

Developing an Indirect ELISA Based on Recombinant Hexon Protein for Serological Detection of Inclusion Body Hepatitis in Chickens

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ABSTRACT. Fowl adenovirus (FAdv) serotype 2 causes inclusion body hepatitis (IBH) disease which adversely affects the broiler industry in Thailand. We developed an indirect ELISA based on the recombinant hexon protein produced by *E. coli*. The recombinant hexon protein was tested with sera, in both infected and noninfected chickens. The recombinant hexon protein was standardized with an antigen concentration of 3.75 µg/ml and test sera. The intra- and inter-assays were repeatable. The cutoff value from TG-ROC curve analysis was 0.106. The specificity and sensitivity were 80 and 80%, respectively. The correlation coefficient (r) of absorbance values from this ELISA compared with the serum neutralization test was 0.76. This ELISA might be helpful for IBH diagnosis and surveillance.

KEY WORDS: chickens, enzyme-linked immunosorbent assay (ELISA), fowl adenovirus, inclusion body hepatitis, recombinant hexon protein.

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Group 1 fowl adenoviruses (FAdv) are divided into 5 species (A–E) with 12 serotypes [1]. FAdv is a nonenveloped icosahedral that is 70–90 nm in diameter. The hexon protein is the major capsid and plays a role in stimulating specific immunity.

Since 2007, the Thai broiler industry has been affected by inclusion body hepatitis (IBH) caused by serotype 2 fowl adenovirus (FAdv) group 1 species D [16, 18]. This disease can be diagnosed by virus isolation, immunohistochemistry, electron microscopy and polymerase chain reaction (PCR) [4]. The infected culture cells show a characteristic round-type cytopathic effect and cell detachment [2]. Double immunodiffusion (DID) is used for classifying the three groups of FAdvs, but it has low sensitivity and probably cross-reacts with other groups [11]. The serum neutralization (SN) test is a standard technique for detecting an antibody against FAdv. However, the SN test is a time consuming and laborious method [10]. The enzyme-linked immunosorbent assay (ELISA) can be used for detecting both group- and

type-specific antibodies against FAdv and is very sensitive [15]. This technique is useful for disease surveillance and monitoring the antibody status. The aim of this study was to develop an indirect ELISA for detecting and monitoring group D serotype 2 antibodies against IBH in chickens.

The viral DNA was extracted from serotype 2 FAdv, namely KU 01/07, isolated from infected chicken liver tissue samples [16] by using a FavorPrep™ Tissue Genomic DNA Extraction Mini kit (Favorgen®, Ping-Tung, Taiwan). A pair of primers was designed according to the published sequence of fowl adenovirus D in GenBank including FAdv from Canada (accession no. AC_000013) and KU 01/07 FAdv (accession no. EU678792.1). The forward primer was AdEx F 5'-CAAATTCAGGCAGACGGTTCG-3', and the reverse primer was AdEx R 5'-GGCTAACATGTACTGGTAAC-3'; these primers were used to perform PCR amplification as described previously [14]. The PCR products were purified with a FavorPrep™ Gel/PCR purification Kit (Favorgen®) and were ligated into T&A RBC (Invitrogen®, CA, U.S.A.) and subcloned into the pQE-31 (Qiagen®, Hilden, Germany) expression vector in frame with a 6×His N-terminal tag. The protein was induced by isopropyl-beta-D-thiogalactopyranoside (IPTG) and purified with an Ni-NTA affinity column (Thermo Scientific®, IL, U.S.A.). Protein was evaluated by using SDS-polyacrylamide gels (SDS-PAGE) and Western blot. The hexon protein immunized mouse or convalescent chicken sera obtained from chickens infected with FAdv

