Genetic analysis of the virulence factors and development of a novel diagnostic antigen and antibody test for Fowl Aviadenovirus

(鶏アデノウイルス病原性の遺伝子解析および診断用抗原と抗体の開発)

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Contents

PREFACE

Before you lies the dissertation “Genetic analysis of the virulence factors and development of a novel diagnostic antigen and antibody test for Fowl Aviadenovirus”, the research of which is a study on the Aviadenovirus gene that cause pathogenic action to the birds and could be use these genes as an alternative tools for disease diagnosis. It has been written to fulfill the graduation requirements of the Ph. D. of Veterinary Science Program at the Azabu University. I was engaged in researching and writing this dissertation from April 2015 to February 2019.

My research questions were formulated together with my supervisor, Professor Satoshi Taharaguchi. The research was difficult, but conducting extensive investigation has allowed me to answer the question that we identified. Fortunately, Associate Professor Kan Fujino was always available and willing to answer my queries. I would like to thank my supervisors for their excellent guidance and support during this process. I also wish to thank all of my colleagues at Laboratory of Microbiology II: I would like to thank you for your wonderful cooperation as well.

I hope you enjoy your reading.

Thanasut Khompakorn

ABSTRACT IN ENGLISH

Fowl Aviadenovirus (FAdV) belong to family Adenoviridae and the genus Aviadenovirus. FAdV is a non-enveloped icosahedral which has a linear, double-stranded DNA. It has been grouped into five species and 12 serotypes. FAdVs infection often cause Hydropericardium syndrome (HPS), inclusion body hepatitis (IBH), egg drop syndrome (EDS), gizzard erosion (GE), and respiratory tract disease. The disease occurs worldwide leading to affect economic losses in poultry industry.

To elucidate factors associated with the virulence of Fowl Aviadenovirus, whole genome sequencing using Next geneneration sequencing of FAdV A strain JM1/1 was carried out. Developing the non-structural protein based Fluorescent antibody virus neutralization (FAVN) and Western Blotting assay (WB) coupled with Immunofluorescence assay (IFA) were studied. The results showed as following 1) The complete genome of FAdV A strain JM1/1 is 43,809 nucleotide length. It revealed 99% nucleotide sequence identical to FAdV A strain CELO (chicken embryo lethal orphan), which is an European apathogenic reference strain. The nucleotide sequence differences were analyzed, interestingly showed multiple sites insertion and deletion. The results will provide an information on the evolution and may help elucidate viral pathogenesis on molecular biology especially on genetic roles. 2) The recombinant DBP was constructed and subjected to used as

primary antibody in the developed FAVN test. A DNA Binding Protein (DBP), non-structural protein, which is detectable early and responsible for initiating DNA replication. DBP was found that more conserved domain region within FAdV serotype 1. The developed FAVN test was compared with a conventional VN test by examining the antibody titer in field chicken sera. The results showed the measured neutralizing antibody titers were high correlated with the VN as correlation coefficient was 0.8. This FAVN test is simple, achieve quickly, easily and could be an alternative test for FAdV infection. 3) Likewise, the recombinant 52K was constructed and subjected to use as primary antibody in the developed test based on WB and IFA. 52K, non-structural protein, involve in capsid assembly and/or genome packaging. It is especially expressed in late stages of viral life cycle. The C terminal region of 52K was found that more conserved domain within all serotypes. WB and IFA analyze revealed that anti-52K antibody can detect FAdVs infection including homologous and heterologous serotypes. Therefore, this antibody has a property which could play a role in an alternative method for FAdVs infection diagnosis.

In conclusion, this research study provide some knowledge, will be useful in investigation, surveillance, and elimination in FAdV diseases.

ABSTRACT IN JAPANESE

鶏アデノウイルス（FAdV）は、アデノウイルス科およびアビデノウイルス属に属する。 FAdV は、線状の二本鎖 DNA を有する非エンベロープ正 20 面体で、5 つの遺伝子型および 12 の血清型に分類されている。 FAdV 感染は、しばしば、鶏の心膜水腫症候群（HPS）、封入体肝炎（IBH）、産卵低下症候群（EDS）、筋胃びらん（GE）および呼吸器疾患を引き起こす。この病気は世界中で起こり、家禽産業における経済的損失を与える。FAdV の病原性を決定する因子を解明するために、筋胃びらん材料から分離した FAdV A 型 JM1/1 株の次世代配列決定を用いた全ゲノムシーケンシング、蛍光抗体法を応用した蛍光抗体中和法（FAVN）およびウェスタンブロッティングアッセイ（WB）を用いて解析した。

1）FAdV A 型 JM1/1 株の完全ゲノムは 43,809 ヌクレオチド長であった。それは、欧州の病原性参照株である FAdV A 型 CELO 株 (chicken embryo lethal orphan)と 99％のヌクレオチド配列が同一であることを明らかにした。興味深いことに、複数の部位の挿入および欠失を示した。これらの結果はウイルスの進化情報を提供し、分子生物学、特に遺伝的役割に関するウイルスの病因を解明するのに役立つだろう。 2）DNA 複製を開始させる DNA 結合タンパク質（DBP）で非構造タンパク質である組換え DBP を構築し、開発した FAVN 試験において一次抗体として使用した。DBP の発現は感染初期に検出可能であり、FAdV 血清型 1 内のより保存されたドメイン領域であることが判明した。開発された FAVN 試験を野外鶏血清中の抗体力価を調べることで、従来の VN 試験と比較した。結果は、測定した中和抗体価が、相関係数が 0.8 であることから VN と高い相関が

あることを示した。この FAVN 検査はシンプルで、迅速かつ容易に達成でき、FAdV 感染の NT の代替試験になる可能性を示した。 3）キャプシドアセンブリおよび/またはゲノムパッケージングに関与する後期蛋白で非構造タンパク質である 52K の組み換え体を構築し、この抗体を得た。これを WB および IFA に基づく開発された試験において一次抗体としての使用に供した。52K の C 末端領域は、すべての血清型内で高度に保存されたドメインであることを見出した。 WB および IFA 分析によって、抗 52K 抗体がホモの血清型および異種血清型を含む FAdV 感染を検出できることを明らかにした。この結果から、52K 抗体は、FAdV 感染症診断の代替方法において役割を果たし得ることを明らかにした。

これらの研究は FAdV の知識を提供し、FAdV 疾患における調査、監視、および撲滅に有用であろう。

CHAPTER 1

Sequence analysis of Fowl Aviadenovirus A strain JM1/1 which caused gizzard erosions in Japan

Abstract

An investigation into the complete genome analysis of Fowl aviadenovirus A strain JM1/1 has been performed by comparing to the reference Fowl aviadenovirus A strain chicken embryo lethal orphan (CELO). This viral strain cause gizzard erosions in broiler chicken isolated from slaughter house in Japan affect economic loss in carcass quality. To validate the strain JM1/1, virus was propagated in chicken kidney cells, for which Phenol-chloroform DNA extraction and next generation sequencing to comprise the complete nucleotide sequence were investigated. To comparison with other Fowl aviadenovirus sequences available in GenBank database was also investigated. Analysis of genome sequencing indicated that the complete genome of Fowl aviadenovirus A strain JM1/1 was found to be 43,809 nucleotides long and most closely identical to Fowl Adenovirus A strain chicken embryo lethal orphan (CELO) which shared 99% nucleotide identity. Interestingly, nucleotide difference compared to CELO showed multiple sites of insertion and deletion included ORF 10, 11, 14, 18, 19, 21 and L4 and L5 gene indicated these positions may serve as non-functional gene and ORF due to change in protein translation. These findings will provide some information on evolution of Fowl aviadenovirus and may possibly clarify Fowl aviadenoviral pathogenesis especially in their genetic role

Introduction

Outbreaks of gizzard erosions were consistent with Fowl aviadenovirus involvement. The first natural outbreak has been reported in layer flocks in Japan (Tanimura et al. 1993). Subsequently, epidemics occurred more recently in East Asia (especially South Korea and Japan) and Europe (Abe et al. 2001, Ono et al. 2001, Ono et al. 2003, Manarolla et al. 2009, Domanska-Blicharz et al. 2011, Grafl et al. 2012, Lim et al. 2012). Fowl aviadenovirus (FAdV) infection is extensive in poultry industry lead to worldwide economic losses relate to meat producing chickens as a result of decreased in growth rate, increased mortality rates and poor carcass quality from affected gizzards. Virus is mainly spread horizontally by the oral-fecal route, but vertical transmission can also occur via secretions and excretions. FAdVs have been isolated from both sick and healthy. In chickens, it has ability to cause various diseases for example inclusion body hepatitis (IBH), hydropericardium syndrome (HPS), egg drop syndrome (EDS), respiratory tract disease and gizzard erosions. In Japan, isolates obtained from poultry showing gizzard erosions belong to family *Adenoviridae* and genus *Aviadenovirus*. Fowl aviadenovirus serotype 1 and 8 were isolated while infection with serotype 8 confirm chickens did not develop lesions in gizzard (Grafl et al. 2017). Within serotype 1, there are several viral strains consist with pathogenic and apathogenic. Presently, only limited data about the difference between both strains are available. The present study describes the difference

between Fowl aviadenovirus A strain JM1/1 and the others Fowl aviadenovirus by means of a Next Generation Sequencing and compare to sequence available in GenBank database.

Materials and methods

Adenovirus

Seeding viral stock in this study obtained from broiler chickens with gizzard erosions was detected at slaughter house in Kagoshima, Japan, in 2000. Viruses were isolated using chicken embryonic liver cell. Isolates were identified as serotypes 1 and 8 using Neutralization method; only JM1/1 isolate strain of serotype 1 induce gizzard erosions in an experimental infection with specific-pathogen-free (SPF) chickens (Yamada et al. 2005).

Virus replication

Viruses were propagated in primary culture monolayer of chicken kidney (CK) cells prepared from 3 to 14 day old SPF chicks, cultured in Eagle minimal essential media (EMEM; Nissui Pharmaceutical Co., Tokyo, Japan) containing 5% fetal bovine serum (MP Biomedicals, Solon, OH, USA), incubated at 37 °C under 5% CO_2 atmosphere until cytopathic effect (CPE) occurred. After freeze-thaw three times and removal of debris by centrifugation (3,000 ×g, 20 minutes, 4°C) (HITACHI CF 16 RX II), the crude virus suspension containing 12% (w/v) of polyethylene glycol 6000 and 6% (w/v) of sodium chloride (NaCl) were precipitated by centrifugation (16,000 ×g, 20 minutes, 4°C) and the virus pellet was resuspened in two milliliters of phosphate buffered saline (PBS).

DNA extraction

Resuspend virus pellet were used for virus DNA extraction. In brief, mixed with DNase I and DNase I buffer (10x) (Takara Bio Inc., Shiga, Japan) incubated at 37°C for an hour, subsequently mixed with 10% Sodium Dodecyl Sulfate (SDS) (Wako Pure Chemical Industries Co., Osaka, Japan) and proteinase K (Takara Bio Inc., Shiga, Japan) incubated at 37°C for overnight. Afterwards, phenol-chloroform was added, supernatant including virus DNA was separated by centrifugation (12,000 ×g, 5 minutes, room temperature), then precipitated DNA by added with 2-propanolol and incubated at -20°C more than 15 minutes. To collect DNA pellets, conducted by centrifugation (12,000 ×g, 4°C, 15minutes), removed supernatant, washed pellets using room temperature 70% ethanol by centrifugation (12,000 ×g, one minute, room temperature), carefully removed supernatant, then air-dry DNA pellet until the tube was completely dry and dissolved by lab grade distilled water. Viral DNA was treated with RNase A (Takara Bio Inc., Shiga, Japan) incubated at 37°C for an hour subjected to extraction with phenol-chloroform and purified with ethanol as previous step. Extracted DNA was stored at 4°C until sequencing.

Whole genome sequencing

For complete genome sequencing, next generation sequencing was used. DNA was fragmented to create smaller strands and then ligated with adaptors and amplified using emulsion PCR. Sequencing was performed using Ion Torrent, the hydrogen ions produced during DNA polymerization were detected, and the whole genome sequences were created. Sequences assembly and alignment were conducted using Integrative Genomics Viewer (IGV) version 2.3.79 (Robinson et al. 2011) and Genetyx program version 12.0.4 (Genetyx Inc., San Francisco, CA, USA). Sequence comparisons were performed using the software BLAST.

Results

The complete genome of Fowl aviadenovirus A strain JM1/1 was found to be 43,809 nucleotides long with 54.31% G+C content. Compared with other Aviadenovirus sequences which available in online GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). The strain JM1/1 revealed 99% nucleotide sequence identical to Avian Adenovirus CELO (GenBank accession number: U46933.1). Nucleotide differences between strain JM1/1 and CELO interestingly showed multiple sites insertion and deletion (Fig. 1) include three nucleotides insertion (GAG) between position 3,354 and 3,355 where is open reading frame (ORF) 14, six nucleotides insertion (GAGGAG) between position 26,491 and 26,492 where is L4 gene noted produce hexon assembly-associated protein, one nucleotide deletion (C) at position 30,511 where is L5 gene noted is fiber splice acceptor site, two nucleotides insertion (CG) between position 32,890 and 32,891 where is open reading frame 21, one nucleotide insertion (G) between position 35,541 and 35,542 where is open reading frame 19 noted produce putative lipase, one nucleotide insertion (G) between position 35,541 and 35,542 where is open reading frame 18, one nucleotide deletion (A) at position 41,020 where is open reading frame 10 and two nucleotides insertion (AT) between position 42,325 and 42,326 where is open reading frame 11. Moreover, tandem repeat sequence was assessed by Tandem Repeats Finder program showed one repeat was found at

position 37,295 to 37,329 where is non coding region with 12 consensus size of CCCCCTGTACAC consensus sequence, the copy number was 2.9 compare with Fowl Adenovirus strain CELO as a reference strain which had two repeats found. There were GCA consensus pattern at position 30,510 to 30,536 with nine copies number and CACCCCCCTGTA consensus at position 37,289 to 37,236 with 3.2 copies number. These finding suggested that positions consist ORF 10, 11, 14, 18, 19, 21 and the L4 and L5 genes may serve as non-functional gene and ORF due to change in protein translation.

