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RESEARCH ARTICLE

Whole Yeast Expressing Recombinant Fiber 2 Protein Vaccine Candidate Protects Chicken against Fowl Adenovirus Serotype 4 Infection

Van Dam Lai[1], Joo-Sung Son[1], Tea-Sik Kim[1], Kang-San Kim[1], Eui-Sung Choi[2] and In-Pil Mo[1*]

[1]Avian Disease Laboratory, College of Veterinary Medicine, Chungbuk National University, Cheongju, 361-763, South Korea; [2]Biotechnology Process Engineering Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Cheongju, South Korea
*Corresponding author: moip@cbu.ac.kr

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A B S T R A C T

Fowl adenoviruses (FAdVs) are prevalent infectious agents in a variety of avian species including chickens worldwide. Among several serotypes, fowl adenovirus serotype 4 (FAdV-4) is known to cause mass economic damages to chicken farms in Korea as a pathogen for the hepatitis-hydropericardium syndrome (HHS). The encoding portions of hexon and fiber genes of FAdV-4 field strain ADL15 0401 were fully sequenced and three constructs, Hexon-L1-His and two constructs of fiber genes Fiber-1-His and Fiber-2-His were ligated with the pYEGa-HIR525α which was optimized for producing complex proteins in *Saccharomyces cerevisiae* yeast systems. The yeast cell containing these recombinant proteins were inoculated in specific pathogen-free (SPF) chickens. Antibodies against FAdVs were identified from all immunized chicken groups by commercial ELISA as early as 1 week post inoculation and better virus neutralization in Fiber-2 group with 2 logs neutralizing ability. Finally, the protective efficacy result showed the full protection after challenge with virulent FadV-4 virus in the SPF chicken from Fiber-1 and Fiber-2 groups. These results suggest that the whole yeast expressing recombinant fiber-2 protein can be a candidate for vaccines against the FAdV-4 infection.

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INTRODUCTION

Fowl adenoviruses are prevalent viral agents in a wide range of avian species including poultry. These viruses were classified into five distinct species named A to E and subdivided into 12 different serotypes (FAdV-1 to 8a and 8b to 11) by cross-neutralization tests (Hess, 2000). Most 12 serotypes of FAdVs were related to being inclusion body hepatitis, whereas only serotype 4 has a role for inducing HHS which was characterized by high mortalities with hydropericardium and hepatic necrosis and hemorrhage, predominantly in broiler chickens, at 3 to 5 weeks of age. In Korea, FAdV-4 has become an important infectious agent since the first report of the outbreak from 2007 by Kim *et al.* (2008).

FAdVs are non-enveloped DNA viruses with 70 to 90 nm in diameter sizes and belong to the genus *Aviadenovirus* under family *Adenoviridae.* The capsid of the virus consists of 240 hexons and 12 penton fibers projected from each penton base. The hexon proteins have responsibilities to elicit the humoral responses in virus neutralizing and serotyping. The fiber proteins have knob head domains containing the receptor-binding sites which help the virus attach to the host cell membranes (Henry *et al*., 1994; Louis *et al*., 1994). Initially, liver homogenates collected from broiler chickens infected with FAdV-4 were used widely in some countries as inactivated vaccine materials for prevention of HHS (Admad *et al*., 1990). After that, the cell-culture derived inactivated vaccines provided quite effective protection against the virulent FAdV-4 isolates with an appropriate quantity of virus and suitable adjuvant (Kim *et al.,* 2014; Du *et al.,* 2017). However, inactivated FAdV-4 vaccines provided short term of immunity with high costs in manufacture and often fail to provide the high protection efficacy in commercial chicken farms in conditions such as infectious immunosuppressive disease infections such as Infectious Bursal Disease (IBD) and Chicken Infectious Anemia (CIA) outbreaks or noninfectious factors such as environmental situation inducing much stress and toxins

(Shivachandra *et al*., 2003; Balamurugan and Kataria, 2006). Recently, the subunit vaccines based on capsid proteins of the viruses have been developed for control of this disease including structural proteins such as hexon, fiber and penton proteins as well as non-structural proteins such as 100k protein (Schachner *et al*., 2014; Shah *et al*., 2016; Wang *et al*., 2018).

