Development and characterization of a potential diagnostic monoclonal antibody against the capsid protein VP1 of chicken anemia virus

Yi-Yang Lien[1Ψ], Chi-Hung Huang[2Ψ], Fang-Jun Sun[3], Tsung-Chi Lu[2], Meng-Shiunn Lee[4], Shu-Chin Hsueh[1], Hsi-Jien Chen[5], Meng-Shiou Lee[6*]

[1] Dept. of Veterinary Medicine, National Pingtung University of Science and Technology, Pingtung, Taiwan.

[2] Graduate school of Biotechnology, Hung kuang University, Taichung, Taiwan.

[3] Dept. of Bioresources, Da Yeh University, Chunghwa, Taiwan

[4]Dept. of Medical Research, Tung's Taichung MetroHarbor Hospital, Taichung, Taiwan

[5]Dept. of Safety, Health and Environmental Engineering, Mingchi University of Technology, Taipei, Taiwan.

[6] School of Chinese Medicine Resources, China Medical University, Taichung, Taiwan

[Ψ]These authors contributed equally to this work.

[*]**Correspondent:** Meng-Shiou Lee, School of Chinese Medicine Resources, China Medical University, Taichung 40402, Taiwan.

E-mail: leemengshiou@mail.cmu.edu.tw; Telephone: +886-4-2205-3366#5208; Fax: +886-4-22078083.

Running title: Development and characterization of a diagnostic monoclonal antibody against CAV VP1 capsid

Abstract

Chicken anemia virus (CAV) is an important viral pathogen and causes anemia and severe immunodeficiency syndrome in chickens worldwide. In this study, a potential diagnostic monoclonal antibody against CAV VP1 protein was developed that can recognize the CAV antigen precisely for diagnostic and virus recovery purposes. The VP1 gene of CAV encoding the N-terminus deleted VP1 protein, VP1Nd129, was cloned into an *E. coli* expression vector. After IPTG induction, VP1Nd129 protein was shown to be successfully expressed in *E. coli*. By ELISA screening using two coating antigens, purified VP1Nd129 and CAV-infected liver tissue lysate, E3 MAb was identified to have higher reactivity against VP1 protein than the other positive clones by the limiting dilution method from 64 clones. Using immunohistochemistry, the presence of the VP1-specific MAb, E3, was confirmed using CAV-infected liver and thymus tissues as positive-infected clinical samples. Additionally, CAV particle purification was also established using an immunoaffinity column set up with E3 MAb. The developed monoclonal antibody, E3 MAb, it will not only be very useful for detecting CAV infection and for histopathology studies of infected chickens but may also be employed to purify CAV particles in the future.

Keywords chicken anemia virus, monoclonal antibody, immunohistochemistry, immunoaffinity column, VP1

Introduction

Chicken anemia virus (CAV), is the sole member of the genus *Gyrovirus* of the *Circoviridae* family and causes severe anemia and an immunosuppressive disease, namely chicken anemia disease [2, 12, 13]. Histopathological studies have shown that CAV infection leads to aplasia of the bone marrow. This results in anemia and severe immunodeficiency syndrome due to the destruction of T lymphoid tissue [8, 18].

The CAV genome consists of a circular single-stranded DNA genome of 2.3 kb; this encodes three viral proteins, VP1, VP2 and VP3 [2, 12, 13]. VP1 protein is the sole structural protein of the CAV capsid. At a very late stage of the virus life cycle, the assembled virus particles created by VP1 protein spread into various other tissues and organs of chicken such as thymus, spleen and liver. Among these tissues and organs, liver tissue has been reported to have the highest accumulation of CAV virions [18]. Up to the present, several conventional methods have been developed to detect CAV infection such as serological tests for the detection of CAV antibodies. Recently, immunohistochemistry (IHC) and immunofluorescence (IF) have been used as alternative methods for the detection of the CAV antigen [1, 10, 11, 15, 16]. For these to be successful, an excellent monoclonal antibody is essential. In this context, antigen preparation is a critical factor when producing such a monoclonal antibody. It has been reported that VP2 and VP3 have been used as target antigen to generate monoclonal antibodies for immunological characterization or for the development of diagnostic ELISA kits [5, 17]. However, VP1 protein has rarely been used as the antigen for generating antibodies or for diagnostic kit development; this is because problems with CAV VP1 protein expression have been reported in several host cell systems [5, 7, 14, 16].

