Journal of Virological Methods 179 (2012) 57–61

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

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

Segmentation expression of capsid protein as an antigen for the detection of avian nephritis virus infection in chicken flocks

W. Zhao[a,b], A.L. Zhu[a], Y. Yu[a], C.L. Yuan[a], C.X. Zhu[a], D.L. Lan[a,b], Z.B. Yang[a], L. Cui[a], X.G. Hua[a,*]

[a] School of Agriculture and Biology, Shanghai Jiaotong University, Room 3-217, Building of Agriculture & Biology, 800 Dongchuan Road, Shanghai, China
[b] Department of Laboratory Animal, Liaoning Medical University, Room 309, Building of Science Technology, No. 40 Songpo Road, Jinzhou, Liaoning Province, China

Article history:
Received 20 March 2011
Received in revised form
22 September 2011
Accepted 28 September 2011
Available online 5 October 2011

Keywords:
Avian nephritis virus (ANV)
Capsid protein
ELISA
Serological diagnosis

A B S T R A C T

Subclinical pathological changes in the kidneys of broiler chickens and suppression of growth caused by the avian nephritis virus (ANV) affect poultry flocks worldwide. A test for detection of virus-specific antibodies in serum would be useful for epidemiological investigations, however the poor propagation in cell cultures has restricted the development of serological tests based on the use of ANV particles as antigens. An enzyme-linked immunosorbent assay (ELISA) was developed for detection of ANV-specific antibodies in chicken serum, using a recombinant protein antigen prepared by segmentation expression of the capsid protein antigen epitope of ANV (HM029238) transfected into *Escherichia coli*. The expressed fusion protein was detected by Western blotting with ANV-positive serum, and the optimal immunoreactive fusion P1 protein was determined. Using the optimized P1-ELISA, ANV-specific antibodies were detected in commercial chicken flocks aged 10–25 days obtained from the Liaoning Province, China. Out of 960 serum samples, 459 (47.8%) were positive for infection with ANV. These results indicate that the P1-ELISA is helpful for preliminary serological diagnosis of ANV infection, and could be used to for screening in ANV infection and for determining antibodies against ANV.

1. Introduction

Avian nephritis virus (ANV) was first isolated in 1976 and is associated with mild growth suppression, kidney lesions, and mortality in young chickens (Yamaguchi et al., 1979). This virus was regarded originally as a Picornavirus, but genome sequence determination and analysis led to the classification of ANV as a member of the genus *Avastrovirus* of the family *Astroviridae* (Shirai et al., 1992; Imada et al., 2000).

ANV is known to cause growth suppression in young chickens as a result of interstitial nephritis (Shirai et al., 1991). ANV infection in chickens ranges from subclinical infection to death (Frazier et al., 1990; Shirai et al., 1991). Serological tests have shown that ANV infections are widespread in commercial chickens in Japan and several European countries (Pantin-Jackwood et al., 2006). ANV has also been detected repeatedly in broiler flocks with runting syndrome and in baby chick nephropathy (Shirai et al., 1992). ANV-specific antibody has also been detected in turkeys and specific pathogen-free (SPF) flocks (Gough et al., 1985; Connor et al., 1987).

Although ANV has been implicated in growth suppression, the nature of the disease and its severity remain unknown, due to the lack of convenient diagnostic tests (Todd et al., 2010). Knowledge of the ANV genomic sequence has allowed the development of reverse transcription-polymerase chain reaction (RT-PCR) for the detection of the virus (Todd et al., 2010; Mandoki et al., 2006; Day et al., 2007). Although PCR is useful for detection of ANV infection, a test for detecting virus-specific antibodies in serum would be more convenient for epidemiological investigations. However, as is the case with other *Astroviridae* members, ANV is difficult to isolate in cell culture due to its poor growth in primary chicken cells. The difficult propagation of ANV virus in cell cultures has restricted the development of serological tests, such as virus neutralization, indirect immunofluorescence, and enzyme-linked immunosorbent assay (ELISA), based on the use of ANV particles as antigens.

In the present study, the development and application of an ELISA that utilizes the recombinant ANV segment expression capsid protein antigen produced in *Escherichia coli* cells for the detection of ANV-specific antibodies in chicken serum are described.