Accession number. Nucleotide sequence of Fowl Aviadenovirus A strain JM1/1 has been submitted to the GenBank database under the accession number MF168407.

Discussion

The strain JM1/1 used in this study was isolated from an outbreak of gizzard erosion in Japan and be classified as serotype 1. Virus genomic sequence showed 99% nucleotide sequence identitcal to Fowl aviadenovirus strain CELO which is the European reference strain. Gizzard erosion Fowl aviadenovirus serotype 1 from Europe (Germany, Poland, Hungary, Austria and Italy), Japan and Korea has share common ancestor from phylogenetic analysis of loop 1 of hexon gene (Mase et al. 2014) consistent with some study showed a divergence is independent of geographic distance, low divergence between strains of the same serotype from different countries from loop 1 sequence of hexon gene (Niczyporuk et al. 2016). Not only serotype 1 found connection with gizzard erosion (Kajan et al. 2013) but also serotype 8 can affect chicken especially in Japan. Nonetheless serotype 8a and 8b were not reported in chickens with gizzard erosion in another countries (Mase et al. 2014). These findings suggested that Fowl aviadenovirus serotype 1 strain JM1/1 from Japan was group together with European Fowl aviadenovirus serotype 1 strain CELO from nucleotide sequence identity.

Since pathogenic Fowl avidenovirus infection diagnosis based on macroscopic lesion of koilin layer inflammation with scoring scheme 0-3 (Nakamura et al. 2002), microscopic lesion of cellular infiltration in lamina propia, submucosa and muscular layer of gizzard (Ono et al. 2003), viral isolation from samples with

high amount of viral DNA as the present higher amount of viable virus usually found maximum viral load in gizzard (Grafl et al. 2013) where has putative Aviadenovirus serotype 1 receptors (Taharaguchi et al. 2007) and biomolecular technique for example real time PCR for detection and quantification (Gunes et al. 2012), typing by polymerase chain reaction with primer pair Hexon A- Hexon B followed by restriction fragment length polymorphism (RFLP) (Meulemans et al. 2004) and followed by DNA sequencing or pyrosequencing analysis of hexon L1 region provide rapid easier differentiation and classification (Pizzuto et al. 2010) Although, all of these technique cannot be find and identity pathogenicity marker in the genome sequence so Next-Generation-Sequencing (NGS) might be useful. The present complete genome sequencing of Fowl aviadenovirus A strain JM1/1 showed multiple sites insertion and deletion. Interestingly, one of all mutation, mutation in open reading frame 19 (ORF19) which coding putative lipase. It has the property in viral cycle in Marek disease virus (MDV) that enhance the replication and pathogenic potential (Kamil et al. 2005) but the role in Aviadenovirus has not been studied. Lipase has homologues not only in Family Herpesviridae but also in Parvoviridae, Poxviridae (Chiocca et al. 1996, Ojkic and Nagy 2000, Washietl and Eisenhaber 2003, Corredor et al. 2006, Corredor et al. 2008) and some Avian Adenoviruses (Kamil et al. 2005). ORF 19 were highly conserved between serotype 1 and serotype 4 in BLASTP analysis (Griffin and Nagy 2011). Fowl aviadenovirus

serotype 4 strain SH95 cause 100% mortality in SPF birds in Mexico showed the important difference between pathogenic and apathogenic using reference strain KR5 and ON1, was identified in ORF 19. It has two stop codons caused by deletions of nucleotides, result in alteration in the reading frame (Vera-Hernandez et al. 2016) consistent with an outbreak of Chinese Fowl aviadenovirus serotype 4 in 2015, revealed deletion of 196 nucleotides compared to serotype 4 reference strains (Ye et al. 2016). Another higher pathogenic Chinese Fowl aviadenovirus serotype 4 strain HB 1510 cause Hydropericardium-Hepatitis Syndrome in chicken also showed 11 amino acid deletion at ORF 29 (Li et al. 2016) which located in the right end of Fowl aviadenovirus genome similar to ORF 19. The role of deletion is still unknown, a recent study indicated that serotype 4 isolates with shortened ORF19 showed higher virulence than isolate with the full ORF 19 (Vera-Hernandez et al. 2016). These findings suggested that Fowl aviadenovirus A strain JM1/1 with the deletions at ORF 19 may predispose the lesion of gizzard erosion and its pathogenicity.

In term of repeat sequence analyses from the complete genome of Fowl avidenovirus A strain JM1/1 showed one repeat was found at position 37,295 to 37,329 where is non coding region with 12 consensus size of CCCCCTGTACAC consensus sequence that is similar to high mortality outbreak caused by serotype 4 in Mexico revealed eight repeated sequence compared to KR5 and ON1 as reference strain (Vera-Hernandez et al. 2016). These findings suggested that the repetitive

sequence change in Fowl aviadenovirus genome may cause higher virulence, however this effect is still unknown and further studied are need to be elucidated.

In summary, the complete genome sequence analyses of Fowl aviadenovirus A strain JM1/1 cause gizzard erosions in broiler chickens in Japan revealed that it is a member of serotype 1 and most closely identical to the strain chicken embryo lethal orphan (CELO) which is apathogenic Fowl aviadenovirus serotype 1 European strain. It also have some multiple site insertion, deletion and repetitive sequence were found. These will provide more information on the evolution of Fowl aviadenovirus and may help elucidate viral pathogenesis on molecular biology especially on its genetic role. Our next studies will be figure out the locus where can cause pathognomonic lesion in gizzard.

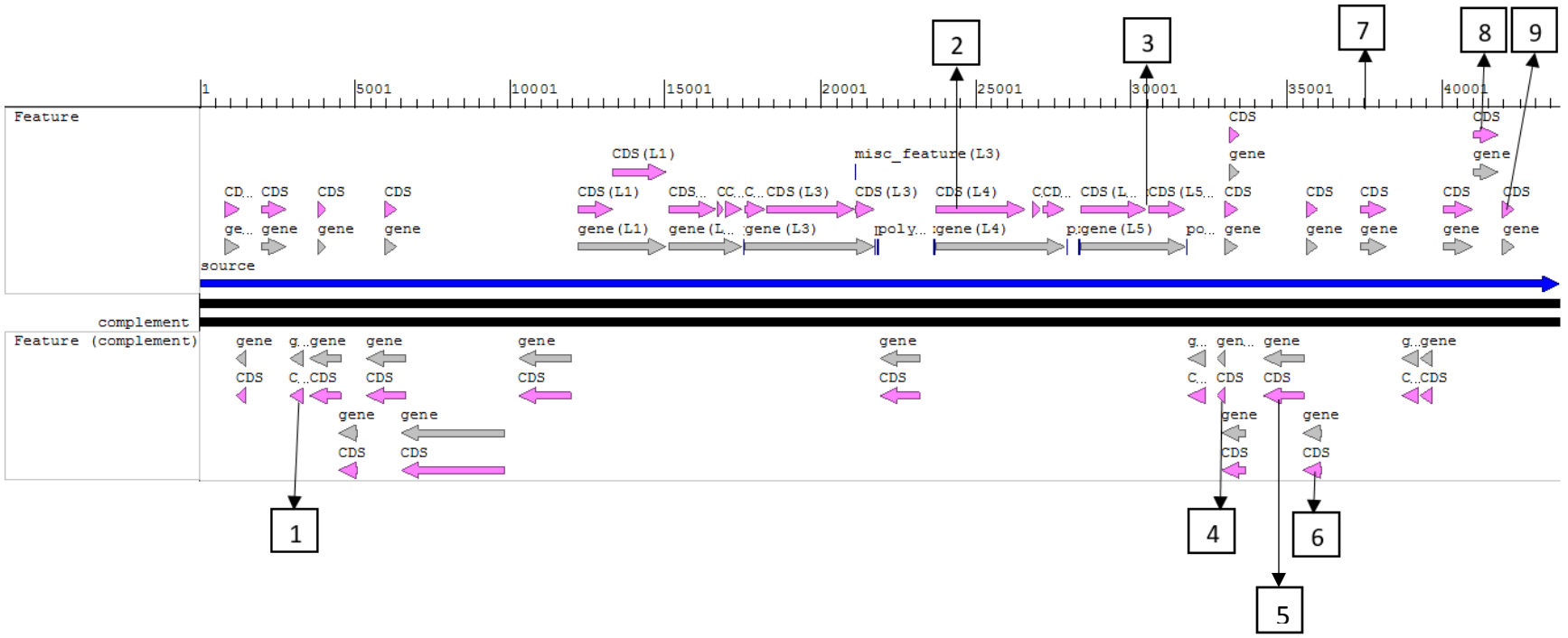

Fig. 1. Comparison of the different positions of Fowl aviadenovirus A strain JM1/1 and pathogenic strain CELO by using CELO map as a reference. (1-8) Different positions are shown in number within square. (1) ORF 14. (2) L4 gene. (3) L5 gene. (4) ORF21. (5) ORF19. (6) ORF18. (7) Tandem repeat sequence of CCCCCTGTACAC consensus sequence. (8) ORF10. (9) ORF 11.

CHAPTER 2

Recombinant DBP antibody production and its application on the diagnosis of Fowl Aviadenovirus infection

Abstract

To investigate Fowl aviadenovirus (FAdV) infection, neutralizing antibody titer is detected by Neutralization test using chicken kidney (CK) cells which is a one of serological assay, widely use in epidemiological survey. By using this assay, it is necessary to perform serial dilution work following incubation for couple days and evidence of cytopathic effect (CPE) was examined, thus it takes time and difficult to concurrently process with multiple specimens. Generally, a neutralizing antibody inhibits viral proliferation by inhibiting adsorption of virus to cells or to neutralize by binding to the virus. In this study, the novel neutralization test was developed to detect the presence of viral protein expression by Fluorescent Antibody Virus Neutralization assay (FAVN).

FAdV serotype 1 strain JM 1/1 and anti-FAdV chicken serum were mixed and incubated for an hour according to the conventional method, subsequently the serum- virus mixture was inoculated into 96 wells of CK cells and incubated for 72 hours. To examine positive fluorescent signal, cells were incubated with In-House anti-DBP antibody for primary antibody incubation and anti-rabbit IgG FITC. In addition, the neutralizing antibody titer was measured and compared the titer of field sera between a conventional and a novel method.

FAVN was successfully detected in an expression, and the titre results were high correlated with the conventional method. The correlation coefficient (R^2) was 0.8, $p < 0.01$ and it can be applicable to field chicken sera. Moreover, the use of 96 wells-plate of CK cells could be used, so that an operation of multiple samples can be achieve quickly and easily. In conclusion, this developed FAVN could be an alternative test for FAdV infection, and it will be useful in disease investigation, surveillance of FAdV infection, and disease elimination.

Introduction

The Family adenoviridae is a linear non-enveloped virus, with an icosahedral nucleocapsid containing a double stranded DNA genome, approximate genome size is 30 to 37 kbp. The capsid has a regular icosahedral structure with a diameter of 70 to 90 nm and consists of 252 capsomeres (Chiocca et al. 1996). 12 of them are penton and 240 are hexon (McFerran et al. 1975). Replication of adenovirus is carried out by expression of an early gene followed by expression of a late gene. The adenoviridae are classified into the genus Mastadenovirus which is a mammalian host, and the genus Aviadenovirus, Atadenovirus and Siadenovirus, which birds are hosts (Buchen-Osmond 2016). Moreover, Fowl adenovirus, Goose Adenovirus and Duck adenovirus which are affect avian species, these virus can be able to propagate in susceptible primary cultured cells derived from avian, subsequently form a CPE in infected cell (Mase et al. 2002, Mirza et al. 2012.

Fowl aviadenovirus (FAdV) has been grouped into five species based on their molecular structure and further divided into 12 serotypes by cross-neutralization assays. FAdVs often isolated from chicken intestinal tract and respiratory tract, more than one serotype commonly isolated from single specimen. FAdV has almost no cross protection among serotypes (Nakamura et al. 1999, Nakamura et al. 2000, Nakamura et al. 2002a, Nakamura et al. 2002b). FAdVs are endemic worldwide and have been found to cause pericardial edema syndrome (HPS), inclusion body

hepatitis, quail bronchitis, hydropericardium syndrome, gizzard erosion and pancreatic necrosis (Nakamura et al. 2000, Okuda et al. 2001, Okuda et al. 2004). Most FAdVs are a low virulent and subclinical infections however they can be induced by immunocompromised condition such as coinfection with infectious bursal disease virus (IBDV) and avian anemia virus (CAV) (Abe et al. 2001, Okuda et al. 2004, Ono et al. 2004).

Gizzard erosion in leghorn chicken by using microscopic findings, electron microscopy, immunohistological examination and virus isolation has been reported, the most common for gizzard erosion is related to FAdV serotype 1 (Goodwin 1993). In addition, the occurrence of gizzard erosion was accompanied by FAdV serotype 1 infection in 17-days-old chicks (Abe et al. 2001). An experimental infection with FAdV serotype 1 isolated from the gizzard erosion lesion was also performed and it has been confirmed that SPF chickens and commercial broilers reproduce lesions of gizzard erosion in many isolates (Okuda et al. 2001, Ono et al. 2003, Ono et al. 2004). Furthermore, Okuda et al. reported that infection of FAdV serotype 8 to 5-days-old SPF chickens showed no clinical signs but mild macroscopic and microscopic lesions were in the gizzard. An intramuscular inoculation of FAdV serotype 8 to day-old SPF chicks showed severe clinical signs (Okuda et al. 2001). Seroepidemiologic studies in Japan have found neutralizing antibody against

serotype 8, suggesting that it is widely circulated in chicken flocks nationwide (Mase et al. 2014)

Therefore, no convenient method for serological survey of FAdVs serotypes infections are provided, neutralization test is used. However, the neutralization test has several problems as a result of the requirement of multistage dilution work, it takes time to process multiple samples and skill is required to visually confirm the occurrence of CPE. Above all, CK cells which are susceptible cells are difficult to culture, they are easy to be detached from the culture vessel thus 96-wells plate cannot be used for multiple specimens. Fluorescent Antibody Virus Neutralization assay (FAVN) which is a neutralizing antibody titration method using fluorescent conjugated antibody was successfully performed on Rabies Virus, which is play an important role in public health, has been established and put to practical use (Cliquet et al. 1998, Hostnik 2000). In the same way it would be applicable to aviadenovirus.