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serotype 2 and horseradish peroxidase (HRP)-conjugated secondary antibodies (Pierce®) were used for detecting hexon protein by Western blot, and 3,3-Diaminobenzidine (DAB) was used as a chromogenic substrate. A total of 150 SN-positive serum samples from 80 chickens that recovered from IBH and 100 SN-negative serum samples from 80 non-infected chickens were used as positive and negative sera, respectively. These sera were collected from commercial chicken farms and were kindly provided by Betagro Science Center (BSC) Co., Ltd., Thailand. Checkerboard titrations were carried out between hexon proteins and positive sera. A SN test was simultaneously performed. Titers from the SN test of 150 convalescent sera were divided into three groups: low titer (<1:4–1:32), intermediate titer (1:64–1:512) and high titer (1:1024–>1:8192). The repeatability and reproducibility were evaluated with the coefficient of variation (CV) [6]. The cutoff was selected by a two-graph receiver operating characteristic (TG-ROC) [3, 9]. The data were used to analyze the relationship between the ELISA test and the SN test using the correlation coefficient (r) statistics [5] and were interpreted as described by Hinkle *et al.* (1998). Eighty negative sera from chickens vaccinated with Newcastle disease (ND), Infectious bronchitis (IB), Egg drop syndrome (EDS) and Infectious bursal disease (IBD) vaccines were specificity tested.

The highest amount of protein expression was induced by 1 mM IPTG and was recorded at 6 hr after induction, which is shown in Fig. 1. The immunized mice and all chicken antisera could detect hexon protein by Western blot as shown in Fig. 2. The optimal conditions for the ELISA test from checkerboard titration are shown in Fig. 3. The optimal optical density (O.D.) values were obtained with the 3.75 μg /well of hexon protein, 1:100 of positive serum and 1:10,000 of goat anti chicken HRP-IgG.

The cutoff value from the TG-ROC curve analysis indicated 0.106 (Fig. 4) that obtained 80% sensitivity and 80% specificity, respectively. The inter-assay and intra-assays showed CVs ranging from 5.10 to 20.78% and from 2.93 to 16.65%, respectively (Table 1).

The r value was 0.76 (Fig. 5). The O.D. values of the ELISA test ranged from 0.001 to 0.408 and were correlated with the SN titers [$<1:4$ ($2 \log_2$) to $>1:8,192$ ($13 \log_2$)]. The ND-, IB-, IBD- and EDS-vaccinated chicken antisera revealed negative results in the ELISA and were specific to serotype 2 and group D of FAdV according to the data obtained from the GenBank.

SDS-PAGE analysis showed two bands; the first was the specific hexon protein band of about 35 kDa, and the other, about 70 kDa, was a possible dimer that formed a disulfide bond from one or more of the cysteine residues in the hexon protein molecules [13]. A previous study showed that a mouse serum against a recombinant hexon protein reacted with native FAdV hexon using an immunohistochemistry technique [7], which performed with infected chicken liver tissues that were confirmed by PCR and nucleotide sequencing (data not shown). The optimal dilution revealed the high reactivity. We found the high background reaction could be decreased by absorbing the sera with donor equine serum.

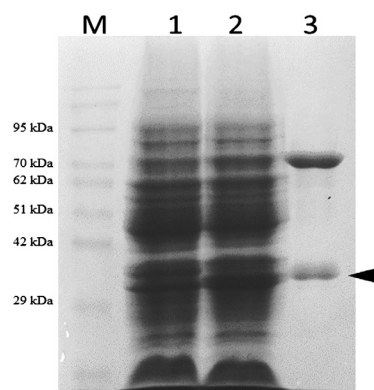


Fig. 1. Coomassie blue-stained SDS-PAGE of *E. coli* pQE31-expressed hexon protein. Lane M, molecular weight marker; lane 1, protein extract from *E. coli* pQE31; lane 2, *E. coli* pQE31-expressed hexon protein; lane 3, the purified hexon protein. The black arrowhead indicates the position of hexon protein. The dimer of hexon protein is shown as the upper band in lane 3.