S. cerevisiae is known as the first eukaryote whose genome was fully sequenced (Goffeau *et al*., 1996). And it is the most common, low cost eukaryotic microbial expression systems used to produce dozens of pharmaceutical products such as vaccine subunit proteins and blood factors including human insulins (Roohvand *et al*., 2017). In this study, we construct new proteins of hexon loop1 region, fiber-1 and fiber-2 genes in *S. cerevisiae* vector in the hope of producing immunogenicity and neutralizing efficacy of these proteins against FAdV-4.

MATERIALS AND METHODS

Viral DNA extraction: Viral DNA of the FAdV-4 strain ADL15 0401 was extracted by using Viral Gene-spin (iNtRON Biotechnology Inc., Seongnam, Korea).

Recombinant plasmid construction: According to the nucleotide sequences of interesting genes, a total of 3 constructs were designed and amplified by PCR of the hexon loop1 region and entire open reading frame of the fiber-1 and fiber-2 gene with specific primers containing *SalI* and *EcoRI* restriction sites extensions homologous to the yeast vector ends and the 6 His-tag sequences for further western blotting (Table 1). The gene constructs were cloned into the linear pYEGa-HIR25a plasmid by the In-Fusion® HD kit (Clontech, CA, USA).

Protein expression: Transformation of the extracted plasmid from the competent *E. coli* DH5α into *S. cerevisiae* with Dimethyl Sulfoxide was conducted by previously described methods (Hill *et al.*, 1991). The selected colonies were pre-cultured on 1ml of Uracil Deficient liquid media before culture on yeast extract peptone dextrose medium in a shaking incubator at 30°C, 200rpm for 48 hours.

Western blotting: The homogenized yeast cells were analyzed by 10% Polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Bio-Rad, CA, USA). After blocking with 5% skim milk, membranes were processed with primary antibody including chicken serum anti-Fadv-4 and the 6xHis-tag monoclonal antibody (Thermofisher Scientific, MA, USA) as a control.

Animal experiment: The 10 weeks-old SPF chickens (ChoongAng Vaccine Laboratories Co. Ltd, Daejeon, Korea) were divided into 5 groups of 5 birds each including three vaccine candidates, IBH-Fiber1-His (Fiber-1), IBH-Fiber2-His (Fiber-2), IBH-hexonL1-His (Hexon-L1); negative control and positive control group. The whole *S. cerevisiae* cells containing their respective recombinant proteins were inactivated at 60°C for 1 hour. Then 0.5ml containing 10^6 cells were mixed with incomplete Freund's adjuvant (Sigma-Aldrich, MO, USA) at the ratio of 1:1 and inoculated in the thigh muscle. Blood was collected from chickens of each group weekly. Birds were challenged at 4wpi (week post inoculation) with $10^{3.5}$ EID_{50} doses and under monitoring for 2 weeks. All animal experimental processes were reviewed and approved by Chungbuk National University Institutional Animal Care and Use Committee with project code CBNUR-1099-17.

Agar gel precipitation test (AGPT): 25µl of yeast cell lysates and two-fold serial dilution of serum samples were added to the center and outer wells in agar gel plates, respectively. The plates were placed in the moisture chamber and then incubated at 37°C for 48 hours for precipitation.

ELISA: The commercial Fowl Adenovirus Group 1 Antibody test kit (BioCheck, Kings Ride Ascot, United Kingdom) was used to determine antibody level in collected sera following the manufacturer manual.