The full-length genome sequence of JM1/1 isolated from a broiler, which caused gizzard erosion (Thanasut et al. 2017) was compared with other FAdV serotypes and explored for a conserved domain. A DNA binding protein (DBP) was found more conserved a domain region of FAdV species A. DBP is a nonstructural protein present in the E2A region, which encodes one of the early proteins. DBP is a protein, which is detectable, early and is responsible for initiating DNA replication (Guo et al. 2013). With the purpose of developing a neutralization test of FAdV, we

selected DBP as the target of the primary antibody in the FAVN and constructed an In-house DBP antibody. In comparison with the FAVN detection, using this antibody and the conventional VN test, we investigated whether the FAVN method which is the novel approach, and could it be an alternative method.

Materials and methods

Genome sequence comparison

Complete genome sequence of FAdV A strain JM1/1 was obtained from GenBank database. The accession number is MF168407. Subsequently, FAdV A strain JM1/1 was compared with available reference complete genome sequence of FAdV species A, B, C, D and E (Accession numbers: NC001720, NC021221, NC015323, AC 000013, and NC014969) by using software BLAST. Nucleotide and amino acid sequence were compared and showed as homology percentage. Moreover, for phylogenetic analysis, the DBP region of E2B gene of the FAdV was used. Phylogenetic analysis, multiple sequence alignment was performed using ClustalW. Phylogenetic tree was generated by the Neighbor Joining (NJ) method as implemented in Genetyx software version 12.0.4 (Genetyx Inc., San Francisco, CA, USA).

DBP gene cloning and expression

A plasmid for synthesizing the GST fusion protein of DBP was constructed. PCR was performed to amplify the DBP region using a primer pair with restriction enzyme cleavage site added as follow: 5 'DBP Flexi (5'gtcagcgatcgccatggaaagaaccccgaaaag-3') and 3' DBP Flexi (5'cttcgtttaaacagcgaacgggtcgtcactgg-3'). PCR mixture included: 100 ng of viral

DNA, 10 µM of each primer, 2.5 µM of each dNTP, 1X reaction buffer containing 1.5 mM $MgCl_2$ and 1.25 unit of Ex Taq polymerase (TaKaRa, Tokyo, Japan), in a total volume of 50 µl. The PCR conditions used were: one cycle of initial denaturation at 95°C for 2 min, 30 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 60 sec, extension at 72°C for 90 sec and one cycle of final extension at 72°C for 10 min. Agarose gel electrophoresis was carried out in order to confirm whether the produced PCR product was the target DNA size on 1% agarose gel. PCR products was gel purification using FastGene™ Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan) according to manufacturer's protocol.

In order to insert the DBP gene into the plasmid pFN2K (GST) Flexi® Vector (Promega, WI, USA), both ends of the plasmid and the DBP gene are cleaved with a restriction enzyme. pFN2K is a plasmid available for inducible expression of GST fusion protein in *E. coli* using the T7 RNA polymerase promoter. Since it has a kanamycin resistance gene, it is possible to select *Escherichia coli* successfully transformed using kanamycin-supplemented LB medium. Plasmid have a barnase gene as a lethal gene in the recombination region and whose recombination was unsuccessful are selected during transformation. In order to clone the recombinant plasmid, the ligated plasmid was transformed into competent cell JM 109 which is suitable for the cloning of a plasmid that is stable in quality by mutation of recA

gene (expressing recombinase) and mutation of endA1 gene (expressing nonspecific endonuclease). FastGene ™ plasmid mini kit (Nippon Genetics, Tokyo, Japan) was used for extraction of plasmids according to manufacturer's protocol. Several colonies were picked up with a sterilized toothpick. Sequence analysis was performed on the extracted plasmid, plasmids which recombination was correctly performed were selected and stored at -25 °C.

Competent OneShot® BL21 Star™ (DE3) cells (Thermo Fisher Scientific, Massachusetts, USA) was used for expression of recombinant protein. BL21 is an *Escherichia coli* strain lacking lon protease and ompT outer membrane protease which is excellent in protein expression stability. The ligation mixture was transformed into competent cells. Plasmids containing DBP genes were identified by restriction pattern analysis using *Sma*I and *Eco*RI and also confirmed by sequencing. Recombinant bacteria were grown in 5 ml LB broth containing 100µg/ml kanamycin overnight at 37 °C with shaking. The culture was then added to 400 ml LB supplemented with 100µg/ml kanamycin and cultured with shaking at 37 °C until the OD600 was 0.4 to 0.6. 1mM Isopropyl-beta-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1% V/V to induce the DBP expression. After incubation for 2 h, cells were centrifuged at 4,800 rpm for 20 min at 4°C and cell pellets were treated with a final concentration of 1 mg/ml lysozyme in lysis buffer. The lysis buffer contain 10x PBS, 10 mg of

lysozyme, one tablet of protease inhibitor, 1M $MgCl_2$ and 100 U of DNAase I in a final volume of 10ml. A protease inhibitor (Roche, Mannheim, Germany) was added so the target protein was not decomposed by *E. coli* protease. To extract protein extraction from *E. coli*, a freeze-thaw method and ultrasonic disruption method were combined following centrifugation at 12,000 xg for 20 min at 4°C. The supernatant (soluble fraction) was collected.

Recombinant protein was purified from the soluble fraction by affinity chromatography column of GSTrap™4B (GE Healthcare, Uppsala, Sweden), following the manufacturer's protocol. Pool eluted purified protein concentration was measured using the NanoDrop™ 1000 and subjected to SDS-PAGE, CBB staining and Western Blotting using anti-GST tag as a primary probing were carried out to confirm the expression of the recombinant protein. The sample was run in 10% SDS-PAGE gel with 200 V of electricity for 40 min to confirm a present of target protein.

Anti DBP antibody production

A SPF New Zealand White rabbit (SLC, Japan) was subcutaneously injected with 0.2 mg purified DBP protein in Complete Freund's Adjuvant (CFA). The rabbit was boosted twice at 3 week intervals with half concentration purified DBP protein in Incomplete Freund's adjuvant (IFA). Serum samples were collected from ear vein

prior each immunization and one week following last immunization. The sera were left at 37 °C for 30 minutes, centrifuged at 3,000 rpm for 15 minutes and stored at -25 °C. Specific DBP antibodies were determined by Western Blotting and Immunofluorescence assay (IFA). In addition, the collected sera were used as primary probing antibody and performed cross reaction activity to other serotypes (strain Ote, SR48, SR49, KR5, TR22, SR119, TR59 and YR36) by Western Blotting and to prove inhibition of DBP expression by anti-FAdV strain JM 1/1 antibody by IFA. A SPF rabbit was kept in our Research Institute of Biosciences in isolator units under positive pressure. Water were provided *ad libitum*. The animal experiments were approved by the Azabu University-Institutional Animal Care and Use committee (Permission No. 150318-2) and conformed to the guidelines on the care and use of animals in research.

FAVN and VN test

Two fold serial dilutions of anti-FAdV strain JM1/1 chicken serum in the range of 400 to 25,600 were prepared and incubated with a 200 $TCID_{50}$/100 µl of FAdV strain JM1/1 for an hour. Following this incubation, 100 µl of virus-serum mixture were added into 48 and 96 wells of confluent CK cells for VN and FAVN test respectively and continue incubated at 37 °C. After this incubation period, direct light microscopic examination of the VN test plate for presence of Cytopathic effect

(CPE) was performed. For the plate of FAVN, 4% paraformaldehyde in PBS was added into each wells for 45 min. After fixation, cells were incubated with 0.05% Triton X-100 in PBS for 30 min. Nuclear membrane permeated cells were wash and blocking was made with 10% nonfat dried milk diluted in PBS to prevent antibodies from nonspecific binding. Primary antibody incubation was performed using the anti-DBP antibody from rabbit serum diluted in 1% nonfat dried milk in PBS and incubated for 30 min. Cells were incubated with anti-Rabbit IgG FITC in 1% nonfat dried milk in PBS for 30 min to develop a fluorescent signal. Visualize the result on a fluorescent microscope. The antibody titre were determined to assess a synchronization between two tests. A maintenance medium mixed with virus and SPF chicken serum mixed with virus were used as positive control. Whereas, a negative control was only inoculated maintenance medium.

Field sera

VN and FAVN test as described previously, were performed on Chicken serum samples, obtained from poultry farms in Japan. The antibody titre from both tests was analyzed for correlation and a graph of an antibody titre distribution by farm were made.

Results

In the DNA extraction from FAdV JM1/1 seeding stock using Phenol-chloroform method, genomic DNA was observed one band on 1% agarose gel (Fig. 2a). The DNA digested with restriction enzyme *Hin*dIII showed the same band pattern as predicted digested genomic DNA of CELO strain by using GENETYX: Search Restriction Fragment (Fig. 2b). FAdV strain CELO which is the apathogenic strain of serotype 1 and it is a European reference strain. It was suggested that full-length genomic DNA of FAdV JM1/1 strain was extracted. This full-length genomic DNA was subjected to next generation sequencing, performed by Ion Torrent to determine the complete nucleotide sequence of FAV JM1/1 strain. In addition, sequences were analyzed by BLAST search in the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/blast). The result showed the complete genome of FAdV strain JM1/1 was found to be 43,809 nucleotides long with 54.31% G+C content. Compared with other Aviadenovirus sequences available in GenBank. The strain JM1/1 revealed 99% nucleotide sequence identical to Fowl Adenovirus A strain CELO (GenBank accession number: NC001720). Sequence has been submitted to the GenBank database under the accession number MF168407 (Thanasut et al. 2017). From find out a conserved domain, there are many conserved domains and DBP region is a one of them. In addition, at 1,326 bases from 21,918 to 23,243 bases encoding the early protein DBP of FAdV JM1/1 was compared to

the complete nucleotide sequence of FAdV which available in GenBank include FAdV species A strain CELO (GenBank accession number NC001720), species B strain 340 (GenBank accession number NC021221), species C serotype 4 strain ON1 (GenBank accession number NC 015323), species E serotype 8 strain HG (GenBank accession number NC014969) and species D serotype 9 strain A-2A (GenBank accession number AC000013). The nucleotide homology of DBP in comparison to other serotype showed 99% identity within serotype 1 which a member of species A, but within species B, C, D and E showed low identity with the range of 60-72%. By comparing the amino acid homology, it also showed high identity within serotype 1 but showed low identity with 53-75% in the other serotype (Table 1). It was also expected that DBP is a serotype specific based on nucleotide sequence and predicted amino acid sequence. Phylogenetic analysis based on the DBP region, a closer genetic relationship between FAdV species A strain CELO and JM1/1 was found (Fig. 3). Similar result was described using nucleotide and amino acid homology.

In the PCR evaluation using purified DNA template, positive DBP amplification was observed and the target amplicon size was generated. Its size was 1,326 base pairs. Amplicons were successfully cloned into pFN2K vector and sequenced. The sequences were analyzed by BLASTN showed an identity with initiator. These plasmids were transformed into BL21 (DE3) competent cell to express recombinant protein by IPTG induction. The expression result showed

bands appeared around 75 kDa by SDS-PAGE and CBB staining. It seems that fusion proteins of DBP (51.5 kDa) and GST (24.2 kDa) are normally expressed. On the other hand, the purified protein sample that passed through GSTrap affinity chromatography column was broken down: two bands appeared around 50 kDa and 25 kDa, it was originally expected that it would appear at 75 kDa as a fusion protein.

A rabbit was immunized with purified GST-DBP fusion protein to obtain antibodies against DBP. Western blotting and IFA were performed using rabbit serum in order to ascertain whether the antibody against DBP was present in the obtained immune serum. Sera were used as the primary antibody. The expected molecular weight from the DBP amino acid sequence is 51.5 kDa. WB analyze showed two bands around 50 kDa and 35 kDa were observed in FAdV JM1/1 infected cell lysate, but no specific and nonspecific bands were observed in uninfected cell lysates (Fig. 4a). Since the expected molecular weight is 51.5 kDa, the band found at 50 kDa on membrane is DBP of FAdV. Likewise, in the CK cells infected with FAdV JM1/1, the positive fluorescent reaction was found in the nucleus by IFA, but fluorescence was not observed in non-infected cells (Fig. 4b). The result showed the rabbit sera contained a specific antibody against DBP that could detect FAdV infection by WB and IFA.

To clarify cross reaction to other FAdV serotypes study using anti-DBP antibody as primary probing, the result showed bands around 50 kDa relate to the

molecular weight of DBP and 35 kDa in the lanes of JM 1/1 and Ote strain. A band of 35 kDa was also observed in KR 5 and TR 22, but no band around 50 kDa was observed in the other strain (Fig. 5). The results suggested that DBP antibody had a cross reactivity only within FAdV serotype 1 and did not respond to other serotypes as stated in the genetic information.

The expression of DBP was suppressed by FAdV JM1/1 immune serum by neutralization. As a result of observation using a fluorescence microscope performed by fluorescent antibody assay using anti-DBP antibody as the primary incubation, specific or nonspecific fluorescence was not observed in the case where anti-FAdV serum reacted with FAdV, but in the reaction of chicken SPF serum with FAdV showed positive fluorescence was inside the CK cells nucleus (Fig. 6). This suggested that FAdV JM 1/1 was neutralized by anti-FAdV serum and DBP expression was suppressed so anti-DBP antibody could be applied to FAVN test.

For evaluation of the synchronization between the two tests, a novel FAVN and a conventional VN test were conducted separately using anti-FAdV JM1/1 antibody and the neutralization antibody titre were measured. In the FAVN test, the result showed specific positive fluorescence was observed in the nucleus at 6,400 times dilution that it is no longer able to neutralize all the virus in the test. Similarly, in the VN test, CPE was only observed at 12,800 times dilution (Table 2). The neutralizing antibody titre from both tests were 6,400, this result suggested that it

has a synchronization of measured antibody titre between the two tests. Furthermore, it was inferred that the neutralizing antibody titre could be measured from the developed FAVN.