Research on VP1 antigen preparation has generally been unsuccessful because of a failure to find a good recombinant protein expression system. The highly rich span

of arginine residues at the N-terminus of the VP1 protein has been proposed to be cytotoxic in an *E. coli* expression system [14]. Thus, there is a need to overcome the difficulties of VP1 antigen preparation. If successful, this would allow the generation of a monoclonal antibody against chicken anemia virus VP1 capsid that may potentially be used diagnostically for the clinical detection of CAV infections. Recently, our group has shown that the VP1Nd129 protein (amino acid residue 130-450 of VP1 protein), which has the first 129 amino acid residues at N-terminus of VP1 protein deleted, is able to be expressed successfully in large amounts in prokaryotic cells [7].

In this study, to develop and produce a number of monoclonal antibodies for immunological applications, the truncated recombinant VP1Nd129 protein was employed as antigen to immune BALB/c mice. One of these monoclonal antibodies, E3, was selected for assessment of the specificity of monoclonal antibody to recognize CAV VP1 protein using clinical samples infected with CAV including liver and thymus tissue, respectively. In addition, immunoaffinity column employing the E3 MAb as ligands for virus particle purification also was explored herein. This will be very useful to develop immunological tool for detecting the CAV pathogen, identifying CAV infection or for CAV histopathology studies on chicken.

Materials and methods

Virus strain, CAV-infected liver tissue

Chicken anemia virus, CIA-89, was provided by Professor Yi-Yang Lein of the National Pingtung University of Science and Technology (Pingtung, Taiwan). Two one day old specific pathogen free (SPF) Hybrid White Leghorn (HWL) chickens purchased from Animal Health Research Institute of the Council of Agriculture (Taiwan, Taipei) were used to propagate the virus by passaging 20% liver

homogenates with 0.1 ml per bird . These were inoculated with CIA-89 orally. At 10-days post-infection, the individual livers of the sacrificed chickens were removed, collected, immersed in formaldehyde, and then stored at room temperature until required.

Plasmid construction and bacterial strain

The plasmid, pGEX-6P-1-VP1 derived from plasmid pGEX-6P-1 (GE Healthcare, Piscataway, NJ), which contains the cDNA encoding the VP1 genes of chicken anemia virus, was provided from Professor Yi-Yang Lien of National Pingtung University of Science and Technology (Pingtung, Taiwan) and initially used as the PCR template [7]. In this study, to amplify the VP1 gene with the first 129 amino acids truncated from the full-length VP1 gene, PCR primers were designed and used as shown in Figure 1. Using pGEX-6P-1-VP1 as the template, the PCR reaction was performed at 95℃ for 5 min, 95℃ for 45 sec, 59℃ for 50 sec and 72℃ for 1 min for 30 cycles. The last cycle of PCR was carried out with a final elongation step of 10 min at 72℃. The amplified DNA fragments were digested with XhoI and EcoRI, and then cloned into the prokaryotic expressing vector pET28a. The constructed recombinant plasmid, pET28a-VP1Nd129, was transformed into One Shot® Top10 and into BL-21 (DE3) competent *E. coli* for the maintenance of the recombinant plasmids and for protein expression, respectively. Transformants with the correct gene size were identified by PCR and checked using restriction enzyme digestion and sequencing.