Serum neutralization test on SPF chicken embryo: The chicken serum was heated at 56℃, 45 minutes for inactivating nonspecific inhibitors and $10^{5.5}EID_{50}$/ml Fadv-4 indicator virus was diluted from 10^{-1} to 10^{-4}. The sera were mixed diluted viruses at ratio 1:1 and incubated for 1 hour at room temperature. After incubation, the mixtures were inoculated into five 10 days old SPF chicken with 0.2 ml/bird by chorioallantoic membrane (CAM) route. Five embryos were inoculated with PBS for negative controls and five embryos were inoculated with 0.1ml diluted viruses for positive controls. All SPF eggs were checked twice daily for viability and chilled at 4℃ overnight after 7 days for evaluation of FAdV-4 pathognomic lesions on CAM and liver.

Statistical analysis: All data were analyzed in Excel and SPSS Subscription software (IBM SPSS Statistics, IBM Corporation, NY, USA). The data of serum neutralizing were performed by o4ne-way ANOVA and statistical significances were considered at p-value <0.05.

Table 1: Primers used for amplification of specific cloning genes

Primer	Sequence	Purpose	References
HxAinf	5' – *GAAAATTCAAGAATTC*ATGAACAAGTTCA GACAGACGGT - 3'	Hexon loop1	Modified after Meulemans *et al.,*
HxBinf	5'- *TTCAAGTCGACTTAGTGATGGTGATGGTGATG* GCGGGACATCATGTC - 3'	Hexon loop1	Modified after Meulemans *et al.,*
Fiber1inf F	5' – *GAAAATTCAAGAATTC*GAACCGTAACATAGC CTG - 3'	Full-length fiber 1 gene	This study
Fiber1inf R	5' – *TTCAAGTCGACTTAGTGATGGTGATGGTGATG* GGGGCC CGGAGCATTGTT - 3'	Full-length fiber 1 gene	This study
Fiber2inf F	5' -*GAAAATTCAAGAATTC*ACGACCGGTCCTATCC CTTTT- 3'	Full-length fiber 2 gene	This study
Fiber2inf R	5' – *TTCAAGTCGACTTAGTGATGGTGATGGTGATG* CGGGACGGAGGCCGCTGG - 3'	Full-length fiber 2 gene	This study

Underline nucleotides: restriction enzymes recognizing nucleotide sequences Bold italic nucleotides: 6x His tag and stop codon.

RESULTS

Expression of recombinant protein: All three recombinant hexon-L1, fiber-1, fiber-2 proteins were successfully expressed and identified using the anti-Fadv-4 chicken serum and 6x His-tag monoclonal antibodies in SDS-PAGE and western blotting procedures. The constructs loaded on lane 1, lane 2 and land 3 showed adequate levels of expressions regarding the band density of 33kDa (Hexon-L1), 46kDa (Fiber-1), and 52kDa (Fiber-2) (Fig. 1). There were no non-specific reactions indicating successful expression of the target proteins. The negative control of the yeast lysates without vector transformation showed no reaction.

Antibody against yeast detection: AGPT titers of Hexon-L1, Fiber-1 and Fiber-2 groups determined the recombinant yeast stimulated immunity in inoculated chicken (Fig. 2). There were no antibody titers detected from all the groups at 0 and 1wpi. At 2wpi, the antibodies were identified in all 3 groups at first, showing Fiber-1 group represented the highest AGPT titers with $2.4\log_2$ and the follows were Fiber-2 group and Hexon-L1 group (at $1.8\log_2$). There was also no difference between Fiber-1 group and Fiber-2 group at 3wpi with $4\log_2$ and $3.4\log_2$ mean value ($P>0.05$) but higher than the titer of Hexon-L1 group at $3\log_2$ ($P<0.05$). At 4wpi, the overall titers of vaccinated groups were maintained in comparison with the titers at 3wpi and no significant difference between three inoculated groups ($P>0.05$).

