-old chickens reared in a specific pathogen-free (SPF) environment. A total of 120 chicken serum samples from different ages were collected in 12 flocks at three chicken farms of Hubei and Henan Provinces. The animals were vaccinated separately at intervals ranging from one month to five months.

Preparation of colloidal gold-McAbFc conjugates: Colloidal gold was prepared using a previously reported method with minor modifications [1, 9]. Briefly, 100 ml of 0.01% chloroauric acid aqueous solution (HAuCl₄) was heated to boiling under a rapid magnetic stirring, and 2 ml of 1% sodium citrate solution was added to the solution. After an additional boiling for 10 min, the colloidal gold was gradually cooled, and was then stored at 4°C in a dark colored glass bottle until use.

To prepare colloidal gold-McAbFc, the McAbFc [10] was first equilibrated with 2 mM borax buffer (pH 9.0) at 4°C. Subsequently, 0.4 mg of the McAbFc in 0.5 ml distilled water was added dropwise in 20 ml of gold colloid solution, and the pH was adjusted to 8.0. The mixture was stirred vigorously for 30 min and then 5 ml of 5% (w/v) bovine serum albumin (BSA) aqueous solution was added to block excess reactivity of the colloidal gold, followed stirring for 30 min. After centrifugation, the resultant McAbFc-CGC pellet was washed and re-suspended in 1 ml of 2 mM borax buffer (pH 9.0) containing 0.1% (w/v) PEG-2000.

Assembly of the gold-immunochromatographic test strip: The GICA test-strip consisted of four main elements: the sample pad, the conjugate release pad, the nitrocellulose membrane, and the absorbent pad. The sample (absorbent paper) and conjugate pads (glass-fiber membrane) were treated with 20 mM phosphate buffer (pH 7.4) containing 2% BSA, 0.5% Tween-20, and 0.05% sodium aside and dried at 37°C. Thereafter, the N protein (0.6 mg/ml) and the rabbit anti-mouse antibody (1 mg/ml) were loaded onto the nitrocellulose membrane to the test and the control line using the BioDot XYZ Platform at a jetting rate of 0.9 ml/cm and then dried at 37°C. The McAbFc-CGC was loaded on the treated conjugate pad at 0.4 ml/cm, and then the pad was lyophilized. The absorbent pad, nitrocellulose membrane, pretreated conjugate pad, and sample pad were assembled to form the strip and attached to a plastic scale-board. The assembly was cut into 60-mm long and 3-mm wide strips. The GICA kit consisted of a bag of gold-immunochromatographic test strips (10 strips/bag), a bottle of sample dilution buffer (10 ml/bottle) and a micropipette.

Test strip result judgments: In the GICA assay, if two red band colors are visible with positive samples, and the intensity of the band color on the test line is proportional to the concentration of the anti-IBV antibody (Fig. 1A, test line OD: 0.251. Read the test strip with Bio-Rad GelDoc2000). If there is no IgG antibody against IBV in the sample serum (negative test), only the band color at the control line will be visible (Fig. 1B, test line OD: 0.000). Evaluation of the test-strip results can be achieved by naked-eye with an assay time less than 10 min.

Specificity, sensitivity, and stability of the GICA: The specificity of the GICA was evaluated with 16 standard negative serum samples from SPF chickens; standard serum samples positive for no-IBV pathogens (the pathogens for ND, IBD, AIV-H5, and AIV-H9); serum samples positive for IBV. The analyses focused on forty-three chicken serum samples at 1:16 ratio by 2 M sodium chloride. 0.1 ml of diluted serum sample was added into the sample pad for analysis by the GICA, as described above. Then, the results were visualized within 10 min at room temperature.

The sensitivity test for GICA was conducted using serially diluted anti-IBV serum. The positive serum with an HI titer of 2⁸ (ELISA OD 450 1.052) was tested over a range of dilutions (from 1:4 to 1:512); sera were diluted with PBS and tested with the GICA as above. The same procedure was repeated three times with different operators.

The stability of the GICA test was determined by using one IBV-positive (HI titer 2⁸, ELISA OD 450 1.031) and one negative sample. Sera diluted at 1:16 ratio were added to sample ports of strips. For both positive and negative samples, 30 strips were stored per time point. Strips were tested after storage at 3, 6, 9, 12, 15, and 18 months at 4°C.

Analysis of consistency among GICA, ELISA, and HI test: The GICA kit was equilibrated at room temperature and
then 0.01 ml of the serum or diluted serum was mixed with 0.15 ml of dilution buffer in the sample pad. The sample pad of an individual testing strip was dipped into the sample solution and left for 10 min until a red band appeared at the control line. The tests were repeated for 5 times in laboratory by different personnel.