Expression and purification of VP1Nd129 protein in recombinant *E. coli*

The recombinant *E. coli* strain BL-21 (DE3) containing pET28a-VP1Nd129 was used for protein induction and expression. The recombinant strains were grown

overnight in LB medium in the presence of kanamycin (50 μg/mL) at 37^{o}C. Then 0.5 mL of overnight culture was inoculated into 50 mL LB medium and grown at 37^{o}C for around 3 hrs by which time the optical density of culture had reached 0.5. At this point, isopropyl-β-D-thiogalactopyronoside (IPTG) at 0.1 mM was added to the culture to induce protein expression, which continued for 6 hrs. The presence of expressed VP1Nd129 protein was confirmed by 12.5% SDS-PAGE followed by Western blotting using monoclonal anti-His antibody. To purify the recombinant VP1Nd129 protein, cell pellet was spun down from 50 mL of the culture supernatant and resuspended in denaturing binding buffer (20 mM NaH_2PO_4, 0.5 M NaCl, 8M urea, pH 7.8). The mixture was then sonicated on ice three times for 3 minutes with a 20% pulsed activity cycle, and then centrifuged for 10 min at 10,000 rpm to remove the cell debris. The resulting cell lysate was poured into an Enco-column with 2 mL of Ni^{2+}-NTA agarose and the resin allowed to settling by gravity. The packed resin in column was washed by gravity with three volumes of denaturing binding buffer and then a similar volume of wash buffer (20 mM NaH_2PO_4, 0.5 M NaCl, 8M urea, pH 6.3). Finally, the bound proteins were eluted with elution buffer (20 mM NaH_2PO_4, 0.5 M NaCl, 8M urea, pH 4). For each fraction, 2 ml of elute was collected. The fractions were monitored at OD_{280} using a U-2001 spectrophotometer with wash buffer as a blank. The putative peaks containing the recombinant CAV viral protein was identified and the eluate collected for analysis. The total protein concentration of each fraction was determined using a Micro BCA kit with bovine serum albumin as the reference protein. The purity of the protein sample was analyzed using aliquots of the concentrated fraction; this was done by 12.5% SDS-PAGE and Coomassie brilliant blue staining.

Generation of monoclonal antibody against CAV VP1 protein

SPF BALB/c mice were immunized by subcutaneous injection of 20 ug purified VP1Nd129 protein emulsified with complete Freund's adjuvant. After immunization, BALB/c mice were sacrificed, the spleens were removed, and the splenocytes were fused with the SP2/0 myeloma cells. Antibodies secreted from the various hybridomas were screened by ELISA. They were subcloned three times by the limiting dilution technique, and then ascitic fluid containing monoclonal antibodies was produced by introducing the cloned hybridomas into Pristane-primed mice. The immunoglobulin class of the hybridoma antibodies were determined by ELISA with a Zymed Mab Kit using rabbit antisera against mouse IgM, IgG1, IgG2a, IgG3, and IgA and goat anti-rabbit IgG serum conjugated with horseradish peroxidase.

Enzyme-linked immunoabsorbent assay (ELISA)

To evaluate the specificity and reactivity of MAb against CAV, ELISA with the antibody was used against purified VP1Nd129 protein or tissue lysate infected with CAV as the antigen. After this, polyvinyl chloride 96-well plates were coated with recombinant VP1Nd129 or CAV containing-tissue lysate. The antigen was buffered and diluted with carbonate-bicarbonate buffer (pH 9.6). Varying amounts of antigen were added to the EIA strip plate wells and incubating overnight at 4℃. After antigen coating, the plate were washed twice with phosphate-buffered saline (PBS), containing 0.05% Tween 20 (PBS-T), and then 0.2 ml of blocking reagent (PBS containing 5% skim milk) was added to each well. After 30 min of incubation at room temperature, the plates were washed twice with PBS. The hybridoma supernatants were then incubated in the CAV antigen or recombinant antigen-coated plate wells at 37℃ for 1 hr to test each individual samples. The bound MAbs were detected using HRP-conjugated rabbit anti-mouse IgG, and color was developed with *o*-phenylenediamine dihydrochloride (OPD).

Histopathology and Immunohistochemistry (IHC)

The CAV-infected and uninfected liver and thymus tissues as positive control and negative control, respectively, were all confirmed from clinical cases by PCR method using specific primers as described as previously test [7]. The obtained liver and thymus were fixed using 30% neutral buffered formaldehyde, and embedded in paraffin. The paraffin-embedded tissues were sectioned, mounted and then stained with hematoxylin and eosin. Immunohistochemistry staining was carried out at room temperature and this is briefly described below. Tissue sections (5 μm) of the paraffin-embedded tissues were washed with phosphate saline buffer containing 0.3% hydrogen peroxide to inactivate endogenous peroxidase. After washing three times with phosphate saline buffer, antigen retrieval was carried out by incubating the tissue sections with 0.1% trypsin solution. The primary antibody was then applied, monoclonal antibody, E3 MAb, which is specific for CAV VP1 protein recognition. The applied E3 MAb was recognized by horseradish peroxidase (HRP) conjugated goat anti-mouse IgG secondary antibody. After IHC staining, the sections were counterstained with hematoxylin, and examined under light microscopy.