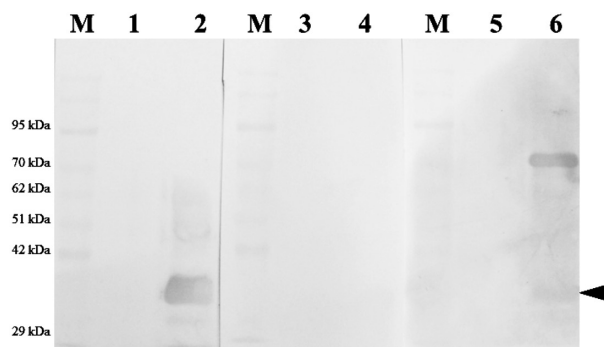


Fig. 2. Recombinant hexon protein analyzed by Western blot with antiserum from a chicken is shown. Lane M, protein marker; lanes 1 and 2, protein extracts from the induced recombinant cells and purified hexon proteins reacted with immunized mouse antiserum to hexon protein; lanes 3 and 4, protein extracts from the induced recombinant cells and purified hexon proteins that were not reacted with a negative chicken antiserum to FAdV; lanes 5 and 6, protein extracted from the induced recombinant cells and purified hexon protein reacted with convalescent chicken antibody against FAdV. The black arrowhead indicates the position of hexon protein.

The optimal hexon protein concentration was 3.75 μg /well purified by affinity column chromatography. The CV% ranged from 5.10 to 20.78% and from 2.93 to 16.65%, respectively, which were values, according to Jacobson *et al.* (1995). The r value showed good correlation. There was no cross-reaction with positive sera for the ND, IB, IBD and EDS vaccines in the ELISA test. By a molecular technique, the Loop 1 (L1) amino acid sequences were used for classifying twelve serotypes. It was found that there was a closed

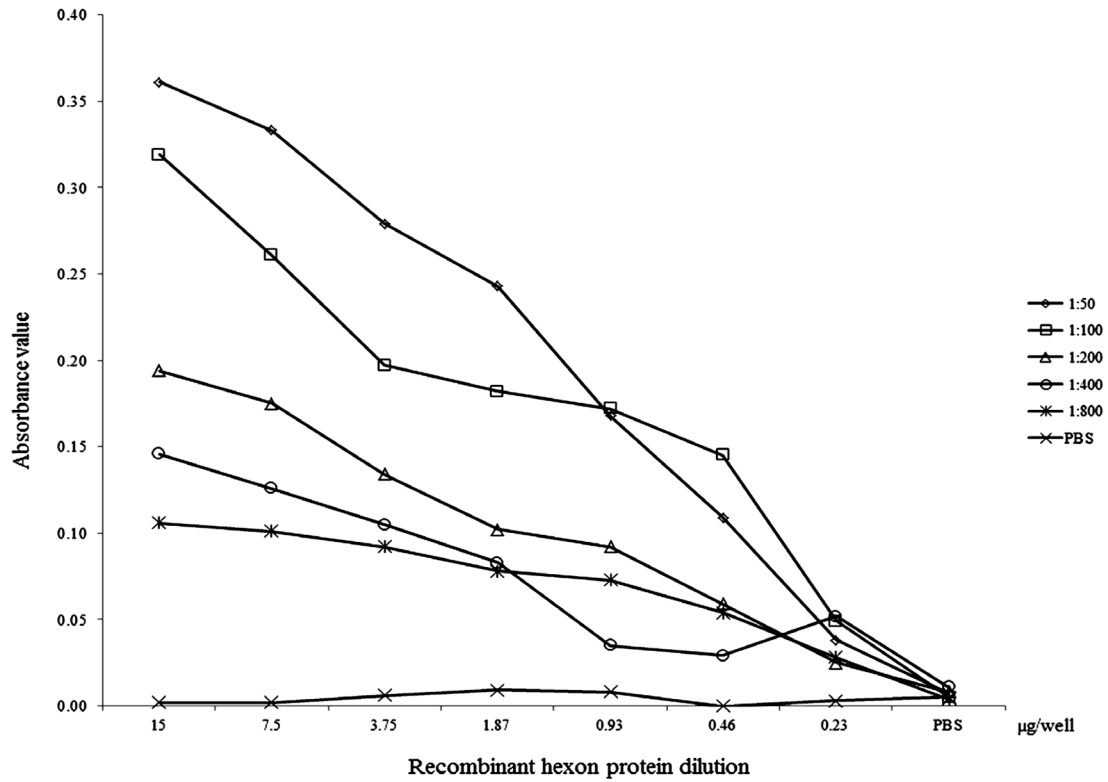


Fig. 3. Checkerboard titration between recombinant hexon protein and convalescent chicken antibody in ELISA. Twofold serum dilution starts with a dilution of 1:50, and hexon protein dilution starts with 15 $\mu\text{g}/\text{ml}$. The negative control reaction is PBS.