ELISA test: The commercial Fowl Adenovirus Group 1 Antibody ELISA test kit was used to detect the specific antibody against FAdVs. The positive antibodies in the serum of the vaccinated chickens were confirmed as early as 1wpi with quite low at mean titer over 1200 (Fig. 3). Then the antibody levels in immunized chickens with Fiber-1, Fiber-2 and Hexon-L1 group were significantly increased until 3wpi at mean value 2417, 2015 and 1859, respectively with no significant difference ($P>0.05$). The anti FadVs antibody titer had slightly changed at 4wpi. The lower titer than the cut off level range from negative control group considered no antibody against FadVs (data not shown).

Challenge protection: After 14dpc (day post challenge), the result showed complete protection against Fadv4 virus challenge without any gross and histopathological lesions in the chicken group vaccinated with whole yeast cell containing fiber-1 and fiber-2 recombinant protein. Two of five and three of five dead chickens associated with hemorrhage liver and intranuclear inclusion body in group Hexon-L1 group and positive control group from 4 to 6dpc (Fig. 4). This indicated hexon loop1 recombinant protein did not protect the birds from the virulent challenge. No mortality and lesion were observed from the negative control group.

Serum neutralizing capacity of antibodies in SPF chicken embryos: The neutralization capacity of antibodies collected at 3wpi of all groups were evaluated based on the severity of the pathognomic gross lesions such as increased opacity and thickening on the CAM of

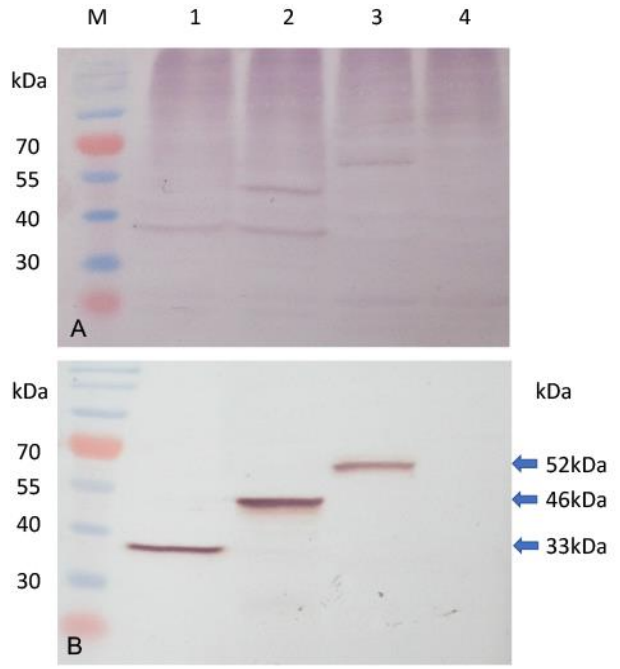

Fig. 1: Western Blotting results of capsid constructs of Fowl adenovirus serotype 4 used in this study. A) Western blot incubated with chicken serum anti-Fadv-4 as the primary antibody. B) Western blot incubated with the commercial 6x His-tag monoclonal primary antibody. Lane M: Protein ladder; Lane 1: IBH-HexonL1-His (33kDA); Lane 2: IBH-Fiber1-His (46kDa); Lane 3: IBH-Fiber2-His (52kDa); Lane 4: *S. cerevisiae* Y2805.

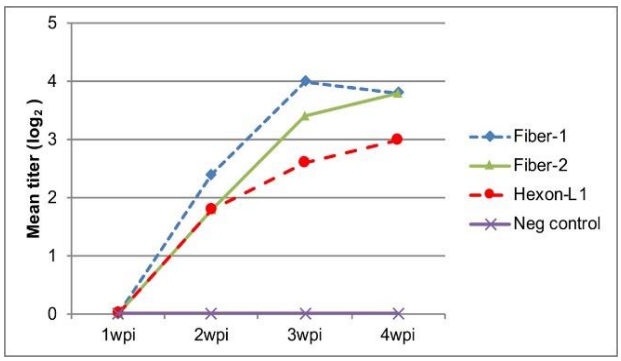

Fig 2: The mean titers of antibodies against yeast cell lysate on an agar gel precipitation test. The abbreviations used in this figure are as follows. Fiber-1: IBH-fiber1-His group; Fiber-2: IBH-fiber2-His group; Hexon-L1: IBH-hexonL1-His group. n=5 chicken serums per group. The significant difference was observed between group Fiber-1 and Hexon-L1 at 3wpi ($P<0.05$). However, the titers of all groups showed a slight variance at 4wpi.