2. Materials and methods

2.1. Biochemical reagents

The restriction enzymes *Eco*RI, *Xho*I, and T4 DNA ligase were purchased from Fermentas Life Sciences (Fermentas, Hanover, USA). Fast*Pfu* DNA polymerase, protein weight markers, BL21 (DE3) chemically competent cells, and *E. coli* DH5a were purchased from

* Corresponding author at: Group of Zoonosis and Comparative Medicine, School of Agriculture and Biology, Shanghai Jiaotong University, Room 3-217, Building of Agriculture & Biology, 800 Dongchuan Road, Shanghai 200240, China.
E-mail address: hxg@sjtu.edu.cn (X.G. Hua).

0166-0934/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2011.09.020

TransGen Biotech (TRANS, Beijing, China). Plasmid pET-30a$^+$ was obtained from Novagen (Madison, WI, USA). Ni-NTA His Bind purification agarose was obtained from GE Healthcare HisTrap^{TM} HP GE Healthcare (Chalfont St Giles, UK). Alkaline phosphatase (AP)-labeled goat anti-chicken serum was purchased from Zymed Laboratories, San Francisco, CA, USA. Positive and negative serum controls were obtained from ANV-infected chicken sera (using PCR for detection ANV RNAs in infected chicken stool) and SPF chickens (from the Shanghai Veterinary Research Institute, CAAS), respectively. Sera for chicken rotavirus, infectious bursal disease virus (IBDV), infectious bronchitis virus (IBV), avian adenovirus, and avian reovirus were stored in our laboratories. PCR tests showed that these were negative for ANV.

2.2. Bioinformatic prediction epitopes of ANV

ANV strain Sichuan54 (GenBank ID: HM029238) was detected from the stool sample of a layer in Sichuan Province, China, in 2009 (Zhao et al., 2011). DNAStar and DNAMAN software were used to predict the epitopes of the capsid protein of ANV. Based on an overall consideration of flexible regions, antigenic index, surface probability, hydrophilicity, and hydrophobicity, four epitope segments of ANV capsid protein were selected; P1: 10–104 aa, P2: 151–306 aa, P3: 359–466 aa, and P4: 555–681 aa.

2.3. Segment cloning and recombinant plasmid constructed

Four pair primers were designed to amplify the four gene segments of ANV ORF2.

The sequences are as follows: P1F: 5′-CCGGAATTCATGGGGGGC-ACCCGCCCTAAAACTCA-3′ (nt 28–50), P1R: 5′-CCGCTCGAGCATT-GAGGACCTGATTGAGAGCG-3′ (nt 317–339); P2F: 5′-CCGGAA-TTCATGGCTACTGCTGATTCTATTGATACC-3′ (nt 451–474), P2R: 5′-CCGCTCGAGCCAGATGGTCTCCGATTTCCCC-3′ (nt 895–918); P3F: 5′-CCGGAATTCATGGCAATGTCCGATCAGCCCATCTT-3′ (nt 1075–1097), P3R: 5′-CCGCTCGAGACCAAAAGGAGAAGTTGTGCCTG-3′ (nt 1376–1398); P4F: 5′-CCGGAATTCATGGACAAATCAACCAGAA-CTACATCT-3′ (nt 1666–1689), P4R: 5′-CCGCTCGAGG-ATAGTGAAGCGCTTTGCGTCGAT-3′ (nt 2017–2040). All upstream primers (F) contained *Eco*RI restriction enzyme sites and all downstream primers (R) contained *Xho*I sites.

The samples were subjected to PCR. Five microliters of the plasmid template were used. PMD18-T-ANV plasmids were constructed for sequence analyses of the representative Chinese-prevalent strain of avian nephritis virus and stored in our laboratories (Zhao et al., 2011). This sample was used in a 50-μL reaction mixture and amplified with PCR TaqMix (TaKaRa, Tokyo, Japan). The amplification program involved an initial 5-min step at 94 °C, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 40 s. At 72 °C, a final elongation step that lasted 5 min was performed. Five microliters of each PCR amplification product were analyzed by 2% agarose gel electrophoresis in the presence of ethidium bromide, and visualized under ultraviolet light. The RT-PCR products were digested with *Eco*RI and *Xho*I restriction enzymes, and inserted into the corresponding *Eco*RI/*Xho*I cloning sites of the digested pET-30a$^+$ vector to form recombinant constructs.