The HI test has been described previously [4]. Briefly, 0.025 ml of PBS was dispensed into each well of a plastic V-bottomed microtiter plate and 0.025 ml of serum was added into the first well of the plate before a 2-fold dilution was made. Four hemagglutination units of virus/antigen (0.025 ml) were added to each well, and the reaction was incubated for 30 min at room temperature. Subsequently, 0.025 ml of 1% (v/v) chicken red blood cells (RBCs) was added to each well and gently mixed; the RBCs were allowed to settle for about 40 min at room temperature. The HI titer was determined as the highest serum dilution leading to a complete inhibition of hemagglutination.

The ELISA (IDEXX Laboratories, Inc.) was realized following the manufacturer’s protocol using an automated microplate reader (Multiskan MK3, Thermo Electron Corporation, Vantaa, Finland). The software provided by the manufacturer was used to determine the antibody titer in each individual sample and to calculate the geometric mean titer (GMT) for the group of serum samples collected from each flock. Positive and negative control antisera were provided in the kit and were used in each run.

RESULTS

Each of the 16 SPF chicken serum samples, reference sera against AIV-H5, H7 and AIV-H9, NDV, and IBDV, were negative for anti-IBV antibodies using the GICA. The results were similar with the blank control, which had only one red band at control line. The known positive sera gave a positive result. The results were similar with the blank control which had only one red band at control line. The sensitivity of GICA test was tested with anti-IBV serum diluted serially. Red bands developed at the test line and at the control line at dilutions up to and including 1:256 (data not shown). This indicates that the GICA test has a high sensitivity and can detect a small amount of anti-IBV antibody.

Storage at 4°C of the prepared test strips did not adversely affect the assay (data not shown). Indeed, samples stored up to 15 months gave identical results to samples tested immediately after collection. What’s more, between 15 months and 18 months, 10% of the previous positives became negative. After 18 months, 18% of positives were negative. False positive was not observed (Fig. 2).

One hundred twenty chicken serum samples were tested by the GICA and by HI test. The HI test indicated 38 positives (inhibition index, >4Log2) and 82 negatives (inhibition index, <4Log2). In the GICA test, 50 samples were positive and 70 were negative. Of the 50 samples that tested positive by the GICA, 35 were positive and 15 were negative by the HI test. Compared with the HI test, the sensitivity of the GICA test was 92.11% and specificity was 81.71%. The agreement between two tests was 85% (Table 1). Compared with the ELISA, the sensitivity of the GICA test was 92.31% and specificity was 97.06%. The agreement between two tests was 95%. The detection of anti IBV IgG using the GICA test strip took was less than 10 min, whereas the HI test and the ELISA required 1-2 hours. On the detection of one sample, the cost of GICA is lower with the

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<th>Table 1. Comparison of the GICA test to the HI test and the ELISA using sera submitted for routine diagnostic evaluation</th>
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In total, the HI test results were positive for 38 samples and negative for 82 samples, whereas the GICA test results were positive for 50 samples and negative for 70 samples. Sensitivity of the GICA test compared to HI was 92.11% (35 of 38). Specificity of the GICA test was 81.71% (67 of 82). Agreement between the GICA and HI test was therefore 85% [(35 + 67)/120].

In total, the ELISA test results were positive for 52 samples and negative for 68 samples whereas the GICA test results were positive for 50 samples and negative for 70 samples. Sensitivity of the GICA test compared to ELISA was 92.31% (48 of 52). Specificity of the GICA test was 97.06% (66 of 68). Agreement between the GICA and ELISA test [(48 + 66)/120] = 95.00%.
ELISA (the GICA: about 0.073 dollars; the ELISA: about 1.459 dollars). The GICA test strip is a reliable and rapid useful tool for the on-site surveillance and rapid diagnosis of anti-IBV antibodies.

DISCUSSION

Currently, vaccination is a key strategy for the prevention and control of avian infectious bronchitis in poultry, but vaccination is not always successful. Therefore, a rapid and simple test is required for routine field monitoring of vaccine-induced antibodies to IBV [7]. In this study, a GICA test using colloidal gold-coated the antibody against IBV was developed and validated. Compared with the HI test, GICA exhibited excellent specificity and good sensitivity for detecting antibodies to IBV in serum samples from vaccinated chickens. The GICA approximated to the sensitivity and specificity ELISA. The HI test and ELISA are common methods for monitoring the antibody titer in vaccinated chickens. Although ELISA and the HI test are simple and easy to perform, there are time-consuming and requires also skilled personnel.

Results of this study demonstrated that the GICA developed was highly sensitive and specific for detecting IBV antibodies in serum samples from vaccinated chickens. The GICA is also rapid and easy to perform and does not require specialized equipments or skilled personnel. Furthermore, the bio-safety of the GICA is higher than other serological methods as the core antigen of the GICA is the GST-N fusion protein. Indeed, with the ELISA kit, typical reagents such as 3,3′,5,5′-tetramethyl benzidine dihydrochloride (TMB) may cause negative health effects to the laboratory personnel. With GICA test kit, only one dilution buffer (10% NaCl solution) was required. In addition to offering portability, its high stability renders it suitable for monitoring antibodies to IBV vaccines in veterinary field.

REFERENCES