The obtain field chicken sera from various farms in Japan were subjected to FAVN and VN test and antibody titre were measured. By comparing the neutralizing antibody titre results from FAVN, and VN, the correlation coefficient between tests was calculated. Coefficient of determination (R^2) was as high as 0.8, $p < 0.01$ (Fig. 7). Additionally, a graph of FAVN and VN antibody titre distribution by each farm was generated (Fig. 8). The graph showed no significant difference between tests. These results suggest that the FAVN and VN both, have a high correlation, so that FAVN could be used as an alternative method to that of VN and it is presumed that the antibody from FAVN could also be measured in the field chicken sera.

Discussion

FAdV infection causes various associated diseases, usually in a subclinical state, leading to serious economic loss in the poultry industry. That FAdV infection causes different lesions and clinical symptoms depending on serotype or strain has been confirmed. FAdV serotype 1 is known to exhibit a lesion by itself and cause clinical symptoms such as quail bronchitis, gizzard erosion, remarkable decrease in weight gain, anorexia and so on (McFerran et al. 2000). And because of it affects serious damage in the poultry industry, so investigation of the infiltration status of FAdV serotype 1 is urgent in the poultry farming industry. In Japan, a FAdV vaccine is not approved, so to cope with this infection, improvement in the feed, and environmental management and on-site vaccination by inoculation with FAdV in chickens, should be implemented.

To identify of FAdV serotypes infection, a neutralizing antibody (VN) test is currently used. This is a method of detecting a neutralizing antibody titre, which inhibits the infection of a virus in the animal sera and makes it possible to identify the virus infection. In recent years, it has been used for examination of infectious diseases that cause severe and significant economic loss such as bovine epidemic fever, Ibaraki disease virus and Akabane disease virus. However, it is not simple to replace the VN test, which requires complicate and skillful technique to assess CPE, time-consuming procedures, and difficulty in culturing sensitive susceptible CK

cells. For this reason, some epidemiological surveys of serotypes have been reported (Kawamura et al. 1964). Previously, using FAVN test for measuring a neutralizing antibody titre has been successfully developed for the rabies virus (Cliquet et al 1998, Hostnik 2000, Smith and Gilbert 2017). It has high reproducibility and reliable results. Therefore, in order to solve the problems of the conventional VN test as previously mentioned, we applied the FAVN technique used in rabies virus for detection of FAdV neutralizing antibody and investigated its usefulness. Compared with the conventional method, it was able to process multiple samples simultaneously. The measurement results of antibody titre to serotype 1 using FAVN test, which is a novel method, showed a high correlation with the VN method which is a conventional test. This FAVN is also applicable to field chicken sera, it is possible to use 96-wells plates, for sera dilution, and to use 96-wells CK cells comparisons quickly and easily and it could able to process multiple samples simultaneously. In addition, it is not necessary to observe CPE, which requires skillful technique that is necessary in the VN test, and it has specificity to FAdV serotype 1 when using anti-DBP antibody. It is considered that the FAVN could be an alternative to the existing VN test.

The use of a rapid ELISA test is increasing, along with the VN test, which are applied only a limited number of virus such as the poliovirus (Edevag et al. 1995 and Ivanov et al. 2005). There are some advantages of ELISA test for example,

decrease time consuming due to lack of culturing CK cells, and viruses, no need for special cell handling techniques and a accurate CPE determination technique is unnecessary. However, the available commercial ELISA kits for FAdV, are only serotype 1 specific antibody titre measurements. Recently, a method to prepare a monoclonal antibody against the FAdV fiber gene, which is a structural protein of FAdV serotype 1 (species A), and FAdV serotype 4 (species C), and measuring the neutralizing antibody titre by ELISA have been established. Since monoclonal antibodies against structural proteins are used, homology other than serotype 1 and type 4 is not high. However, they respond to other serotypes due to a similarity of epitopes that may occur. Also, it is considered impossible at the present, to distinguish between a natural infection and an autologous live vaccination by targeting structural proteins (Feichtner et al. 2017).

The results provided by this present study demonstrate that the FAVN test has some advantages over current VN test and ELISA. The simplicity of FAVN, it is a simple procedure with no need for highly skilled workers, and its properties that correlate with the VN test make it an alternative diagnosis test for FAdV infection. To our knowledge, this developed FAVN test will be useful in disease investigation, surveillance of FAdV infection and disease eradication. Further studies focused on the other conserved regions of FAdV, that can be used as antibody in FAVN test to measure the neutralizing antibody titre of all serotypes or serotype specific are

required. If it becomes possible, epidemiological survey of FAdV infection in Japan can be carried out easily in poultry farming industry. Development of tests will be help in rapid flock assessment, prevention and control of the disease.

Acknowledgement

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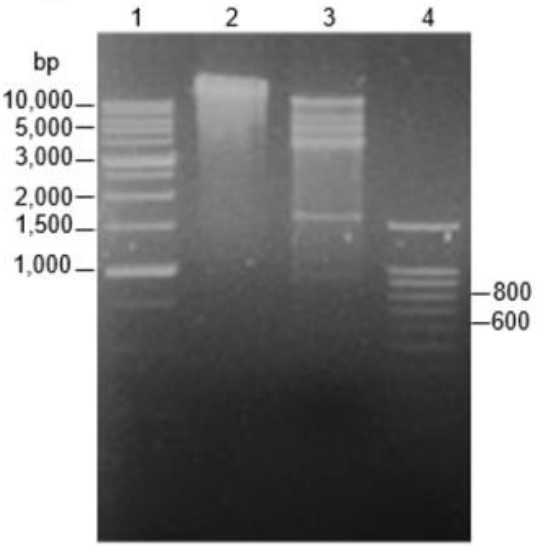
a



b

Location	Fragment size (bp)
5,648-17,900	12,253
27,086-33,935	6,850
17,901-23,346	5,446
33,936-38,742	4,807
38,743-42,850	4,108
1,603-5,647	4,045
23,347-27,085	3,739
1-1,602	1,602
42,851-43,809	959

Fig. 2. Genome of FAdV JM1/1 restriction analysis by *Hin*dIII. Complete genome DNA was extracted from FAdV JM1/1 seeding stock. DNA was digested by *Hin*dIII. on 1% agarose gel (a): lane 1, 1Kbp DNA marker; lane 2, Undigested FAdV JM1/1 DNA; lane 3, digested FAdV JM1/1 DNA; lane 4, 100bp DNA marker. The theoretical DNA band patterns of complete genome of FAdV strain CELO digested by *Hin*dIII was predicted by using Genetyx (b).

Table 1. Homology analysis of DBP region between FAdV serotype 1 strain JM1/1 used in this study and all available complete genome sequence of FAdVs in GenBank database.

a
Nucleotide homology (%)

(%)	species A (JM1/1)	species A (CELO)	species B	species C	species D	species E
species A (JM1/1)[a]	*					
species A (CELO)[b]	99	*				
species B[c]	65	60	*			
species C[d]	64	61	64	*		
species D[e]	64	64	67	64	*	
species E[f]	66	64	68	66	72	*

b
Amino acid homology (%)

(%)	species A (JM1/1)	species A (CELO)	species B	species C	species D	species E
species A (JM1/1)[a]	*					
species A (CELO)[b]	99	*				
species B[c]	59	59	*			
species C[d]	60	55	53	*		
species D[e]	62	62	64	61	*	
species E[f]	61	58	64	61	75	*

[a]FAdV species A strain JM1/1: GenBank accession number MF168407.
[b]FAdV species A strain CELO: GenBank accession number NC001720.
[c]FAdV species B strain 340: GenBank accession number NC021221.
[d]FAdV species C strain ON1: GenBank accession number NC015323.
[e]FAdV species D strain A-2A: GenBank accession number AC000013.
[f]FAdV species E strain HG: GenBank accession number NC014969.

Nucleotide sequence homology (a) and Amino acid sequence homology (b) were performed using Genetyx.

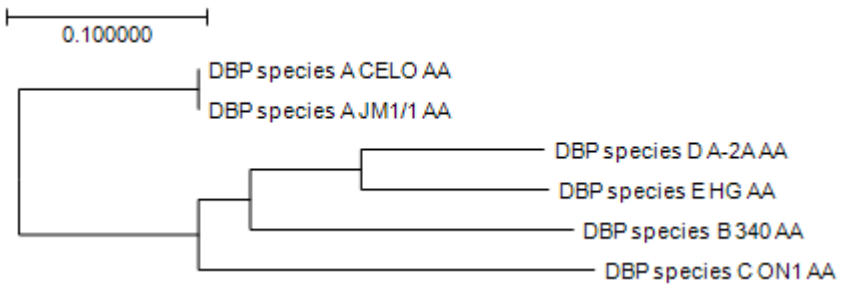


Fig. 3. Phylogenetic tree based on the DBP region of E2B gene of Fowl Aviadenovirus (FAdV). The reference nucleotides and amino acid sequences representing species A to E same as previous homology comparison, including FAdV A JM1/1 were analyzed. The phylogenetic tree were generated by the NJ method.

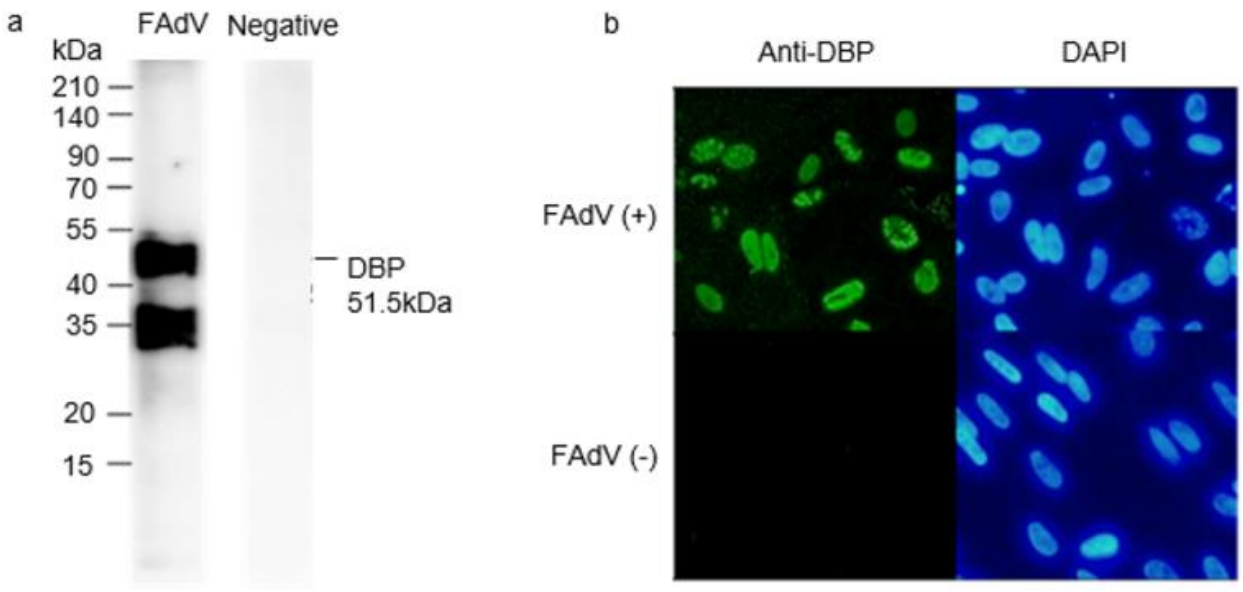


Fig. 4. Expression of DBP protein. The immunized rabbit serum was subjected to Western Blotting and used as primary antibody probing (a). Fluorescent Antibody and DAPI counterstaining were also performed (b).

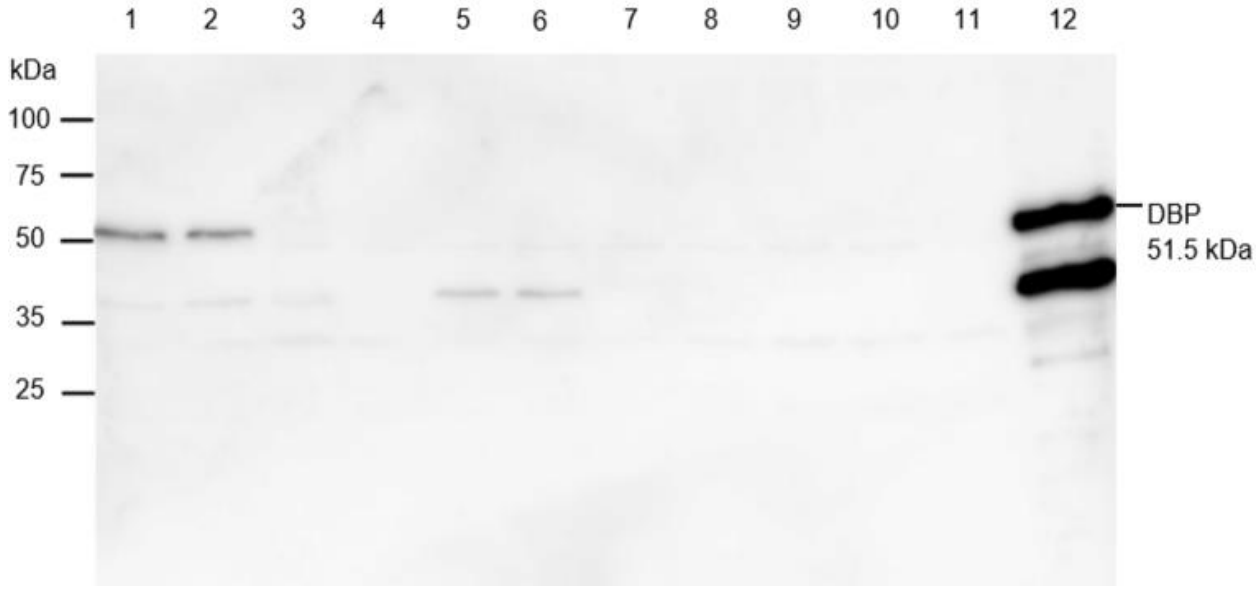


Fig. 5. Cross reaction of anti-DBP antibody to various FAdV serotypes was performed by Western Blotting. Lane 1, strain JM1/1(serotype 1); lane 2, Ote(serotype 1); lane 3, SR48 (serotype 2); lane 4, SR49 (serotype 3); lane 5, KR5 (serotype 4); lane 6, TR22 (serotype 5); lane 7, SR119 (serotype 6); lane 8, YR36 (serotype 7); lane 9, TR59 (serotype 8a), lane 10, CK cells lysate infected with MM (mock control); lane 11, uninfected CK cells lysate (negative control); lane 12, CK cells lysate infected FAdV strain JM 1/1 (positive control).

