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Recombinant UL16 antigen-based indirect ELISA for serodiagnosis of duck viral enteritis

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In this study, a recombinant fusion antigen of duck enteritis virus (DEV) UL16 protein was expressed in *Escherichia coli* Rossetta (DE3). This target protein was used as a coating antigen to establish an indirect ELISA for detecting anti-DEV antibodies in serum samples from ducks. In the optimal method for the UL16-ELISA, the fusion protein was coated at 1.25 μg/ml and duck serum samples were diluted at 1:160. The endpoint cut-off value of this assay was 0.598. The inter-assay and intra-assay coefficients of variation (CVs) were both lower than 10%. There was no cross-reaction with duck positive sera of either DHBV, DHV, RA, *E. coli*, *Salmonella anatum*, H5N1 or DSHDV. The assay was applied successfully to examine the suspected duck serum samples and showed 95.5% (73/76) identity with the serum neutralization test (SNT). The results showed that recombinant DEV UL16 protein could be used as a coating antigen and the developed UL16-ELISA approach was rapid, specific, sensitive and repetitive.

1. Introduction

Duck viral enteritis (DVE) is a highly lethal, acute and contagious disease caused by duck enteritis virus (DEV) that exerts a serious impact on the duck industry worldwide (Sandhu and Metwally, 2008). The clinical characteristics of DVE are fever, depression, nasal discharge, adherent eyelids, photophobia, ataxia, tremors, diarrhea and reduced egg production. In addition, DVE can cause vascular lesions, nervous impairments and tissue hemorrhage (Campagnolo et al., 2001; Shawky et al., 2000). Currently, no methods or drugs are available for controlling or curing this disease. The traditional diagnosis of DVE was based on the clinical and pathological characteristics. Final diagnosis can be made by viral isolation and identification. However, routine examination may be inaccurate and time consuming. In recent years, loop-mediated isothermal amplification (LAMP) (Ji et al., 2009), electron microscopy negative staining (Guo et al., 2008), polymerase chain reaction (PCR) (Cheng et al., 2004), and antigen-capture enzyme-linked immunosorbent assay (AC-ELISA) (Jia et al., 2009) have been developed. These methods are sensitive and quick. Recently, an indirect ELISA assay was established with the whole DEV virion as coating antigen for detection of antibodies against DEV (Qi et al., 2007). The assay was specific, repetitive and the kit could be stored for 10 months at −20 °C. However, the virion is very difficult to purify. An indirect ELISA based on recombinant thymidine kinase (TK) protein as coating antigen to detect anti-DEV antibodies was developed (Wen et al., 2010). The TK-ELISA is simple, specific, sensitive, and repetitive. In this study, based on the expressed and purified recombinant UL16 protein, an indirect ELISA (UL16-ELISA) method for serodiagnosis of duck viral enteritis was developed and evaluated.

2. Materials and methods

2.1. Strains, sera and reagents

Escherichia coli DH5a, Rossetta (DE3), pET32b (+) vector, DEV CHv strain, rabbit anti-DEV, and sera positive and negative for DEV were all kept in the Key Laboratory of Animal Disease and Human Health of Sichuan Province. The Ni^{2+}-chelating column, DEAE–Sepharose column, 96-well ELISA microplates, and Bio-Rad model 860 plate reader were purchased from Bio-Rad (USA). HindIII, XhoI, and T4 ligase were purchased from TaKaRa

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Biotechnology Company (China). Horseradish peroxidase-labeled goat anti-duck IgG (HRP-goat anti-duck IgG) and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from KPL (USA). Horseradish peroxidase-labeled goat anti-rabbit IgG (HRP-goat anti-rabbit IgG) was purchased from Zhongshan. Plasmid purification kits and agarose gel DNA purification kits were purchased from Axgene Company. Bovine serum albumin (BSA) was purchased from Promega.

2.2. Recombinant protein expression and purification

Expression and purification were performed as described previously (He et al., 2011). The recombinant plasmid pET32b–UL16 was transformed into *E. coli* Rossetta (DE3). The positive single clone was cultured in LB medium with 100 μg/ml ampicillin at 37 °C to an optical density (OD_{600}) of 0.6. The cells were then induced with 0.2 mM IPTG and allowed to grow further for 6 h at 37 °C. Cells were harvested, freeze thawed, lysed, purified and renatured. The recombinant UL16 protein was obtained and used for western-blot analysis.

2.3. Western-blot assay

To confirm antigenicity and immunogenicity, the recombinant fusion DEV UL16 protein was subjected to 12% SDS-PAGE and electro-transferred onto PVDF membrane as described previously (Kano et al., 2008; Towbin et al., 1979). The membrane was then blocked with 3% BSA in PBST (0.2% Tween-20 in PBS, PH 7.4) at 37 °C for 1.5 h. Subsequently, the membrane was incubated with rabbit anti-DEV IgG at a dilution of 1:100 with 0.5% BSA in PBST at 4 °C overnight. The membrane was incubated further with HRP-goat anti-rabbit IgG at a dilution of 1:3000 at 37 °C for 1 h. The reaction was developed with diaminobenzidine substrate buffer and terminated by washing with distilled water.