Immunofluorescence

For immunofluorescence microscopy, the infected and un-infected MSB-1 cells on glass coverslips were fixed in 4% formaldehyde, washed with phosphate buffered saline (PBS) and permeated with 0.01% Triton X-100. After washing with PBS, samples were incubated with blocking solution for 1 h, followed by 1 h of incubation with an anti-VP1 monoclonal antibody E3. Fluorescein isothiocyanate-conjugated

goat anti-mouse immunoglobulin G (IgG) was used to react with E3 mAb. Cell nuclei of cultures were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich Corp., St Louis, MO, USA) and fluorescence images were captured using a Leica laser scanning confocal microscope.

Immunoaffinity column purification of CAV particles

To create an immuno-affinity column, cytoplasmic extracts from chicken liver tissue infected with CAV were prepared by centrifugation at 15,000 rpm for 30 min at 4℃. Next, 100 μl of cell extract was mixed with 50 μl of ascitic fluid and incubated overnight at 4℃; then 50 μl of protein A agarose was added and incubated overnight at 4℃ with gentle rotation. The agarose beads were washed three times with buffer containing 50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 0.2% NP-40, 0.05% sodium deoxycholate. Finally, viral protein and/or CAV virus particles were eluted from the protein A agarose beads by boiling with SDS-PAGE sample buffer. The extracted protein was then subjected to SDS-PAGE followed by Western blotting. In addition, CAV genomic DNA from the CAV virus particles was detected by PCR using CAV genome-specific primers. The sequences of the CAV VP1 gene PCR primers were VP1F: 5'- ATGGCAAGACGAGCTCGCAGACCGAGAGG-3' and VP1R: 5'-CTAACCATGGTGATGGTGATGGTGGGGCTGCGTCCCCCAGTA-3'. The sequences of the CAV VP3 primers were VP3F: 5'-CCGCTCGAGCAGTCTTATACACCTTCTTG-3' and VP3R: 5'-GCGAATTCATGAACGCTCTCCAAGAAGATAC-3'.

Results

Expression, purification and characterization of CAV VP1Nd129 protein using a recombinant *E. coli* system

To express the CAV VP1 protein as antigen for immunization, the VP1Nd129 gene was created by PCR using VP1 cDNA of CAV as the template DNA. As illustrated in Figure 1, the VP1 cDNA overlapped partially with the VP2 gene of the CAV genome, which had been cloned into pGEX-6P-1 earlier [4]. By PCR using the primers VP1-388FE and VP1-RHX, the VP1Nd129 gene was amplified and cloned into pET28a using *EcoR*I and *Xho*I sites, which created a protein with in frame His-tag. This plasmid, pET28a-VP1Nd129, was transformed into *E. coli* BL-21 (DE3). This strain was examined for protein expression after 4 hrs induction with IPTG. The VP1Nd129 protein was successfully expressed in *E. coli*, producing the correct size band on a Coomassie blue gel and these proteins were also recognized by anti-His tag antibodies (Fig. 2A and 2B). The estimated molecular weight of pET28a-VP1Nd129 was 40 kDa for VP1Nd129. VP1Nd129 in *E. coli* was expressed at a high level and reached 26.2 mg/L when 0.1 mM IPTG was added to the culture broth. After affinity chromatography purification, purified denatured VP1Nd129 protein was confirmed to be present as shown in Figure 3. The purity of the VP1Nd129 approached homogenicity in elution fractions E1 to E7 at pH 4.0. Purification of His-tag fused VP1Nd129 is therefore feasible by Ni-NTA column. Additionally, the identity of this Ni-NTA purified VP1Nd129 protein was confirmed by mass spectrometry. (data not shown).