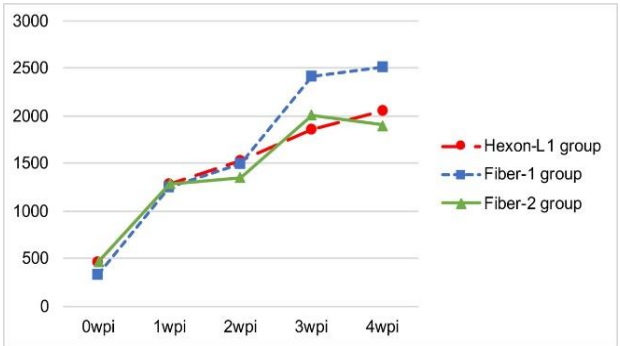

Fig. 3: Results of antibody titer by commercial FAdV Group 1 ELISA. The titer range greater than 1071 was considered positive. The specific antibody were determined in all Hexon-L1, Fiber-1 and Fiber-2 groups with no significant statistical difference ($P>0.05$).

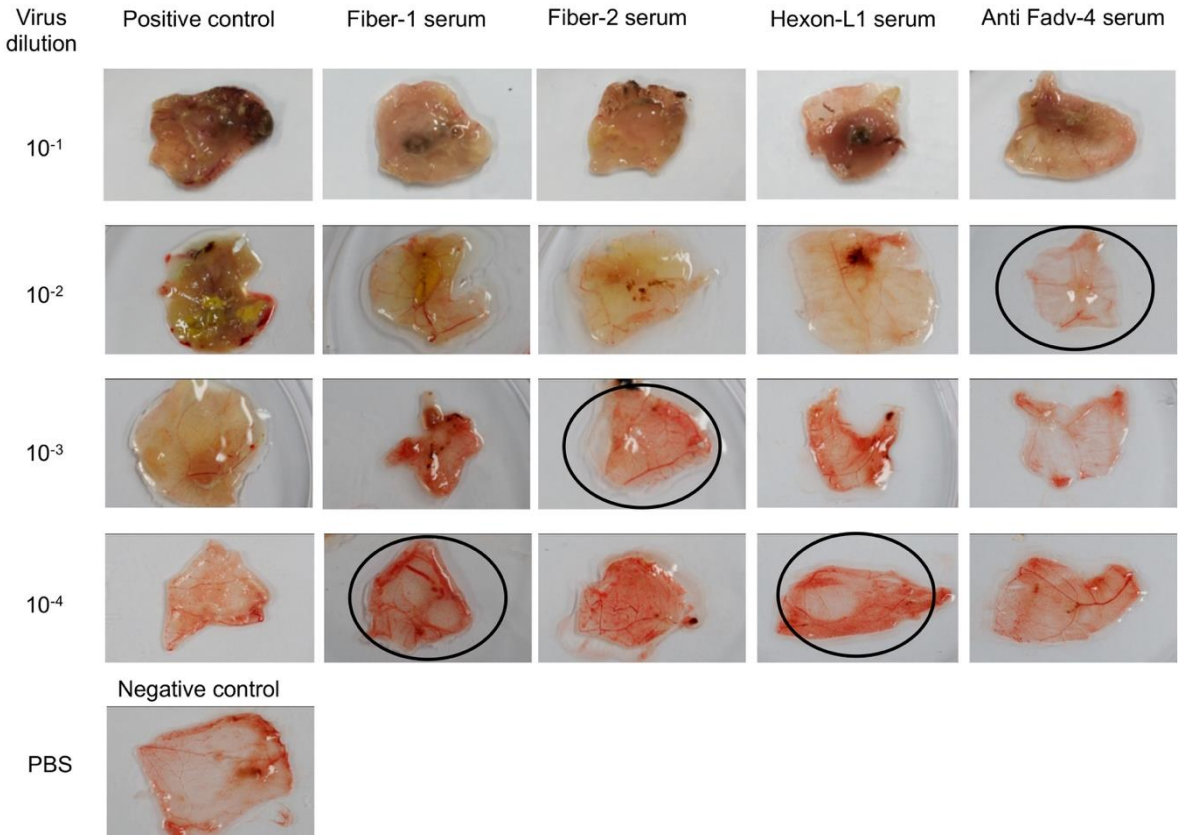

Fig. 5: The gross lesions of chorioallantoic membrane of SPF eggs in serum neutralization test. The abbreviations used in this figure are as follows. Fiber-1: IBH-fiber1-His group; Fiber-2: IBH-fiber2-His group; Hexon-L1: IBH-hexonL1-His group. The circles marked the maximum dilution rates of neutralization based on gross lesions on CAM.

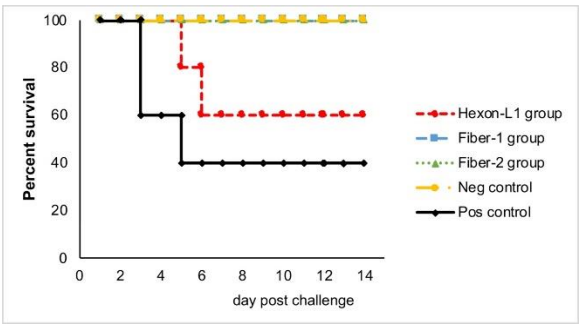

Fig. 4: The survival of chickens immunized with different vaccine products in the challenge experiment. n=5 SPF chickens per group.

SPF chicken embryo eggs (Fig. 5) and necrosis lesion on embryo liver. At the 10^{-2}, antibodies from chickens of all groups reduced the severity of gross lesions on CAM of embryos in comparison with positive control. There are no lesions on CAM and liver of embryos inoculated with mixtures of the serum of group Fiber-2 and 10^{-3} dilution of $10^{5.5}$ indicator virus. In group Fiber-1 and Hexon-L1 serum neutralized lower virus concentration at 10^{-4} dilution of indicator virus. These no gross lesion liver showed the negative PCR result with fowl adenovirus group 1. The EID_{50} titer calculation of virus after neutralization with Fiber-2, Fiber-1 and Hexon loop 1 group are $10^{3.5}EID_{50}$, $10^{4.5}EID_{50}$ and $10^{4.5}EID_{50}$, respectively. The neutralizing ability of each group determined by subtracting the log of the test serum titer from the indicator virus titer is 2logs (Fiber-2) and 1logs (Fiber-1 and Hexon loop 1). Overall, Fiber-2 showed higher abilities of serum neutralization than Fiber-1 and Hexon-L1 group.