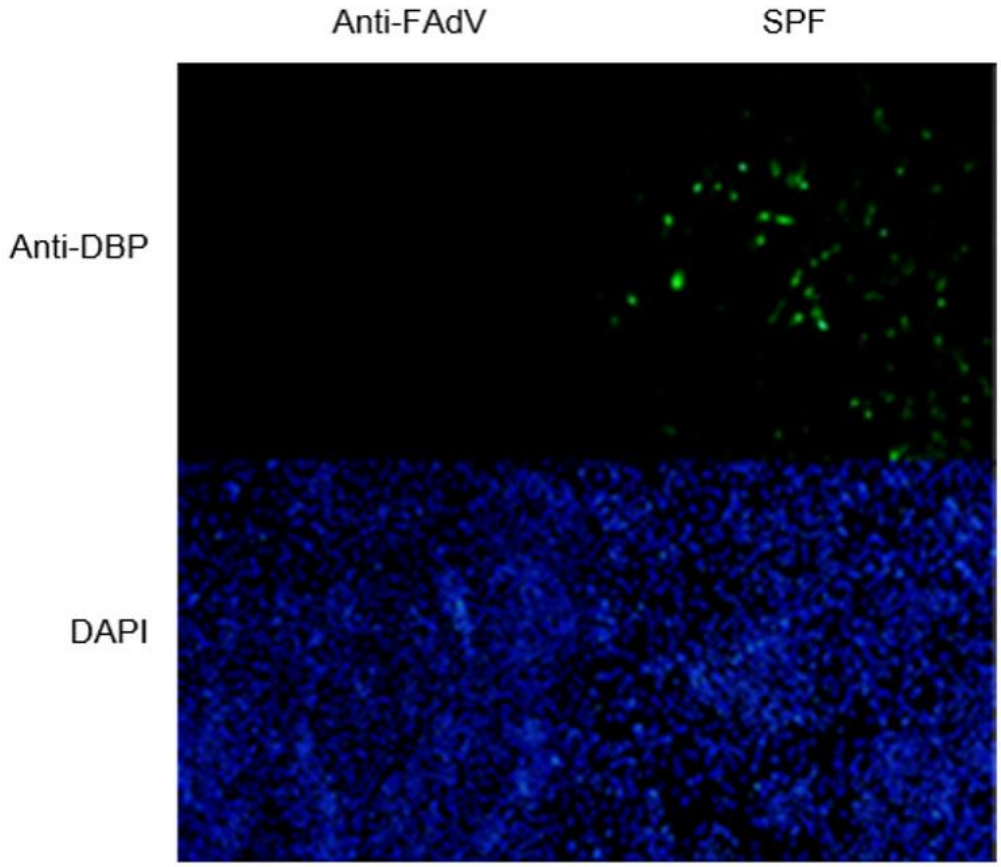


Fig. 6. DBP expression was suppressed by anti-FAdV JM1/1 antibody. Neutralized FAdVs by anti-FAdV suppressed DBP expression by performing Fluorescent Assay using anti-DBP as primary probing antibody and counterstaining by DAPI. SPF chick serum used as negative control.

Table 2. Synchronicity of neutralizing antibody titre of test sera measured between performed by the FAVN and VN test.

	Serum dilution									
	400	800	1,600	3,200	6,400	12,800	25,600	Positive	Negative	SPF
FAVN	-	-	-	-	-	+	+	+	-	+
VN	-	-	-	-	-	+	+	+	-	+

The antibody titre measured by diluting the serum concentration in the range of 400 to 25,600. A maintenance medium mixed with virus (100 $TCID_{50}$) and SPF chick serum (1/40 dilution) mixed with virus were used as positive control. Whereas, a negative control was only inoculated maintenance medium. SPF serum diluted 40-fold was used. Diluted FAdVs 100$TCID_{50}$/100µl was inoculated. Plus sign indicates presence of expression or CPE whereas minus sign indicates absence of expression or CPE.

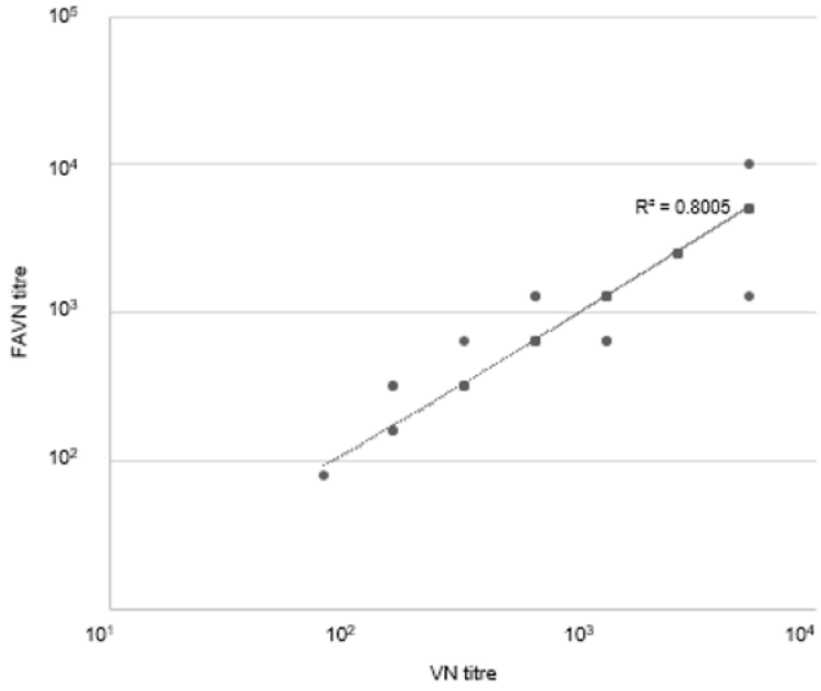


Fig. 7. Positive correlation of chicken sera antibody titre between performing the FAVN and the VN test. Chicken sera from different regions of Japan (n=53) were perform both tests, correlation graph was created for analyzing the correlation coefficient ($R^2 = 0.8005$) ($p < 0.01$).

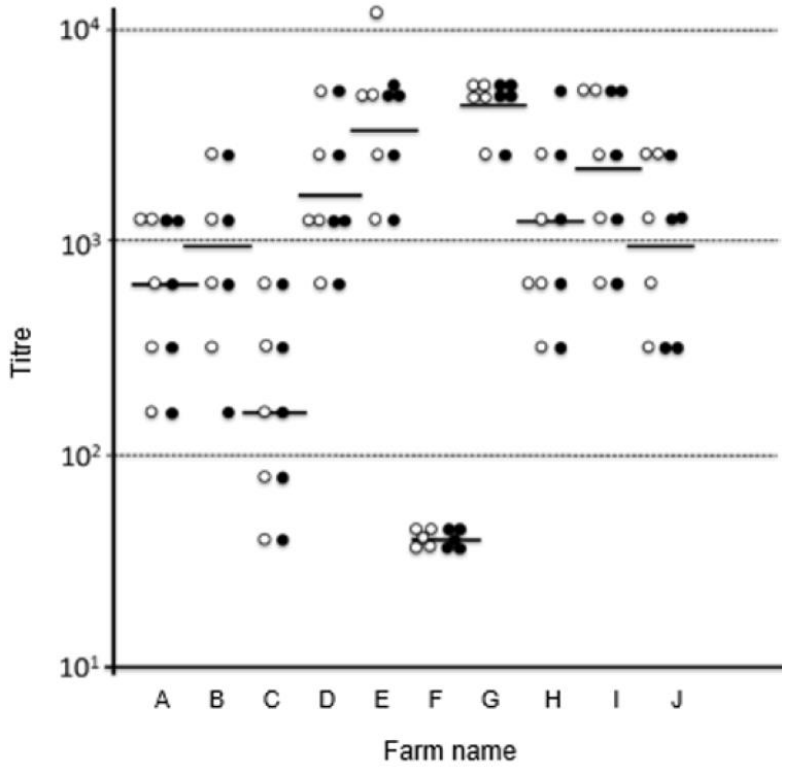


Fig. 8. Neutralizing antibody titre distribution obtained from FAVN and VN test by farm in Japan. ● : Fluorescent antibody virus neutralization test (FAVN), ○ : Virus neutralization test (VN).

CHAPTER 3

Recombinant 52K antibody production and Non-structural 52K protein as an alternative tool for Fowl Aviadenovirus diagnosis

Abstract

The recombinant 52K protein of FAdV strain JM1/1 showed promise as a diagnostic marker for FAdV infection. 52K was found as a conserved domain within all serotypes. It was also a one of late gene and specific on FAdV. The C terminal region, more conserved, was cloned and expressed the 52K non-structural protein gene in the pFN 2k plasmid vector. Purified 52K was used as an antigen, subjected to immunization in a rabbit. Antibody against 52K could be detected in the rabbit sera and used as a primary antibody incubation in Western Blotting (WB) and Immunofluorescence assay (IFA). To further validate the cross reactivity of the anti-52K antibody, CK cells were cultured and inoculated with FAdV serotype 1-8. WB and IFA were performed in detecting FAdVs protein expression and positive fluorescent signal, respectively. The results showed that the anti-52K antibody could be detected all FAdV serotypes used in this study. Overall, the recombinant 52K could be served as an alternative clinical diagnostic tool for FAdV infection.

Introduction

Fowl Aviadenovirus (FAdV) infections are extensive in the poultry industry and leads to worldwide economic losses result of decrease in growth rate, increase in mortality rates and poor quality of gizzards in broiler chickens. FAdV cause inclusion body hepatitis (IBH), Hydropericardium-hepatitis syndrome (HHS), and gizzard erosion (GE) in chickens (Adair et al. 2008). FAdV species D and E have been isolated from IBH, and species C from HHS cases (Hess et al. 1999, Ojkic et al. 2008, Steer et al. 2011). Mostly FAdV species A has been related with GE which has been found in Europe, Korea, and Japan (Ono et al. 2001, Domanska-Blicharz et al. 2011, Manarolla er al. 2009, Marek et al. 2010, Lim et al. 2011).

FAdV belong to the family Adenoviridae and the genus Aviadenovirus. It is non-enveloped icosahedral virus which has a linear, double stranded DNA. Nowadays, it has been classified into five species (FAdV A to FAdV E) from a molecular structure (Zsak and Kisary 1984) and into 12 serotypes (FAdV 1 to 8a and 8b to 11) by the results from cross neutralization test (Hess 2000). Its genome code for a few structural and non-structural proteins (Griffin and Nagy 2011, Shah et al. 2011, Davison et al. 2003). The capsid consists of three major structural proteins include Hexon, Fiber and Penton base (Russel 2009).

Viral structural proteins commonly stimulate host immune response. They have been widely used for vaccine and diagnostic reagent development. On the other hand, some of non-structural proteins for example P27: HIV protein (Bahraoui et al. 1990), NS1: a Dengue virus protein (Zheng et al. 2011), NS3: hepatitis virus protein (Diepolder et al. 1995, can be trigger a humoral and cellular immunity. Viral non-structural proteins can accelerate unique immune responses during particular infectious processes (Falconar 2007). This encourages vaccine design and clinical diagnosis of viral infections (El-Gogo et al. 2008). A non-structural 52K is a one of the important non-structural proteins of the FAdV as involved in capsid assembly and/or genome packaging. 52K expressed in both the early and late stages of infection which suggests that it could play multiple roles in the viral life cycle and interacts with the viral Iva2 protein and is required for DNA packaging (Ahi and Mittal 2016).

For an immunological diagnosis, a structural protein, the structural protein hexon is widely used. The other FAdV proteins have not been fully studied. Therefore, the aim of this study was to investigate the usefulness of the non-structural 52K for an immunological diagnostic development and detection of all FAdV species. The full-length genome sequence of JM1/1 isolated from a broiler, which caused gizzard erosion (Thanasut et al. 2017) was compared with other FAdV serotypes and explored for a conserved domain. The 52K protein was found more

conserved a domain region of all FAdV species. With the purpose of developing an alternative test of FAdV infection diagnosis, 52K has been selected as the target of the primary antibody and constructed an 52K antibody for the Western Blotting and Immunofluorescence assay.

Materials and methods

52K genome homology

L1 52K genome sequence from complete genome sequence of FAdV A strain JM1/1 was derived from GenBank database under the accession number MF168407. Nucleotide and amino acid sequence of 52K were compared to other reference serotype obtained from GenBank include serotype 1 strain CELO (NC001720), serotype 2 strain SR48 (KT862806), serotype3 strain SR49 (KT862807), serotype 4 strain KR5 (HE608152), serotype 5 strain 340 (NC021221), serotype 6 strain CR119 (KT862808), serotype 7 strain YR36 (KT862809), serotype 8 strain TR59 (KT862810), and serotype 9 strain A-2A (AF083975) by using Genetyx program version 12.0.4 (Genetyx Inc., San Francisco, CA, USA). Nucleotide and amino acid sequence homology were showed as homology percentage and a table of homology was made. Moreover, for phylogenetic analysis, the 52K region of L1 gene of the FAdV was used. Phylogenetic analysis, multiple sequence alignment was performed using ClustalW. Phylogenetic tree was generated by the Neighbor Joining (NJ) method as implemented in Genetyx software version 12.0.4 (Genetyx Inc., San Francisco, CA, USA).

One cycle model of 52K expression

CK cells were cultured in 24 wells plate. When the cells were confluent, inoculated with FAdV JM1/1 at MOI=1. At each time point of 0, 6, 12, 24, 36 and 48 h post inoculation, cells were harvested and collected in sample buffer and boiled at 98°C for 5 min. The sample were loaded onto a 10% SDS-PAGE gel and subjected to Western Blotting. CK cells inoculated with maintenance medium used as a control. An anti-52K antibody from rabbit sera was used for primary antibody incubation.