Screening and characterization of Mabs for specific recognition of VP1 protein

To establish monoclonal antibodies, spleen cell removed from five mice immunized with Ni-NTA purified recombinant VP1Nd129 protein and these were fused with SP2/0 myeloma cells. After 14 days, hybridoma cell lines secreting MAbs

specific for VP1Nd129 were screened for by ELISA using the Ni-NTA purified VP1Nd129 protein as the coating antigen. Four MAbs active against VP1Nd129, B1-1, B1-5, D4 and E3, were identified out of 64 clones; these were then screened by subcloning at least three times using the limiting dilution method. The MAb from the E3 hydridoma cells showed the greatest reactivity compared to the other Mabs from the B1-1, B1-5, and D4 hydridomas (Figures 4A and 4B). Even after the supernatant was diluted with 50 fold, E3 still reacted. In addition, the specificity and reactivity of the E3 MAb were also confirmed by Western blotting against purified recombinant VP1Nd129 and against *E.coli* cell lysate. As illustrated in Figure 5A, the E3 MAb showed good specificity and reactivity against the loaded antigen and purified recombinant VP1Nd129, while showing no reaction against *E. coli* cell lysate harboring the blank pET28a vector. When the specificity of the E3 MAb against different amounts of antigens was tested, it was constant, and the reactivity of the E3 MAb increased along with increasing amounts of antigen. There were very few background bands that reacted with E3 MAb, either in the samples of purified VP1Nd129 or in the *E. coli* cell lysate. Moreover, 1 ng of purified VP1Nd129 was sufficient to give a reaction with E3 MAb (Figure 5B). Thus E3 MAb confers good specificity and reactivity against CAV antigen. Additionally, the IgG subtype analysis showed that E3 MAb was IgG2b, and the light chain was κ chain. At this point, the E3 hydridoma was selected to produce MAb in mice and mouse ascitic fluid was then used for further diagnostic applications and for characterization.

Diagnostic application of the E3 MAb for CAV detection

To evaluate the possible clinical application of E3 MAb to CAV diagnosis, E3 MAb was used for CAV detection by immunohistochemistry (IHC) and immunochromatographic assay. First, four CAV infected chicken livers were analyzed

using IHC and all the paraffin-embedded tissues slides were positive. The positive control slides demonstrated strong immuno-reactivity against CAV antigen (Fig. 6B). In addition, four CAV mock-infected chicken livers were analyzed as the negative control group and these were all negative for immunoreactivity against CAV antigen, (Fig. 6A). Moreover, the similar results of immune-reactivity were showed in the thymus tissues. The CAV-infected thymus demonstrated in Fig. 7B was recognized by E3 MAb using IHC in contrast with negative case (Fig. 7A). As to lymphoblastoid cells, MSB-1, the CAV-infected MSB-1 cells and CAV-infected MSB-1 with cytopathic effect, the presence of VP1 antigen in the cells can all be recognized by E3 MAb using immunofluorescence (IF) assay (Fig. 8). Among above, these results indicated that E3 MAb is able to discriminate CAV infected tissues or cells from CAV uninfected tissue or cells in experimental samples using IHC staining and IF, respectively. Furthermore, E3 MAb was used to interact with CAV VP1 protein present in the lysate of CAV-infected liver tissue. As illustrated in Figure 9A, CAV VP1 protein with a molecular weight of 50 kDa was eluted from the protein A agarose beads. In contrast, no VP1 protein from CAV was detected from the eluted sample of CAV mock-infected liver lysate. Detection was by Western blotting assay using E3 MAb as the primary antibody. In addition, Figure 9B shows that CAV specific VP1 and VP3 genes with length of 1.35 kb and 348 bp, respectively could be amplified by PCR using the eluted fraction of the immunochromatographic column as template. This suggests that the eluted fraction from immunochromatographic column includes CAV VP1 capsid.