DISCUSSION

Since HHS has recently been increasing the serious damage in several countries, the vaccine is the effective method to control HHS including inactivated vaccine prepared from liver homogenates of infected chickens (Ahmad *et al.*, 1990) and derived cell-culture (Kim *et al.*, 2014; Du *et al.*, 2017). The live vaccine against HHS was developed by adapting the virulent FAdV-4 strain to the cell lines (Schonewille *et al.*, 2010) and chicken embryos (Mansoor *et al.*, 2011). Even proving immunogenic for the control of HHS, the doubt is risks of spread of HHS for inactivated vaccine or the possibility of virus vaccine strains converting back to virulent forms for live vaccines. In addition, the decreased protection efficacy of these vaccines was reported in poor hygienic conditions (Khan *et al.*, 2005). More recently, the development of safety subunit vaccines based on virus proteins sequences utilizing *E. Coli* and baculovirus has been found effective (Shah *et al.*, 2012; Schachner *et al.*, 2014; Shah *et al.*, 2016). Moreover, the fiber 2 subunit vaccine showed the advantage in more rapid and stronger than inactivated vaccine and provide 100% protection against FadV-4 challenge with the lowest dose as 2.5 µg/bird (Ruan *et al.*, 2018). However, these subunit vaccine utilizing *E. coli* and Baculovirus system are in the order to process the purification with the effective adjuvant as well as multiple immunizations which increase the vaccine price while the