2.4. Development of the UL16-ELISA

A checkerboard titration was performed to determine the optimal working dilution of the coating antigen, serum and HRP-goat anti-duck IgG using a 96-well ELISA plate. A 96-well microtiter plate was coated with 100 μl of purified UL16 protein and incubated at 4 °C overnight. The plates were then blocked for 1 h with 1% BSA/PBST at 37 °C and washed three times with PBST. Subsequently, 100 μl of duck sera were added and incubated at 37 °C for 1 h. The samples were washed, and then incubated for 1 h with 100 μl of HRP-goat anti-duck IgG diluted 1:5000 in 0.1% BSA/PBST at 37 °C, washed again, and detected with 100 μl of TMB/H_2O_2 for 30 min at room temperature (RT) and away from light. The reaction was then stopped by the addition of 50 μl of 2 M H_2SO_4. Optical density (OD) values were measured at 450 nm. Test sera included positive, negative and blank-sample controls. The dilutions that gave the maximum between positive and negative sera (P/N) with the lowest levels of background readings by absorbance at 450 nm were selected for the optimal antigen-coating concentration and serum dilutions. The optimal antigen-coating concentration and serum dilutions to determine the optimal HRP-goat anti-duck IgG dilutions were performed. Twenty-four negative sera from duck were used to determine the cut-off value according to the optimization of the ELISA procedure. The cut-off value was determined by titration as the mean OD_{450} value plus 3 SDs of the antibody levels of the negative controls. The serum sample was regarded as positive if the OD_{450} value was higher than the cut-off value; or, it was considered to be negative.

2.5. Repeatability of the UL16-ELISA

Three serum samples and two batches of UL16 protein were selected to validate the test and evaluate the repeatability of the assay. With the optimal working concentration of the coating antigen, the three serum samples were detected by the positive serum and HRP-goat anti-duck IgG. Each serum sample was tested in eight different replicates to calculate intra-assay variation. The other batch of UL16 protein was coated and the OD_{450} values of the three serum samples were measured. Each serum sample was tested in eight different plates to calculate inter-assay variation.

2.6. Sensitivity of the UL16-ELISA

In order to determine the sensitivity of this method, sera were diluted 1:320, 1:640, 1:1280, 1:2560, 1:5120 and 1:10,240. The other operating conditions were performed as the optimal working procedure.

2.7. Specificity of the UL16-ELISA

The cross-reaction test and the inhibition-reaction test were performed to determine the specificity of the UL16-ELISA method as described previously (Jia et al., 2009). Based on the cut-off value, duck antisera of duck hepatitis B Virus (DHBV), duck hepatitis virus (DHV), *Riemerella anatipestifer* (*R.A*), influenza virus (H5N1), *Salmonella anatum* (*S. anatum*), *E. coli* and duck swollen head hemorrhagic disease virus (DSHDV) were detected in two replicates according to the method above. According to the optimal concentration, an equal volume of fusion UL16 protein and anti-DEV (or negative) sera were mixed and incubated at 37 °C for 1 h to perform the inhibition-reaction test. These mixtures were tested with the UL16-ELISA. Furthermore, negative and positive sera without any treatment were used as control sera. The percentage of inhibition was calculated as reported previously (Ko et al., 2009). The fusion protein was regarded as positive for DEV and the UL16-ELISA was specific when the percentage of inhibition was higher than 50%.

2.8. Comparison of UL16-ELISA and serum neutralization

Ninety suspected serum samples were collected from ducklings from Sichuan Province and kept in the Key Laboratory of Animal Disease and Human Health of Sichuan Province. These serum samples were examined using the developed UL16-ELISA assay as described above. In addition, these serum samples were tested by the traditional serum neutralization test (SNT) as described previously (Shaoying et al., 2006; Wolf et al., 1974). The correlation was carried out by comparing results of the two methods (Chaudhuri et al., 2010).

3. Results

3.1. Recombinant protein expression and purification

The UL16 gene was subcloned successfully into the prokaryotic expression vector pET32b (+) and induced by IPTG to produce recombinant UL16 protein. The size of the purified fusion protein was about 60 kDa by SDS-PAGE analysis. The results of western-blot analysis suggested that the recombinant fusion UL16 protein possessed high levels of antigenicity and immunogenicity.

3.2. Development of an UL16-ELISA

By checkerboard titration tests, the OD_{450} value gave the maximal difference between the positive sera and negative sera (P/N value, data not shown) when the dilutions of antigen and serum