52K late gene and protein expression

CK cells were grown in 6 wells plate until they were confluent. At the FAdV inoculation time, cells were treated with or without 50 µg of cycloheximide. A mock control was only inoculated with a maintenance medium. To examine the expression of mRNA of an early gene DBP and a late gene encoding the 52K protein in FAdV JM1/1-infected CK cells, RNA were isolated from infected cells after 24 h incubation periods using Tripure Isolation Reagent (Roche). cDNA was synthesized from purified DNase-I-treated total RNA using MMLV reverse transcriptase and random primers (Invitrogen, USA) at 30°C for 10 min and 40°C for 60 min according to the manufacturer's protocol. For DBP and 52K transcript amplification, the forward primer DBP (5'-GCTGTGCTCCTGATCCTTGT-3'), the

reverse primer DBP (5’-GTTCCTGGCAGCCGTTATCT-3’), forward primer 52K (5’-GTATCCGCACGGCTCTGTTA-3’) and the reverse primer 52K (5’-TCATGCTCAACTCGACCACC-3’) were used respectively. The PCR products were visualized on 1% agarose gel by gel electrophoresis. In addition, Western Blotting was performed to confirm protein expression.

52K gene cloning and expression

DNA extraction from FAdV A strain JM1/1 was performed as described previously (Thanasut et al. 2017). For the 52K gene, primer pair with the restriction enzyme cleavage site added, the forward primer (5’-gtcagcgatcgccatggagccggctttcacgccggc-3’) and the reverse primer (5’-cttcgtttaaacgatgaagtcatcctcgtcgt-3’) were amplified the 684 base pairs of 52K encoding-gene by PCR. PCR amplification was performed with 100 ng of viral DNA, 10 µM of each primer, 2.5 µM of each dNTP, 1X reaction buffer containing 1.5 mM $MgCl_2$ and 1.25 unit of Ex Taq polymerase (TaKaRa, Tokyo, Japan), in a total volume of 50 µl. The PCR condition with the following cycling times and temperatures: one cycle of initial denaturation at 95°C for 2 min, 30 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 60 sec, extension at 72°C for 90 sec and one cycle of final extension at 72°C for 10 min. Agarose gel electrophoresis was carried out in order to confirm whether the produced PCR product was the target DNA size on 1% agarose gel. PCR products was gel

purification using FastGene™ Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan) according to manufacturer's protocol.

The purified PCR products was digested with restriction enzymes, and the ligated into digested plasmid pFN2K (GST) Flexi® Vector (Promega, WI, USA) by following the manufacturer's protocol. pFN2K is a plasmid available for inducible expression of GST fusion protein in *E. coli* using the T7 RNA polymerase promoter. Since it has a kanamycin resistance gene, it is possible to select *Escherichia coli* successfully transformed using kanamycin-supplemented LB medium. Plasmid have a barnase gene as a lethal gene in the recombination region and whose recombination was unsuccessful are selected during transformation. The ligation mixture was transformed into competent cell JM 109 which is suitable for the cloning of a plasmid that is stable in quality by mutation of recA gene (expressing recombinase) and mutation of endA1 gene (expressing nonspecific endonuclease). FastGene ™ plasmid mini kit (Nippon Genetics, Tokyo, Japan) was used for extraction of plasmids according to manufacturer's protocol. Several colonies were picked up with a sterilized toothpick. Plasmids containing 52K gene was identified by restriction pattern analysis using KpnI and also confirmed by DNA sequencing and stored at -25 °C.

Competent OneShot® BL21 Star™ (DE3) cells (Thermo Fisher Scientific, Massachusetts, USA) was used for expression of recombinant protein. BL21 is an

Escherichia coli strain lacking lon protease and ompT outer membrane protease which is excellent in protein expression stability. The ligation mixture was transformed into competent cells. Recombinant bacteria were grown in 5 ml luria broth (LB) broth containing 100µg/ml kanamycin overnight at 37 °C and cultured with shaking. The culture was then added to 400 ml LB supplemented with 100µg/ml kanamycin and cultured with shaking at 37 °C until the OD600 was 0.4 to 0.6. 1mM Isopropyl-beta-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1% V/V to induce the expression of cloned 52K. After incubation for 2 h, cells were centrifuged at 4,800 rpm for 20 min at 4°C and cell pellets were treated with a final concentration of 1 mg/ml lysozyme in lysis buffer. The lysis buffer contain 10x PBS, 10 mg of lysozyme, one tablet of protease inhibitor, 1M $MgCl_2$ and 100 U of DNAase I in a final volume of 10ml. A protease inhibitor (Roche, Mannheim, Germany) was added so the target protein was not decomposed by *E. coli* protease. To extract protein extraction from *E. coli*, a freeze-thaw method and ultrasonic disruption method were combined following centrifugation at 12,000 xg for 20 min at 4°C. The supernatant (soluble fraction) was collected.

Recombinant 52K protein was purified. The supernatant containing the soluble protein was subsequently bound to GST-agarose resin, affinity chromatography column of GSTrap™4B (GE Healthcare, Uppsala, Sweden), according to the manufacturer's protocol. Pool eluted purified 52K protein

concentration was determined using the NanoDrop™ 1000. To further confirm the expression of the recombinant GST fusion protein, SDS-PAGE, CBB staining and Western Blotting using anti-GST tag as a primary probing were performed.

Anti-52K antibody production

Immunization to the animal

A SPF New Zealand White rabbit (SLC, Japan) was subcutaneously injected with 0.2 mg purified 52K protein in Complete Freund's Adjuvant (CFA). The rabbit was boosted twice at 3 week intervals with half concentration purified 52K protein in Incomplete Freund's adjuvant (IFA). Serum samples were collected from ear vein prior to each immunization and one week following the last immunization. The sera were left at 37 °C for 30 minutes, centrifuged at 3,000 rpm for 15 minutes and stored at -25 °C. Specific 52K antibodies were determined by Western Blotting. A SPF rabbit was kept in our Research Institute of Biosciences in isolator units under positive pressure. Water were provided *ad libitum*. The animal experiments were approved by the Azabu University-Institutional Animal Care and Use committee (Permission No. 150318-2) and conformed to the guidelines on the care and use of animals in research.

Expression of 52K in the rabbit serum by Western Blotting and Immunofluorescent assay

CK cells were cultured on 100 mm dish until cells were confluent. Then, the cells were inoculated with FAdV strain JM1/1 (MOI=5) and continue cultured at 37°C. One day post infection, cells were harvested by a scraper and collected in sample buffer (62.5mM Tris-HCl, pH 6.8), 5% 2- mercaptoethanol, 2% sodium lauryl sulfate, 5% sucrose, 0.005% bromophenol blue) and boiled at 98°C for 5 min. The sample were loaded onto a 10% SDS-PAGE gel. The proteins were transferred onto a polyvinylidene fluoride membrane, then, blocked in 5% nonfat dried milk diluted in PBS. After blocking period, the membrane was incubated with the serum obtained from the immunized rabbit diluted 1:1000 in PBS with 5% nonfat dried milk diluted in PBS for an hour at room temperature and wash three times with PBS-0.1% Tween 20. The incubated membrane was then reacted with a horse radish peroxidase (HRP)-conjugated goat anti- rabbit secondary antibodies (Bio rad, Hercules, California, USA). The membrane was visualized with ECL prime Western Blot detection reagents (GE Healthcare, Buckinghamshire, England) following the manufacturer's protocol. A control was only inoculated maintenance medium.

For confirmation of expression by Immunofluorescent assay, CK cells were prepared into 24 wells plate with immunofluorescence slide and cultured at 37°C,

5% $C0_2$. When cells were confluent, cells were infected with FAdV JM1/1 at MOI 5. One day later, 4% paraformaldehyde in PBS was added into each wells for 45 min. After fixation, cells were incubated with 0.05% Triton X-100 in PBS for 30 min. Nuclear membrane permeated cells were wash and blocking was made with 10% nonfat dried milk diluted in PBS to prevent antibodies from nonspecific binding. Primary antibody incubation was performed using the anti-52K antibody from rabbit serum diluted in 1% nonfat dried milk in PBS and incubated for 30 min. Cells were incubated with anti-Rabbit IgG FITC in 1% nonfat dried milk in PBS for 30 min to develop a fluorescent signal. Visualize the result on a fluorescent microscope. A control was only inoculated maintenance medium.

Cross reactivity to other serotypes

CK cells were cultured on 24 wells plate until cells were confluent. Then, the cells were inoculated with various serotypes of FAdV include strain JM1/1, Ote, SR48, SR49, KR5, TR22, SR119, TR59 and YR36 at MOI=1 and continue cultured at 37°C. One day post infection, cells were harvested by a scraper and collected in sample buffer as describe above and boiled at 98C for 5 min. The sample were loaded onto a 10% SDS-PAGE gel and subjected to Western Blotting assay. The collected rabbit serum was used as primary probing antibody. A horse radish peroxidase (HRP)-conjugated goat anti- rabbit was used as secondary antibody. A control was only inoculated maintenance medium.

52K expression in various viruses

Various virus stock include FAdV strain JM1/1, Feline calicivirus, Influenza virus strain PR8, Bornavirus and Pseudorabies virus, were mixed with sample buffer and boiled at 98°C for 5 min. The sample were loaded onto a 10% SDS-PAGE gel. Subsequently, Western Blotting was performed using anti-52K antibody from rabbit sera as primary antibody incubation. The protocol for detection of expression same as Western Blotting as describe above. The negative control sample was CK cells mixed with maintenance medium and the positive control was CK cells inoculated with FAdV JM1/1.

Results

The 52K C-terminal domain is highly conserved amongst FAdVs

To find out a conserved domain, using GenBank database areas of the FAdVs polyproteins which are conserved across serotypes of the FAdVs are defined. A 684 nucleotides long encoding the late protein 52K of FAdV JM1/1 was compared to the available complete nucleotide sequence of FAdVs in GenBank. The nucleotide homology of 52K in comparison to other serotype showed the range of 70-99% homology within serotype 1-9 as describe previous. Similarly, the amino acid homology showed the range of 66-99% homology. The highest homology was 99% between within serotype 1 strain JM1/1 and Ote which is a member of species A. Otherwise, the homology showed approximately 70% within species B, C, D and E (Table 3). Phylogenetic analysis based on the 52K region, the most closest genetic relationship between FAdV species A strain CELO and JM1/1 was found and the C terminal was found more closer genetic relationship more than N terminal (Fig. 9). Similar result was described using nucleotide and amino acid homology. In addition, by comparing the amino acid sequence of 52K in serotype 1, 4 and 9, it was found a conserved domain especially at C terminal region by Genetyx software (Fig. 10). It was also assumed that 52K is a serotype 1-9 or species A to E specific based on nucleotide and amino acid sequence homology. C terminal region of 52K was also selected to design a primer for DNA amplification and cloning.

Temporal expression pattern of 52K during FAdV infection

Western Blotting analyze the expression time of 52K, the result showed 52K was expressed start from 36 h post inoculation until 48 h in this study (Fig. 11a). Confluent CK cells treated with or without cycloheximide at the time of FAdV inoculation in order to investigate gene and protein expression. By performing RT-PCR, DBP and 52K mRNA expression were detected in untreated CK cells with cycloheximide. DBP was used as an early protein for a control. The intensity of a specific appeared band of DBP was more intense than a band of 52K in lane of cycloheximide treatment. Neither DBP nor 52K expression was detected in mock control and CK cells with no reverse transcriptase enzyme (Fig. 11b). Likewise, by observation of protein expression using Western Blotting technique, DBP and 52K expression were observed in untreated CK cells with cycloheximide, but no expression was observed in mock control and treated CK cells (Fig. 11c).

52K gene cloning and expression

In the PCR evaluation using purified DNA template, positive 52K amplification was observed and the target amplicon size was generated. Its size was 684 base pairs. Amplicons were successfully cloned into pFN2K vector and sequenced. The sequences were analyzed by BLASTN showed an identity with initiator. These plasmids were transformed into BL21 (DE3) competent cell to

express recombinant protein by IPTG induction. Unfortunately, overexpressed protein formed an inclusion body as shown in SDS-PAGE with CBB staining and Western Blotting (Fig. 12a). The *E. coli* cells pellet still has aggregated protein. Consequently, the protein from SDS-PAGE gel was washed with PBS, roughly minced, homogenized and also recheck a correction protein before injection to the laboratory animal. The expression result showed bands appeared around 50 kDa by SDS-PAGE and CBB staining (Fig. 12b). It seems that fusion proteins of 52K (26.5 kDa) and GST (24.2 kDa) are normally expressed.

Immunization induce a 52K specific antigene

A rabbit was immunized with purified GST-52K fusion protein to obtain antibodies against 52K. Western blotting was performed using rabbit serum in order to ascertain whether the antibody against 52K was present in the obtained immune serum. Anti-GST tagged antibody were used as the primary antibody in antibody incubation step. The expected molecular weight from the 52K amino acid sequence is 26.5 kDa combined with a molecular weight of GST is 24.2 kDa. Western Blotting analyze showed a specific band around 50 kDa was observed in serum from the first and the second bleeding, at 3 and 6 week post immunization respectively, but no specific and nonspecific bands were observed in serum from pre-immunization bleeding (Fig. 13). Since the expected molecular weight is 50.7 kDa, the band found at 50 kDa on membrane is 52K of FAdV. It was found that

subcutaneous immunization with purified 52K induced specific serum antibody that could detect FAdV infection by Western Blotting.

Cross reactivity to other serotypes

To clarify cross reaction to other FAdV serotypes using anti-52K antibody. These antibodies were used as a primary probe antibody recognized the 52K protein in Western Blotting and IFA assay to display their immunological cross reactions with other FAdV serotypes i.e. serotype 1 to 8. Interestingly, the 52K antibody of serotype 1 could be specifically recognized by all using FAdV serotypes in this study including homologous and heterologous serotypes, although a band specific for uninfected cell lysate used as negative control was not detected. Moreover, the appeared band had a particular pattern within FAdV species (Fig. 14). To determine their immunological cross reaction by IFA, CK cells were prepared and inoculated with FAdV serotype 1-8, the 52K antibody was used as primary probe antibody and then incubated with FITC-conjugated secondary antibody and nuclear counterstaining by DAPI. The result showed positive signal in the cytoplasm of CK cells but no fluorescent was detected in uninfected cells (Fig. 15). The results suggested that 52K antibody had a cross reactivity within all FAdV serotypes (serotype 1-8).

52K homolog expression in various viruses

By performing Western Blotting, to detect expression of 52K in various viruses, the expression was only detected in FAdV strain JM1/1 seeding stock. Otherwise, the remains were not expressed a 52K. It was presumed that 52K could be found especially in FAdV (Fig. 16).