2.4. Protein expression and purification

The recombinant plasmids were transfected into *E. coli* BL21 (DE3) by heat shock and inoculated overnight in 10 mL of Luria–Bertani (LB) medium that contained kanamycin (100 mg/mL) at 37 °C on a shaker set to 250 rpm. Protein expression was carried out; 1 mL bacterial culture was inoculated into 100 mL LB and the suspension was incubated at 37 °C on a shaker until it reached and optical density (OD) = 0.6–0.8. Isopropyl-β-D-thiogalactoside (IPTG) was added subsequently to the mixture at a final concentration of 1 mM to induce the expression of protein and the culture was incubated for an additional 5 h at 37 °C.

The transfected *E. coli* cells (200 mL) were harvested by centrifugation at 10,000 × *g* for 10 min at 4 °C. The bacterial pellet was disrupted by sonication in a lysis buffer (20 M Tris–HCl, 0.5 M NaH_2PO_4, 6 M guanidine hydrochloride), followed by centrifugation at 12,000 × *g* for 20 min at 4 °C. The supernatant was passed through a 0.45-nm syringe-end filter and loaded onto a His Bind column pre-charged with Ni^{2+}. The purified protein used was obtained from a GE Healthcare HisTrap^{TM} HP column (GE Healthcare, Chalfont St Giles, UK) and processed in according to the manufacturer's protocol. The purified protein was analyzed by SDS–PAGE and stained with Coomassie blue R-250. The recombinant protein was stored at −80 °C.

2.5. Western blot analysis and determination of the optimal immunogenic protein

Western blot analysis was performed by following an established protocol. Briefly, protein expression products were quantitated using a BCA protein assay kit (Beyotime, Beijing, China), separated by SDS–PAGE, and transferred to a nitrocellulose membrane (GE Healthcare, Chalfont St Giles, UK). The membrane was blocked with 5% non-fat dried milk in phosphate-buffered saline (PBS) for 1 h at room temperature with shaking, and washed with PBS that contained 0.01% Tween-20 (PBST). Protein detection in the ANV-positive serum was performed at a dilution of 1:50 in PBS at 37 °C for 2 h. Afterwards, the membrane was washed three times with 0.05% Tween-20 in PBS for 15 min, followed by incubation at 37 °C for 1 h with AP-labeled rabbit anti-chicken immunoglobulin at a dilution of 1:1000. After three washes, antibodies bound to the membrane were detected by incubation with a mixture of 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt and nitroblue tetrazolium chloride. After Western blot analysis, the optimal immunogenicity protein was determined.

2.6. P1-ELISA procedure

Ninety-six-well plates (Corning, Corning Costar., MA, USA) were coated with 100 μL of protein antigen diluted with 0.05 M carbonate buffer (pH 9.6), and incubated for 24 h at 4 °C. Samples were washed three times with PBST and blocked with 5% non-fat milk in PBS for 2 h at 37 °C; next, additional washing with PBST was performed three times. Approximately 100 μL of the ANV serum (diluted in the dilution buffer) were added to the wells and incubated for 2 h at 37 °C. The plates were washed three times, then 100 μL of 1:1000 horseradish peroxidase (HRP)-labeled goat anti-chick IgG (diluted in PBS) were added to the wells, and the plates were incubated at 37 °C for 1 h. The peroxidase reaction was visualized using tetramethyl-benzidine (TMB) (Sigma, Sigma–Aldrich Chemie, Munich), by incubation in the dark for 20 min at 37 °C. The reaction was stopped by the addition of 2 M H_2SO_4, and absorbance was read at 450 nm.

2.7. Standardization and optimization of P1-ELISA

A checkerboard titration that involved serial dilutions of coating antigen and antibody (ANV-positive serum) was performed under the ELISA conditions described above. SPF chicken sera (as a negative control) were used to optimize the detection system. The coating antigen and reference sera were used at two-fold dilutions from 1/25 to 1/400 ng/mL and from 1 to 128 ng/mL, respectively. The optimal serum dilution and P1 protein concentration for the antigen coating was defined at the largest ratio between the positive and negative sample ODs.