Discussion

In this study, we have successfully generated a specific monoclonal antibody, E3 MAb, which is active against VP1 protein, the sole structural protein of CAV. To

generate specific monoclonal or polyclonal antibodies against VP1, the purified CAV particle is usually used as the main antigen for the production of antibodies. However, virion purification is very tedious and time-consuming. Therefore, DNA recombinant technology was chosen as a better method of producing VP1 protein. Previously, several expression systems have been employed to express CAV VP1 protein, including *E. coli*, baculovirus-insect cells and plant cells [5, 6, 14]. However, there has been difficulty expressing full-length VP1 protein because of cytotoxicity [14]. Moreover, production of the recombinant full-length VP1 protein has generally been unsuccessful because of a failure to express a span of amino acids at the N-terminus of the VP1 protein that is highly rich in arginine residues [7, 14]. This might because these amino acids are encoded by codons that are rarely used by *E. coli*. Most of viral capsid protein of circovirus such as porcine circovirus, pigeon circovirus spanning the rare codons of *E. coli* at N-terminus have been investigated in previous studies. The truncation of the N-terminus of capsid proteins, therefore, has become an alternative way to perform to produce these recombinant viral protein. [3, 9]. The inhibition of the use of recombinant antigen to generate monoclonal antibodies and detection kit for diagnostic purposes will be resolved using above strategy [3, 4, 5, 9, 12]. Lee *et al.* reported that the expression of VP1 in a host system can be vastly improved when the arginine residues at N-terminus of VP1 are removed [7]. Using this N-terminus truncated clone of VP1 expressed in *E. coli*, we have produced and purified the VP1Nd129 protein successfully. After using this antigen to create the E3 MAb against VP1 protein, this antibody was investigated in terms of its application to immunohistochemistry, immunofluorescence assay and immunoaffinity chromatographic column purification.

Several diagnostic methods have been developed and refined to detect CAV infection, including electron microscopy, nucleic acid based analysis and antigen-antibody

based assay [10, 11, 15, 16]. An immunofluorescence assay has been most commonly used for the microscopic diagnosis of CAV infection. By combining immunofluorescence and confocal laser scanning microscope, this would be a highly convenient way to directly investigate CAV biology at a subcellular level.

It is well known that VP1 is a sole structural protein of CAV, thus this protein is a highly useful marker for the detection of CAV. In the life cycle of CAV, VP1 encapsidates the CAV genome into the VP1 capsid during virus particle assembly [19]. The fact that CAV VP1 capsid was eluted from the protein A agarose beads in this study implies that the CAV genome is co-eluted with the VP1 protein. When CAV virion purified by immunoaffinity column was tested by PCR, there was CAV specific gene amplification, which confirms the presence of assembled virus particles.

The epitope mapping of VP1 protein in terms of MAb recognition was not investigated in this study. However, MAb E3 might recognize a specific epitope that is located on the surface of VP1 particles, as shown as Figure 9A. Thus it is possible that E3 MAb might recognize a neutralizing epitope and the antibody acts as a virus neutralizing antibody; this can be determined in the future by means of a virus neutralization assay. This would help with the development of a peptide vaccine for the control of CAV infection.

In conclusion, the present study describes the generation of monoclonal antibodies against recombinant VP1Nd129 protein by the mouse hybridoma system. The most immunodominant epitope, the VP1 protein of the CAV capsid, was detected immunologically using one of these monoclonal antibodies, E3 MAb. This monoclonal antibody enables the visualization of the spread of the viral antigen in formalin-fixed tissue by immunohistochemistry. In the absence of virus recovery, the diagnosis of CAV infection can be achieved by an immunological approach using E3 MAb for antigen detection. Therefore, the generated immunological probe represents

a powerful tool that will help the development of CAV diagnostic systems for epidemiological investigation. In addition it will assist with the measurement of the immunization efficacy of vaccines, with the study of CAV biology and with general surveillances of CAV infection.

References

1. **Brentano L, Lazzarin S, Bassi SS, Klein TAP, Schat KA.** Detection of chicken anemia virus in the gonads and in the progeny of broiler hens with high neutralizing antibody titers. Vet Microbiol 2005, **105**, 65-72.

2. **Ducatez MF, Owoade AA, Abiola JO, Muller CP.** Molecular epidemiology of chicken anemia virus in Nigeria. Arch Virol 2006, **151**, 97-111.

