Journal of Chromatography B 1095 (2018) 59–64

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/jchromb

Development of immunochromatographic test strips for rapid, quantitative detection of H9AIV antibodies☆

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ARTICLE INFO

Keywords:
H9 subtype of avian influenza virus
Double-antigen sandwich method
Colloidal gold
Immunochromatographic assays
Quantitative detection of antibodies

ABSTRACT

To improve viral antibody detection and disease control, laboratories need faster and simpler methods for direct detection of H9AIV in clinical samples. In this study, test strips were developed for rapid detection of H9AIV antibodies in poultry serum by a sandwich method with double-line detection. The hemagglutinin protein was labeled with colloidal gold as a detection reagent and was blotted on the test lines, while goat anti-rabbit IgG was utilized on the control line of the nitrocellulose membrane. The test strips have high specificity, sensitivity, and stability, with a correlation coefficient of 0.9656 and coefficients of variation below 10%. Application of the kit to quantitative detection of H9AIV antibodies in 504 samples collected from chickens showed a coincidence rate of 80.56% with previously run HI assays.

1. Introduction

In 1994, H9AIV was first isolated from chickens in Guangdong province in mainland China. Afterwards, the virus spread rapidly in China and has become the most prevalent avian influenza virus (AIV) in poultry. Most infected chickens show symptoms, such as edema, diarrhea, and mild respiratory signs. Vaccine immunization is an effective means of prevention, and antibody surveillance of the poultry population after vaccination is indispensable to avoiding occurrence of the disease. Antibody surveillance is the most direct and effective method for evaluating the immune effect and assessing epidemic dynamics. Therefore, antibody level monitoring has become routine in poultry farms and diagnostic laboratories [1,2].

Hemagglutinin (HA) is an important protective viral surface antigen that induces production of neutralizing antibodies and is utilized in many serological assays, such as agar gel immunodiffusion and ELISA. The sequence of the HA protein used in this study can be found under the id AKD00891.1 in GenBank [3].

Detection of antibodies in sera by colloidal gold immunochromatographic assays only provides qualitative or semi-quantitative results, and cannot determine specific antibody titers, detect infection, or recognize when secondary immunity is acquired. Presently, antibody levels in vaccinated animals are measured by the hemagglutination inhibition assay (HI) or ELISA, and these methods are quantitative; however, they are inconvenient and unsuitable for rapid, on-site detection of antibodies. These methods require skilled experimental operating personnel and several hours to complete [4–6].

In this study, the H9AIV HA protein was used as the capture reagent on a detection line to develop quantitative test strips for detecting H9AIV antibodies. The strips were integrated with a quantitative immune detector to provide an easy method of monitoring antibody levels. Real-time, quantitative detection of antibody levels was realized by measurement of the signal ratio between the detection and control lines. The more antibodies in the sample, the more gold–antigen complex bound to the capturing antigen, leading to higher signals. The test strips provide scientific and technical support for the prevention and

Abbreviations: AIV, avian influenza virus; HI, hemagglutination inhibition assay; BSA, bovine serum albumin; HA, hemagglutinin; HN, hemagglutinin-neuraminidase; NDV, Newcastle disease virus; IBV, infectious bronchitis virus; IBDV, infectious bursal disease virus; ELISA, enzyme-linked immunosorbent assay; SPF, specific pathogen-free; CV, coefficient of variation

☆ Declarations of interest: none.

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https://doi.org/10.1016/j.jchromb.2018.07.024
Received 26 March 2018; Received in revised form 7 July 2018; Accepted 20 July 2018
Available online 21 July 2018

control of AIVH9 and also provide a reference basis for rapid and quantitative monitoring of antibodies against other diseases.

2. Material and methods

2.1. Material

Hydrogen tetrachloroaurate hydrate, sodium citrate, and bovine serum albumin were purchased from Sigma-Aldrich (St. Louis, MO, USA). HRP-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were purchased from Wuhan Boster Biological Technology Co., Ltd. (Wuhan, China). Chloroauric acid was purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Nitrocellulose membranes, Fusion 4 glass fibers, Ahlstrom8964 sample pads, and H5073 absorbent pads were purchased from EMD Millipore Co. (Billerica, MA, USA). The UV-2910 ultraviolet spectrophotometer was purchased from Hitachi (Tokyo, Japan). HM3035 HYZ three-dimensional dispensing platform, and CTD300 guillotine cutter were purchased from Shanghai Kinbio Tech Co., Ltd. (Shanghai, China). The HR8000 quantitative immune detector was purchased from Hubei Huada Real Science & Technology Co., Ltd. (Wuhan, China).

2.2. Expression and purification of H9AIV hemagglutinin

The cDNA for the H9AIV hemagglutinin-neuramidinase gene was obtained from H9N2 AIV by RT-PCR and cloned into an expression vector in *Escherichia coli* to generate the recombinant plasmid pSmart-H9-HA.

Potential positive clones were examined with BamH I and *Xho* I digestion. The recombinant protein was induced in a large amount and purified. The protein concentration was calculated using BSA as a standard, and the protein solution that reached the required concentration was identified by SDS-PAGE and western blot assays.