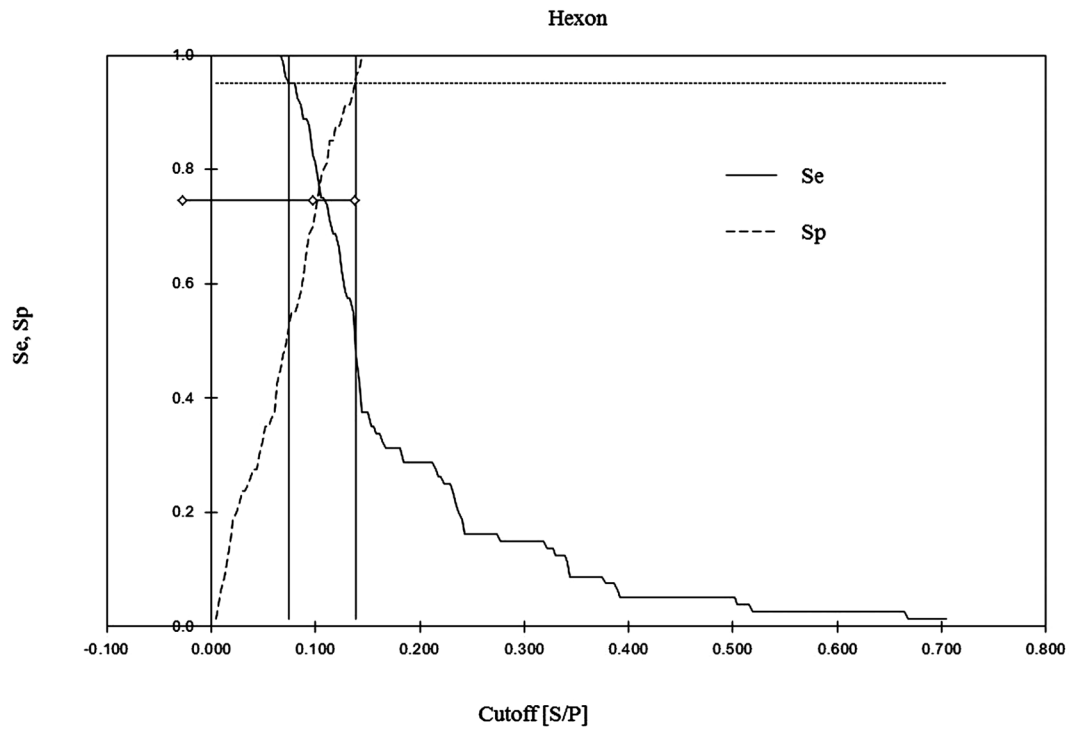


Fig. 4. The intersection point of the TG-ROC curve analysis shows the data from the negative and positive groups; the cutoff point was 0.106. The sensitivity and specificity are 80.0 and 80.0%, respectively.

Table 1. The repeatability of the ELISA test kit for intra- and inter-assays with two replications using infected and noninfected chicken sera

Samples	Mean O.D. \pm SD		Intra-assay CV%	Inter-assay CV%
	Intra-assay	Inter-assay		
Negative sera	0.018 \pm 0.003	0.024 \pm 0.004	16.65	20.78
	0.062 \pm 0.006	0.068 \pm 0.012	10.76	18.27
	0.052 \pm 0.006	0.050 \pm 0.009	12.46	18.39
	0.044 \pm 0.002	0.034 \pm 0.006	5.39	17.78
	0.034 \pm 0.002	0.036 \pm 0.003	6.52	9.07
	0.038 \pm 0.003	0.040 \pm 0.008	10.32	18.88
Positive sera	0.228 \pm 0.036	0.201 \pm 0.043	16.08	12.19
	0.265 \pm 0.017	0.254 \pm 0.028	6.45	10.73
	0.204 \pm 0.010	0.225 \pm 0.018	5.09	7.85
	0.438 \pm 0.012	0.420 \pm 0.021	2.93	5.1
	0.734 \pm 0.031	0.696 \pm 0.058	4.25	8.39
	0.101 \pm 0.008	0.112 \pm 0.014	8.44	12.42

The results are shown as the mean of O.D. \pm SD. O.D.: Optical density. SD: Standard deviation. CV: Coefficient of variation.