3. **Daum I, Finsterbusch T, Hartle S, Gobel TW, Mankertz A, Korbel R, Grund C.** Cloning and expression of a truncated pigeon circovirus capsid protein suitable for antibody detection in infected pigeons. Avian Pathol 2009, **38**, 135-141.

4. **Huang CH, Lai GH, Lee MS, Lin WH, Lien YY, Hsuch SC, Kao JY, Chang WT, Lu TC, Lin WN, Chen HJ, Lee MS.** Development and evaluation of a loop-mediated isothermal amplification assay for rapid detection of chicken anaemia virus. J Appl Microbiol 2010, **108**, 917-924.

5. **Iwata N, Fujino M, Tuchiya K, Iwata A, Otaki Y, Ueda S.** Development of an enzyme-linked immunosorbent assay using recombinant chicken anemia virus

proteins expressed in a baculovirus vector system. J Vet Med Sci 1998, **60**, 175-80.

6. **Lacorte C, Lohuis H, Goldbach R, Prins M.** Assessing the expression of chicken anemia virus proteins in plants. Virus Res 2007, **129**, 80-6.

7. **Lee MS, Lien YY, Feng SH, Huang RL, Tsai MC, Chang WT, Chen HJ.** Production of chicken anemia virus (CAV) VP1 and VP2 protein expressed by recombinant Escherichia coli. Process Biochem 2009, **44**, 390-395.

8. **Lucio BA, Schat KA, Shivaprasad HL.** Identification of the chicken anemia agent, reproduction of the disease, and serological survey in the United States. Avian Dis 1990, **34**, 146-153.

9. **Lou Z, Li X, Li Z, Yin X, Li B, Lan B, Yang B, Zhang Y, Liu J.** Expression and antigenicity characterization for truncated capsid protein of porcine circovirus type 2. Can J Vet Res 2011, **75**, 61-64.

10. **McNulty MS, Connor TJ, McNeilly F, Spackman D.** Chicken anemia agent in the United States: isolation of the virus and detection of antibody in broiler breeder flocks. Avian Dis 1989, **33**, 691-694.

11. **McNulty MS, Curran WL, Todd D, Mackie DP.** Chicken anemia agent: an electron microscopic study. Avian Dis 1990, **34**, 736-43.

12. **Meehan, BM, Todd D, Creelan JL, Earle JA, Hoey EM, McNulty MS.**

Characterization of viral DNAs from cells infected with chicken anemia agent: sequence analysis of the cloned replicative form and transfection capabilities of cloned genome fragments. Arch Virol 1992, **124**, 301-319.

13. **Noteborn MH, de Boer GF, van Roozelaar DJ, Karreman C, Kranenburg O, Vos JG, Eurissen SHJ , Hoeben RC, Zantema A, Koch G.** Characterization of cloned chicken anemia virus DNA that contains all elements for the infectious replication cycle. J Virol 1991, **65**, 3131-9.

14. **Pallister J, Fahey KJ, Sheppard M.** Cloning and sequencing of the chicken anaemia virus (CAV) ORF-3 gene, and the development of an ELISA for the detection of serum antibody to CAV. Vet Microbiol 1994, **39**, 167-78.

15. **Todd D, Creelan JL, McNulty MS.** Dot blot hybridization assay for chicken anemia agent using a cloned DNA probe. J Clin Microbiol 1991, **29**, 933-939.

16. **Todd D, Mawhinney KA, McNulty MS.** Detection and differentiation of chicken anemia virus isolates by using the polymerase chain reaction. J Clin Microbiol 1992, **30**, 1661-1666.

17. **Wang X, Gao H, Gao Y, Fu C, Wang Z, Lu G, Cheng Y, Wang X.** Mapping of epitopes of VP2 protein of chicken anemia virus using monoclonal antibodies. J Virol Methods 2007, **143**, 194-199.

18. **Yuasa N, Taniguchi T, Imada T, Hihara H.** Distribution of chicken anemia agent (CAA) and detection of neutralizing antibody in chicks experimentally inoculated with CAA. Natl Inst Anim Health Q (Jpn) 1983, **23**, 78-81.