Discussion

In this study, 52K, a non-structural Fowl aviadenovirus protein, was shown to be a potential diagnostic tool for FAdV diagnosis. However, the disease diagnosis using 52K have not been studied well. FAdV can be detected by virus isolation combined with virus neutralization though they are laborious and take a certain amount of time. Consequently, other option is needed, Western Blotting and Immunofluorescence assay using anti-52K antibody is the alternative method of choice.

Cycloheximide is a protein synthesis inhibitor by interfering with the translocation step in protein synthesis, thus blocking translational elongation. Cycloheximide is widely used in in vitro studies. Following cycloheximide treatment, the mRNA and protein of 52K cannot be detected by RT-PCR and Western Blotting, respectively. These results suggested that 52K encodes one of a late gene in FAdV. Similar findings were reported as L1 late region expressed protein 52K are involved in capsid assembly and/or genome packaging (Ahi and Mittal 2016). Both steps occur at the last step of viral life cycle. HAdV-C5 mutants unable to express 52/55K or possess a two amino acid substitution (EL334-335GP) fail to package DNA (Gustin and Imperiable 1998) so that it confirmed an important role in the incorporation of the viral DNA into empty capsid. Furthermore, the 52K was expressed from 24 h post inoculation with FAdV JM1/1. It was suggested that

52K is a late expression and might be one of late gene of FAdV that expressed early and has a long time expression, it might be useful in earlier FAdV diagnosis and sensitivity will be increased.

The detection of GST tagged protein having approximate size of 50.7 kDa, during SDS-PAGE following CBB staining and Western Blotting analysis verified that the L1 52K gene was successfully expressed. Although, the prokaryotic expression of recombinant 52K protein was observed both in supernatant and pellet fractions of *E. coli* strain BL21. Overexpressed protein has been occurred and it was difficult to purify by GST tag affinity chromatography column. The protein was subjected to SDS-PAGE to an alternative purification method. The expected band size position on gel was cut, wash with PBS, homogenized and confirmed the protein purification by SDS-PAGE concurrent with CBB staining prior to use in immunization. The result of this purification showed a correct protein size of 52K and it was suggested that an alternative purification method could be used. An immunized rabbit sera showed it contained a specific antibody against 52K that could detect FAdV infection by Western Blotting. The result indicates that 52K is a strongly immunogenic and can stimulate immune response. Certainly, some non-structural proteins have been reported, recombinant DBP and 100K act as immunogenic as they can stimulate laboratory animal antibody titer (Guo et al. 2013, Shah et al. 2016).

Diagnosis of FAdV infection is currently based on direct examination on EM, histopathological examination, virus isolation, antigen and antibody detection, and nucleic acid technology for diagnosis (Hess 2000). The major structural protein for example hexon and fiber are a candidate for examination of infection and are often used for serum antibody diagnosis. Nevertheless, the other protein especially non-structural proteins have been studied. Some have been used in immunodiagnosis for virus infection. For examples, the influenza virus NS1protein combined with NP and M sandwich ELISA could improve sensitivity of the test (Jian-umpunkul et al. 2012). Dengue virus NS1 protein has been used for early diagnosis and the sensitivity of the NS1 IgM ELISA was higher than RT-PCR (Gowri Sankar et al. 2012). In the anti-HCV IgG detection, the NS4 was used, which could identified anti-HCV IgG in approximately 80% of patients with post-transfusion hepatitis (Barrera et al. 1991). Similarly, in this study, immunological cross reactions between anti-52K antibodies and FAdVs serotypes 1-8 were observed. It indicates that recombinant 52K protein antibody can detect FAdVs infection including homologous and heterologous serotypes. Moreover, 52K gene has been used in the development of Real-time PCR assay for universal detection and quantitation of all five species of FAdV (Gunes et al. 2012, Gunes et al. 2013). In contrast, previous study of recombinant non-structural DBP on FAdVs detection, revealed that it act as serotype specific by the result of cross reactions were observed only within serotype

1. Therefore, this anti-52K antibody has a property which could play a role in an alternative method for FAdVs diagnosis in different serotypes for screening procedure.

In conclusion, this study presents the techniques for detection of FAdVs based on Western Blotting and Immunofluorescence assay by using recombinant 52K antibody. All of FAdV serotypes can be detected, it was shown that this is an applicable and nonstructural protein 52K could be served as an alternative clinical diagnostic tool for FAdV infection. These findings also provide a better understanding of the non-structural protein of Fowl aviadenoviruses.

Table 3. The 52K was a conserved region between serotypes. Homology analysis of 52K region between FAdV serotype 1 strain JM1/1 used in this study and reference complete genome sequence of FAdVs in GenBank database.

a
Nucleotide homology (%)

(%)	JM1/1	CELO	SR48	SR49	KR5	340	CR119	YR36	TR59	A-2A
JM1/1[a]	*									
CELO[b]	99	*								
SR48[c]	70	70	*							
SR49[d]	70	70	99	*						
KR5[e]	71	71	70	71	*					
340[f]	71	71	75	75	71	*				
CR119[g]	72	72	81	81	72	76	*			
YR36[h]	72	72	81	81	72	76	98	*		
TR59[i]	72	72	81	81	72	76	98	98	*	
A-2A[j]	70	70	99	98	71	75	81	81	81	*

b
Amino acid homology (%)

(%)	JM1/1	CELO	SR48	SR49	KR5	340	CR119	YR36	TR59	A-2A
JM1/1[a]	*									
CELO[b]	99	*								
SR48[c]	69	69	*							
SR49[d]	69	69	99	*						
KR5[e]	70	70	68	68	*					
340[f]	71	71	77	77	66	*				
CR119[g]	71	71	82	82	68	77	*			
YR36[h]	72	67	82	82	68	77	98	*		
TR59[i]	71	71	82	82	68	76	98	98	*	
A-2A[j]	67	67	96	97	66	75	80	80	80	*

[a]FAdV A serotype 1 strain JM1/1: GenBank accession number MF168407.
[b]FAdV A serotype 1 strain CELO: NC001720.
[c]FAdV D serotype 2 strain SR48: KT862806.
[d]FAdV D serotype 3 strain SR49: KT862807.
[e]FAdV C serotype 4 strain KR5: HE608152.
[f]FAdV B serotype 5 strain 340: NC021221.
[g]FAdV E serotype 6 strain CR119: KT862808.
[h]FAdV E serotype 7 strain YR36: KT862809.
[i]FAdV E serotype 8 strain TR59: KT862810.
[j]FAdV D serotype 9 strain A-2A: AF083975.

Nucleotide sequence homology (a) and Amino acid sequence homology (b) were performed using Genetyx and represented as percentage of homology.

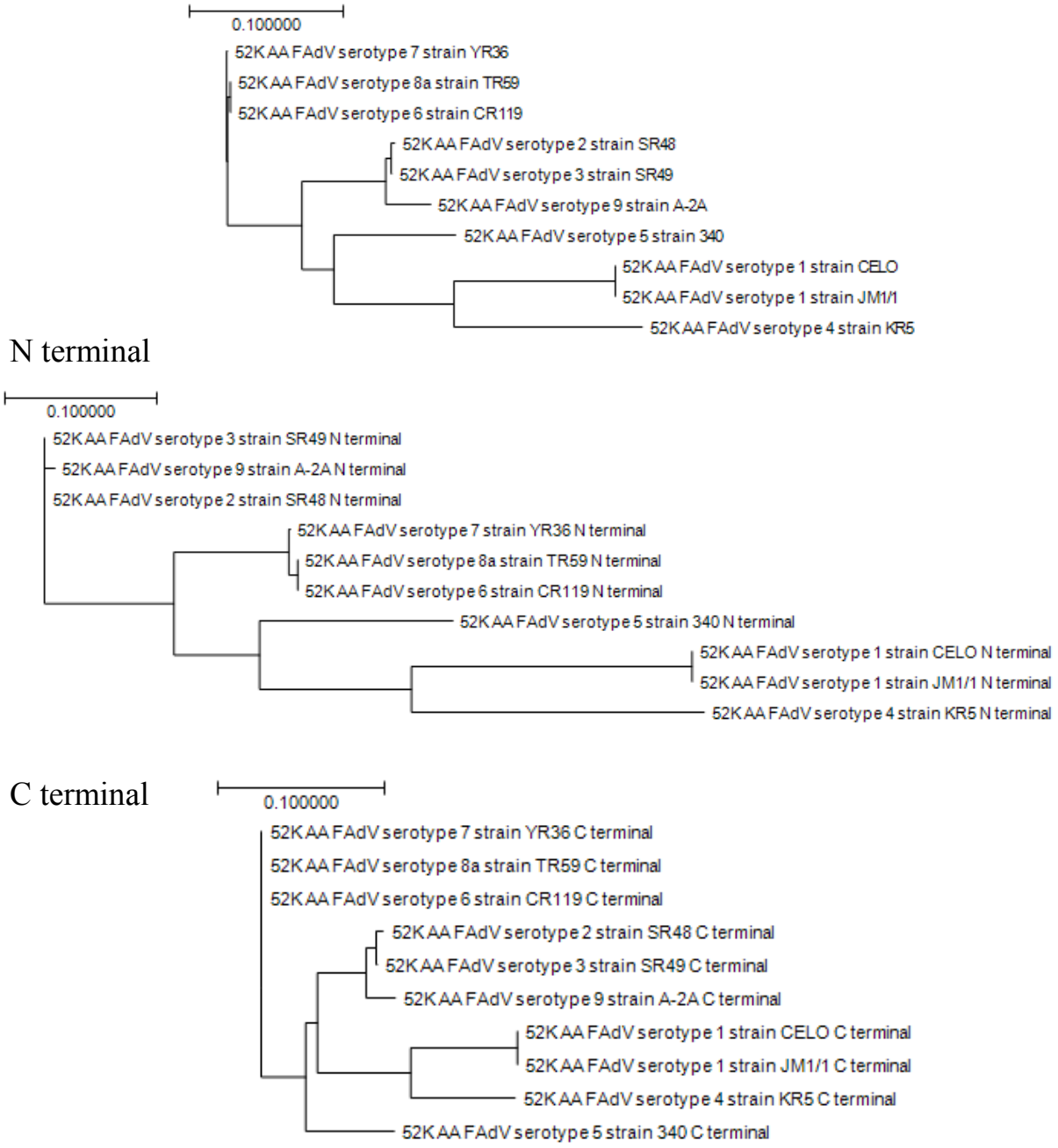


Fig. 9. Phylogenetic tree based on the 52K region of L1 gene of Fowl Aviadenovirus (FAdV). The reference nucleotides and amino acid sequences representing serotypes 1 to 9 same as previous homology comparison, including FAdV-1 JM1/1 were analyzed. The phylogenetic tree were generated by the NJ method.

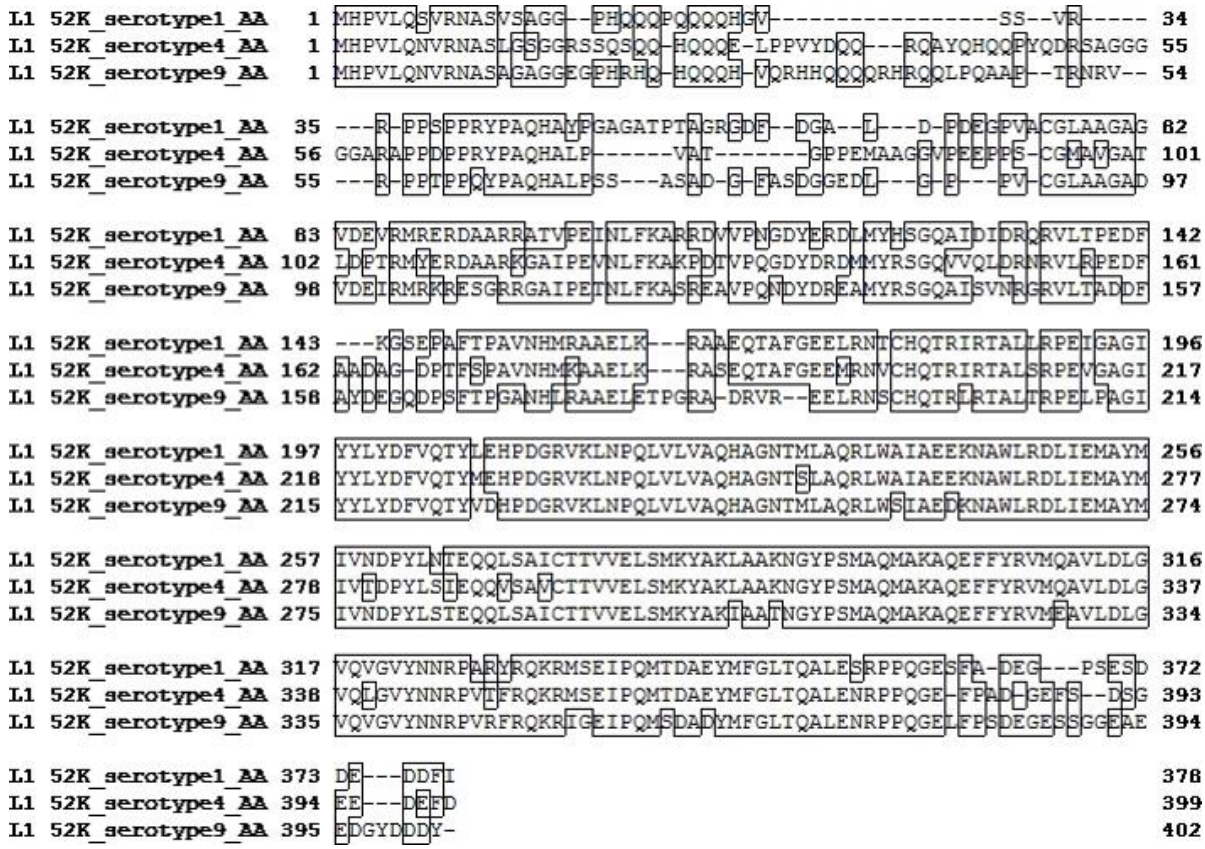


Fig. 10. C-terminal of 52K was more conserved in FAdVs. Amino acid sequence of 52K of FAdV serotype 1, 4, and 9 were compared by Genetyx. The more conserved was showed in square.