2.3. Preparation of quantitative test strips for H9AIV antibody detection

A test strip consisted of an absorbent pad, nitrocellulose membrane, conjugate pad, sample pad, adhesive backing, and base cover, and was based on a sandwich method with double-line detection (Fig. 1).

Nitrocellulose membranes were coated with 0.5 mg/mL goat anti-rabbit IgG on the control line and 1.0 and 0.8 mg/mL recombinant HA on the test lines (T1, T2), respectively, using HYZ three-dimensional zoned film spray at a rate of 0.9 μL/cm and dried at 37 °C. The conjugate pad was treated with a desired volume of Tris buffer (pH 8.2) and sprayed with the gold-labeled goat anti-rabbit IgG and HN at a rate of 6.5 μL/cm. The absorbent pad, nitrocellulose membrane, conjugate pad, sample pad, and adhesive backing were assembled into test strips, which were cut into 4 mm wide section.

The test results were judged according to the signal ratio between the detection and control lines as measured by a quantitative immune detector. Comparing the color depth, lateral wicking rate, striations or intensity of lines, membrane background color, and other properties of different test strip formulations, we chose pure water for sample dilution, Na_2HPO_4 as the basic coating solution, TBS buffer as the blocking solution, Fusion4 as the conjugate pad, Ahlstrom8964 as the sample pad, and H5073 as the absorbent pad for further study. When positive samples were placed on the sample pads, the tracer in the conjugate pads dissolved and was taken up by capillary action, and three red bands appeared on the NC membrane. If the samples contained no target antibodies, no bands were observed in the test lines.

2.3.1. Preparation of colloidal gold solution and colloidal gold–HA conjugate

The colloidal gold solutions were prepared by reduction of chloroauric acid. The size and shape of the gold particles depends on the amount of reducing agent. The colloidal gold appears wine red to the naked eye, transparent with no precipitate or floating particles. The optimum concentration of antigen for colloidal gold labeling was 6 μg/mL. When the pH value is 7.0, colloidal gold and antigen bound with high optical density.

2.3.2. Quantitative immune detector

As a combination of colloidal gold immunochromatography and modern automated detection technology, the HR8000 quantitative immune detector accurately quantifies signals by converting color density of test and control lines into optical density, using a signal acquisition module, signal processing module, communication module, and additional modules. To account for changing parameters such as analyte concentration, immunoreaction time, sample matrices, and operation temperature, the signal ratio between the detection line and the control line was measured.

2.3.3. Establishment of quantitative standard curve

To prepare the standard curve of the test strips for H9AIV antibodies, H9AIV-positive reference samples were prepared in pure water at varying concentrations (HI titer) 2^{10}, 2^9, 2^8, 2^7, 2^6, 2^5, 2^4, 2^3, 2^2 and 2^1. The color intensities on the test lines were measured using the quantitative immune detector, and the data are expressed as relative optical densities. Quantitative test results show that the standard curve of the test strip refers to the same kind of strip at different concentrations (x), there is a color ratio [(T1 + T2) / C] (y). Collecting multiple points (x/y) can be fitted test strip concentration curve, when the sample is detected, the corresponding x value is calculated by obtaining the optical density ratio so as to achieve the purpose of quantitative detection.

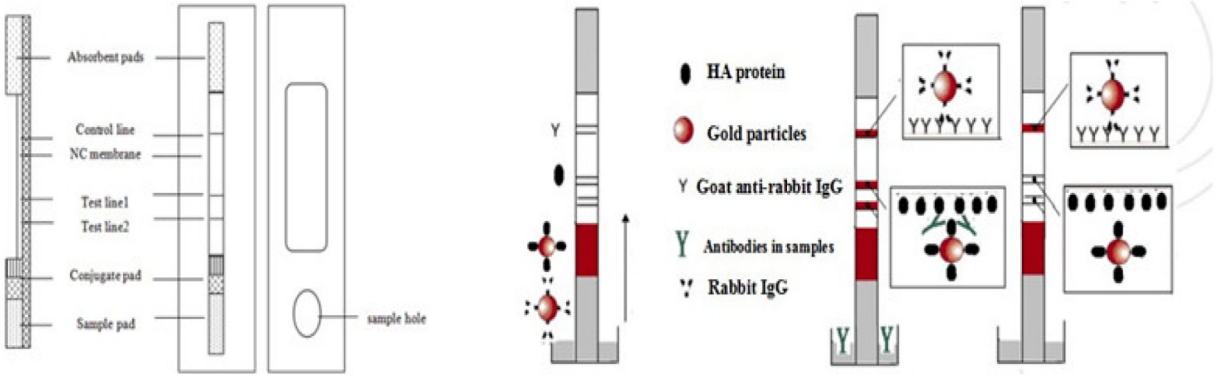

Fig. 1. Schematic diagram of the test strips. The test strip consists of a sample pad, a conjugate pad, a nitrocellulose membrane, and an absorption pad. The conjugate pad contains gold-labeled goat anti-rabbit IgG and HA (colloidal gold conjugate) and the nitrocellulose membrane is used as a chromatographic support on which the HN and goat anti-rabbit antibody are immobilized.