19. **Schat KA**. Chicken anemia virus. Curr Top Microbiol Immunol. 2009, **331**, 151-83.

Legends

Figure 1. Schematic diagram of the genome structure of CAV. A region of the VP1 gene was used as template DNA for the amplification of the VP1Nd129 cDNA using the specifically designed primers, VP1-388FE and VP1RHX.

Figure 2. Analysis of VP1Nd129 protein expressed in recombinant *E. coli* by SDS-PAGE (A) and Western-blot assay (B). The symbols of "－" and "＋" presented pre-induction and post-induction with 1mM of IPTG in *E. coli*, respectively. Anti-His tag monoclonal antibody was used for the recognition of VP1Nd129 protein.

Figure 3. Analysis of Ni-NTA purified VP1Nd129 protein by SDS-PAGE. The recombinant *E. coli* expressing VP1Nd129 was extracted and then subjected to Ni-NTA column protein purification under denaturing conditions using a pH gradient buffer system. Lane M, prestained protein marker; FT, flow through of the cell lysate obtained from column; W, collected fraction after treating with wash buffer; E1-E7, collected fraction 1-7 after treating with elution buffer. The arrow symbol presented the VP1Nd129 protein which was detected by SDS-PAGE.

Figure 4. Analysis of the reactivity of the antibody-secreting hybridoma cell lines against VP1Nd129 protein (A). Serially diluted MAbs secreting by the hybridoma cell lines were screened by ELISA using the Ni-NTA purified VP1Nd129 protein as the coating antigen. Four MAbs, B1-1, B1-5, D4 and E3, out of 64 clones were reacted against VP1Nd129 and were screened in this experiment. (B) The affinity of the E3 MAb against VP1Nd129 at various fold dilution fold of hybridoma cultured medium.

Figure 5. Analysis of specificity and reactivity of E3 MAb against antigen by Western blotting assay (A) and ELISA (B). Different protein amounts of purified VP1Nd129 or *E. coli* cell lysate were used to react with E3 MAb in both assay of (A) and (B).

Figure 6. Immunohistochemical labeling of CAV-infected chicken liver tissues. The E3 MAb was used as the primary antibody to recognize CAV antigen in liver tissue. (A) Negative control. (B) CAV-infected chicken liver tissue. Bar=100 nm.

Figure 7. Immunohistochemical labeling of CAV-infected chicken thymus tissues. The E3 MAb was used as the primary antibody to recognize CAV antigen in the thymus tissue. (A) Negative control. (B) CAV-infected chicken thymus tissue.

Figure 8. Immunofluorescence assay of CAV-infected chicken MSB-1 cells. The E3 MAb was used as the primary antibody to recognize CAV VP1 antigen in the MSB-1 cells. The fluorescent agent, 4',6-diamidino-2-phenylindole (DAPI) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (IgG) were respectively used to counterstain the cell nuclei of cultures and to react with E3 MAb. CPE, CAV-infected MSB-1 with cytopathic effect.

Figure 9. Western blotting assay of immuno-column purified CAV VP1 by E3 MAb (A). CAV-infected liver lysate was subjected to react with E3 MAb and then the immunoaffinity resin was used to precipitate the antigen bounded or unbounded MAb. Lane M, prestained protein marker (The molecular weight of respective bands were 116, 66, 45, 35, 25 and 18 kDa); lane 1, CAV-uninfected liver lysate; lane 2, CAV-infected liver lysate; lane 3, co-precipitated VP1 protein with E3 MAb from tissue lysate of lane 2; lane 4, E3 MAb only; lane 5, protein were precipitated by E3 Mab from tissue lysate of lane 1. The H and L indicate the heavy chain and light chains of IgG, respectively. Identification of the viral genome of the CAV particle by PCR (B). Sample containing CAV VP1 capsid that was coprecipitated with E3 MAb and then used to as DNA template for PCR amplification using specific primers for the VP1 and VP2 genes. Lane M, 1 kb DNA ladder marker; lane 1, positive control for PCR; lane 2, CAV-uninfected liver lysate; lane 3, CAV-infected liver lysate; lane 4, E3 MAb co-precipitated sample from CAV-infected liver lysate.