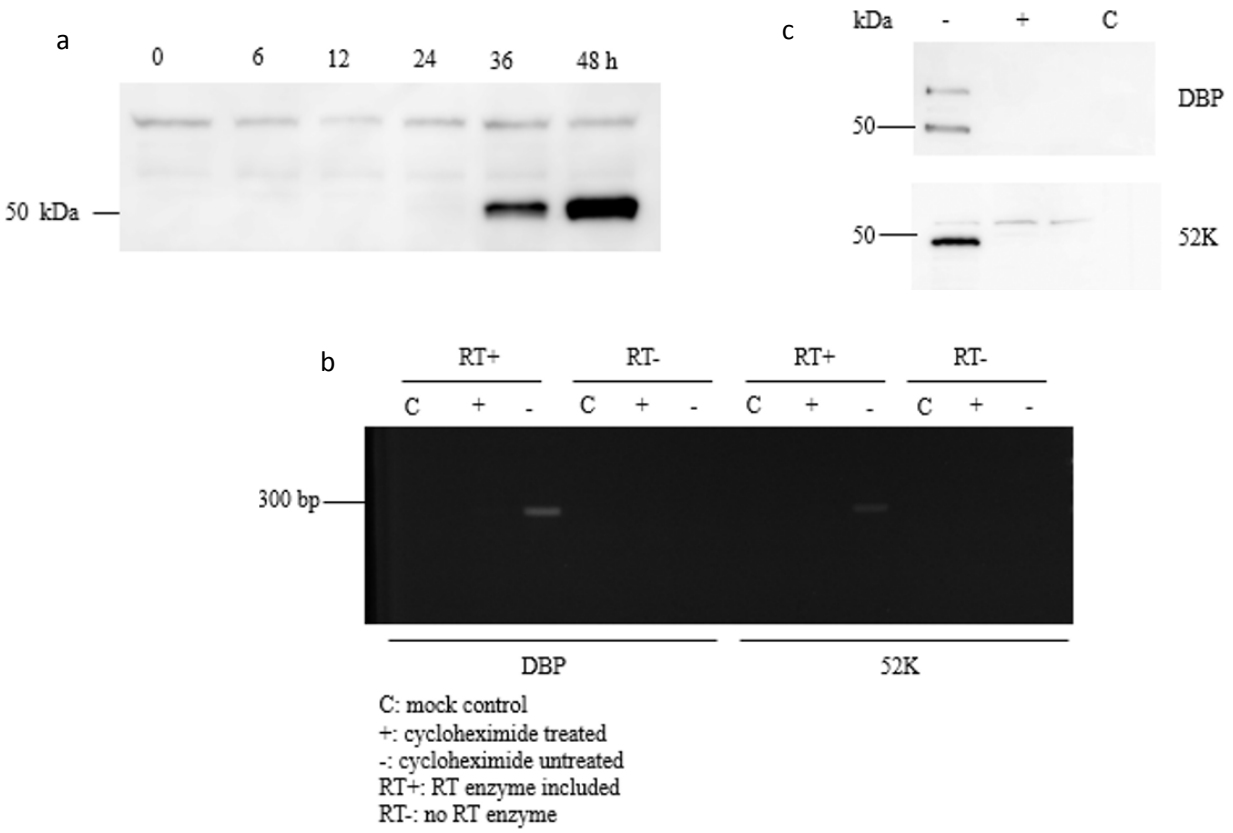


Fig. 11. Late gene and protein expression of 52K. Western Blotting was performed to detect 52K expression, CK cells were infected with FAdV and were collected at 0, 6, 12,24,36, 48 hours post-inoculation (a). By performing 20 cycles of RT-PCR, DBP and 52K mRNA expression with and without cycloheximide treatment (b). Protein expression of DBP and 52K was also performed by Western Blotting (c).

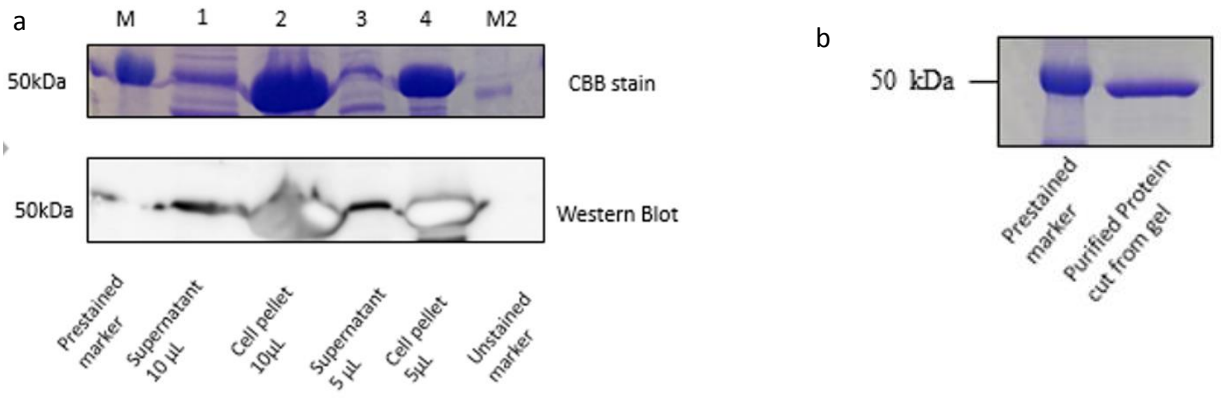


Fig. 12. The recombinant 52K expression. Overexpressed protein was occurred after IPTG induction, performed by SDS-PAGE with CBB staining and Western Blotting (a). The protein was purified by alternative method and confirmed the expression by SDS-PAGE with CBB staining (b).

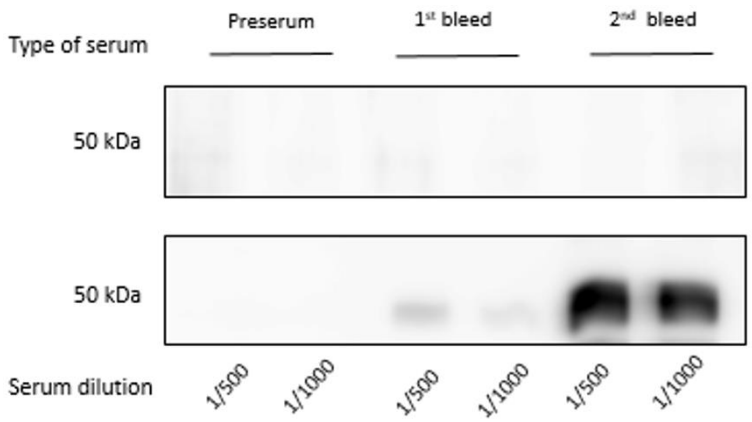


Fig.13. Expression of 52K in the rabbit serum by Western Blotting. Immunized rabbit sera were collected at 0, 3, and 6 week post-immunization and subjected to confirm the antibody against 52K. Anti-GST tagged was used as primary probing. The upper panel is uninfected CK cells lysate.

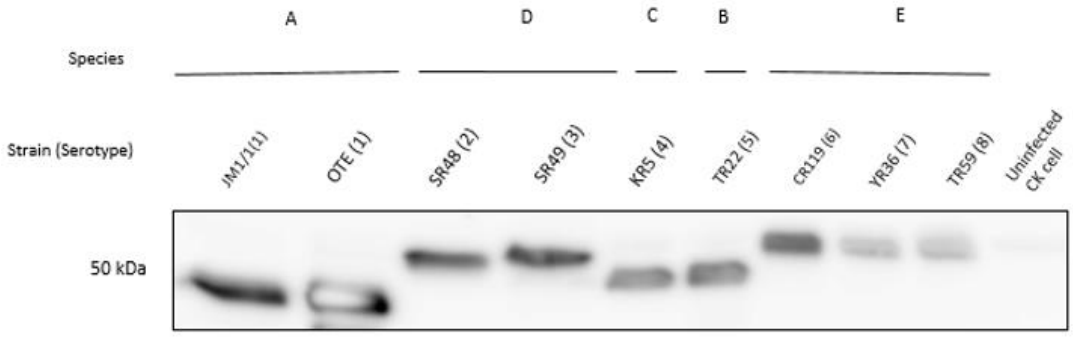


Fig. 14. Cross reaction of anti-52K antibody to various FAdV serotypes was performed by Western Blotting. The FAdVs use in this experiment were strain JM1/1(serotype 1); Ote(serotype 1); SR48 (serotype 2); SR49 (serotype 3); KR5 (serotype 4); TR22 (serotype 5); CR119 (serotype 6); YR36 (serotype 7); TR59 (serotype 8a), and uninfected CK cells lysate as negative control.

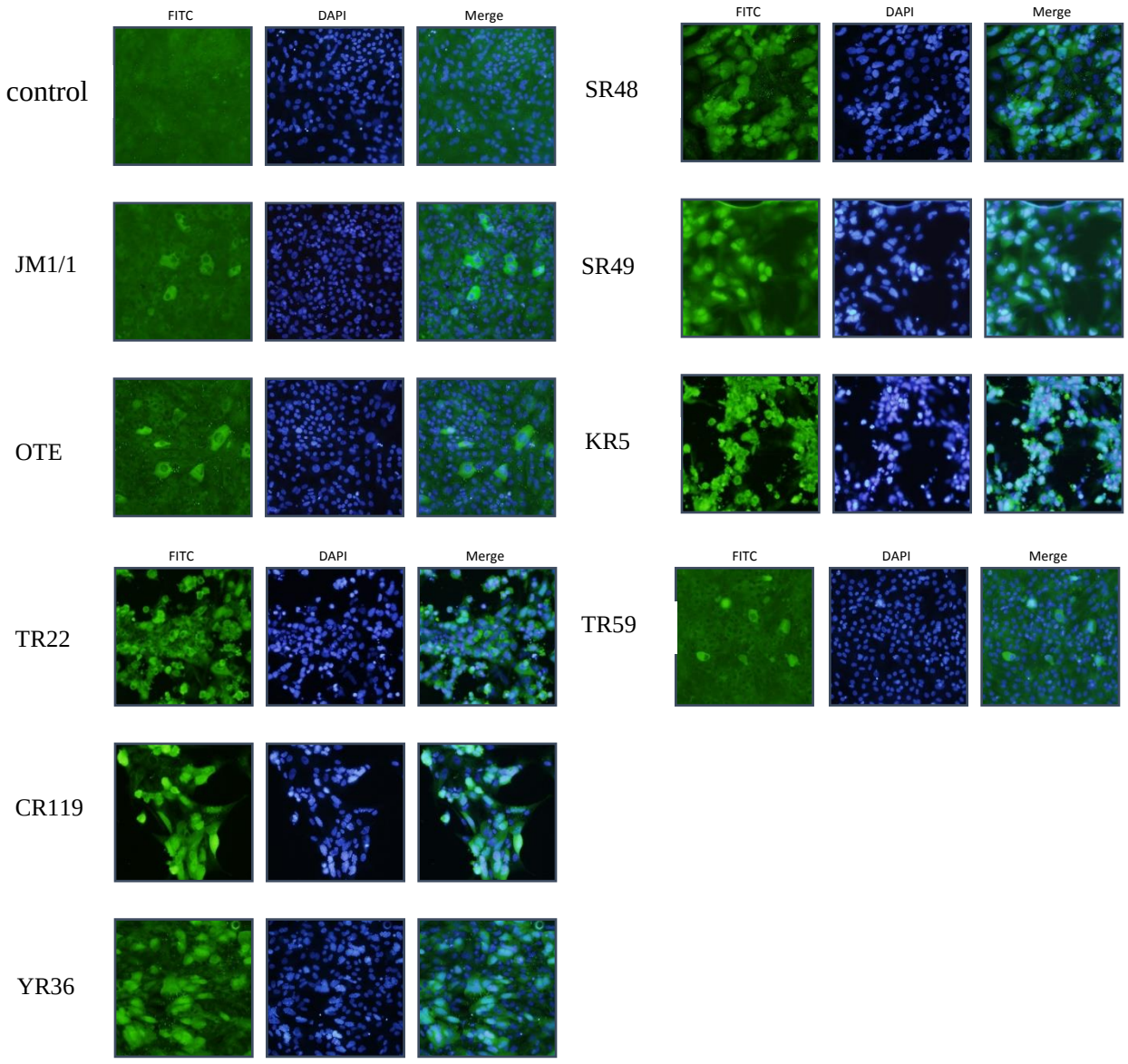


Fig. 15. Cross reaction of anti-52K antibody was performed by Immunofluorescence assay. CK cells were prepared and inoculated with FAdV serotype 1-8. The rabbit serum was used as primary probe antibody and then incubated with FITC-conjugated secondary antibody and nuclear counterstaining by DAPI.

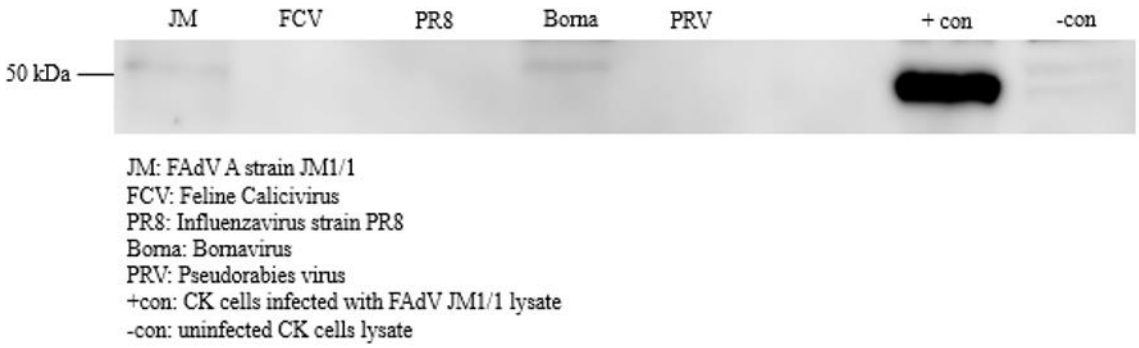


Fig. 16. 52K was only expressed in FAdV strain JM1/1. Western Blotting was performed using various virus seeding stocks.

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