young broiler is a typical association. Therefore, the cost-effective vaccine against FAdV-4 with the desired effect are urgently required.

To resolve this issue, we used the *S. cerevisiae* yeast expression system, known to process the complex proteins due to their post-translation modification ability like other eukaryotic systems (Roohvand *et al.*, 2017). In the Western blot result, the expected size product of hexon loop-1 (33 kDa), fiber-1 (46 kDa) and fiber-2 (52 kDa) were determined by His-tag antibody and chicken serum anti-Fadv-4. AGP test results showed that humoral immunities were induced in chickens by recombinant yeast cell lysates. According to previous literature, beta-glucans, a heterogeneous group of natural polysaccharides anchoring to the surface membranes of yeast species including the *S. cerevisiae,* are known for their immunological effects (Stier *et al.*, 2014). Their structures were discovered to enhance the host innate immunity (Abbott *et al.*, 2015). *S. cerevisiae* and other yeast species initiate immune responses by inducing maturation of dendritic cells and expected the presentation of yeast-expressed antigen via MHC (Major histocompatibility complex) class II and to MHC class I proteins (Stubbs *et al*., 2001) so *S. cerevisiae* was used as a vaccine vehicle in several studies. Vaccination with the whole recombinant *S. cerevisiae* has been used as a concept to protect mice tumors and H5N1 influenza as well (Wansley *et al*., 2008; Tanaka *et al*., 2011; Lei *et al.*, 2016). In the case of avian species, chickens vaccinated with IBD recombinant proteins expressed from *S. cerevisiae* host system showed (Arnold *et al*., 2012). Oral vaccination with recombinant yeast induced a stronger humoral immune response than purified viral capsid antigen (Kim *et al*., 2014). Thus, instead of purifying recombinant proteins, the yeast cells containing proteins were evaluated for protective efficacy as vaccine candidates in our study.

As ELISA results, the commercial ELISA kits (BioChek, Kings Ride Ascot, United Kingdom) detected the positive level of antibodies as early as 1 wpi while it is failed to detect antibodies before the challenge in a previous study (Schachner *et al.*, 2014). The contrast result could be plained by using the whole recombinant *S. cerevisiae* yeast cell do not change the recombinant protein folding. In terms of confirmation of neutralizing capacity of antibodies against FAdV-4 virus, the diminishing gross lesions on CAM of chicken embryo eggs were observed in all challenged groups in comparison with the serum positive and negative control. Therefore, the protein constructs in our study have the possibility of neutralizing the FAdV-4 viruses.

The challenge result showed the different protection level between 3 vaccinated groups with fiber-1 and fiber-2 products induced the 100% survival while hexon loop-1 did not produce the desired protection. Wang *et al.* (2018) indicated that higher dose of proteins gave less challenge mortality, such as increasing recombinant hexon from 50μg to 200μg reduced mortality from 30% to 5%. This change could be due to the number of hexon protein and it's structure and function. The higher level of antibody neutralizing ability and full protection indicated fiber-2 is the best candidate recombinant vaccine following is fiber-1 and the result is similar to the previous study (Schachner *et al.*, 2014; Wang *et al.*, 2018).

Conclusions: The whole *S. cerevisiae* expressed fiber-2 capsid proteins is an eligible candidate for the development of an effective and safe recombinant vaccine to control the HHS in the poultry.

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