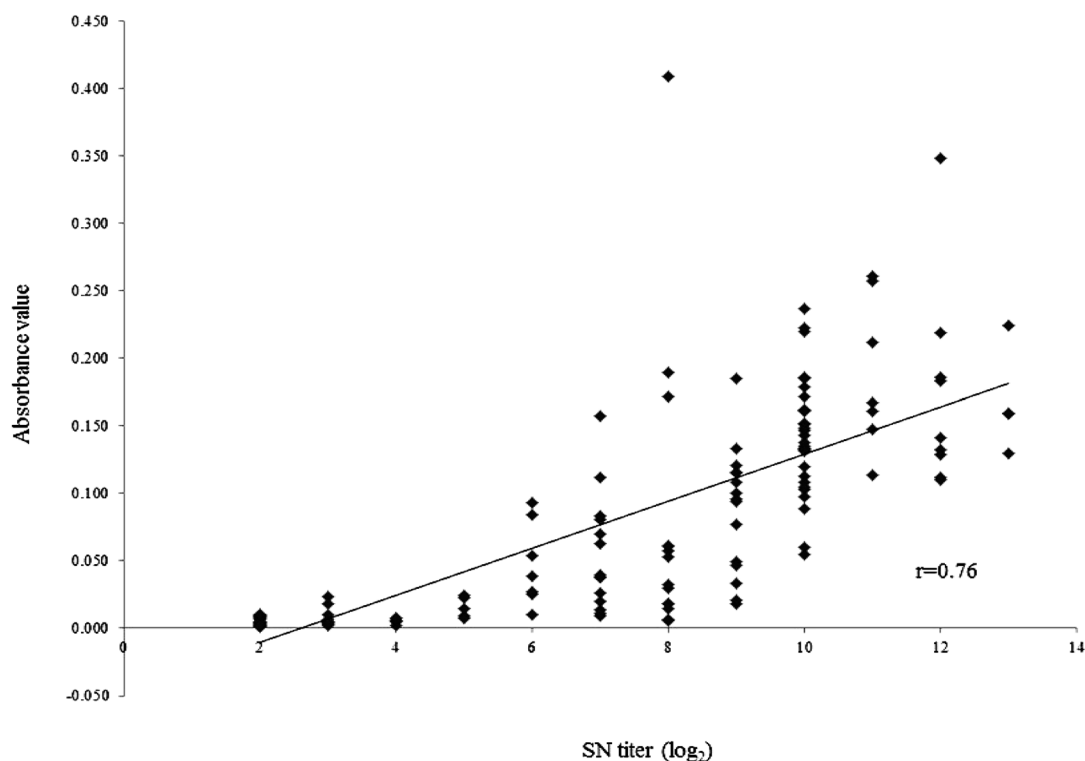


Fig. 5. Correlation coefficient of the ELISA and SN titers for 150 serum samples from vaccinated farm chickens.

genetic relationship between FAdv2 and FAdv12 [12]. In addition, serologic cross-reaction between serotypes 2 and 3 of group D was reported [8]. Since FAdv group D consists of serotypes 2, 3, 9 and 11 [1], the recombinant hexon protein probably cross-reacted with the other serotypes within this group. Although there are no other serotype infections or FAdv vaccination programs presently in Thailand, the specificity of the ELISA test needs to be improved by

performing cross-reaction tests with other serotypes in the future. Also, although the dot-ELISA test kit was previously developed using hydropericardium syndrome virus (HPSV) antigen [17], it is based on the color intensity of the dot. The indirect ELISA is easier to use and is more accurate and appropriate for screening and monitoring large numbers of samples. In this study, an indirect ELISA based on hexon protein produced using *E. coli* as an antigen was successfully

developed.

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