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Recombinant UL30 antigen-based single serum dilution ELISA for detection of duck viral enteritis

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A B S T R A C T

A recombinant UL30 antigen-based single serum dilution enzyme linked immunosorbent assay (ELISA) was developed to measure specific antibody in the sera of ducks against duck enteritis virus (DEV). The partial UL30 gene of DEV was cloned, expressed, purified and tested for its diagnostic use by designing a single serum dilution enzyme linked immuno-sorbent assay (ELISA). A total of 226 duck sera samples were tested using the assay. A linear relationship was found between the predicted antibody titres at a single working dilution of 1:100 and the corresponding serum titres observed as determined by the standard serial dilution method. Regression analysis was used to determine a standard curve from which an equation was derived which demonstrated this correlation. The equation was then used to convert the corrected absorbance readings of the single working dilution directly into the predicted ELISA antibody titres. The assay proved to be specific, sensitive and accurate as compared to the virus neutralization test with a specificity, sensitivity and accuracy being 96%, 95% and 95% respectively.

1. Introduction

Duck viral enteritis, is an acute, contagious infection of ducks, geese, swans of all age and species. The disease has been responsible for significant economic losses in domestic and wild water fowl as a result of mortality, and decreased egg production (Sandhu and Shawky, 2003). The disease is caused by duck enteritis virus (DEV), and is characterized by vascular damage, tissue haemorrhage, eruptions on the digestive mucosa lesions of lymphoid organs and degenerative changes in parenchymatous organs (Barr et al., 1992; Shawky et al., 2000). The disease is difficult to monitor and control because the virus establishes an asymptomatic carrier state in water fowl that is detectable only during periods of intermittent virus shedding (Burgess et al., 1979). The diagnostic procedures that are currently used to identify DEV antigen include virus isolation and identification (Burgess and Yuill, 1981; Hwang et al., 1975), reverse passive hemagglutination test (Deng et al., 1984), histopathology (Shawky et al., 2000; Xuefeng et al., 2008a), immunofluorescence (Proctor, 1975), immunoperoxidase staining (Malmarugan and Sulochana, 2002), immunohistochemistry (Islam et al., 1993; Xuefeng et al., 2008b), electron microscopy (Yuan et al., 2005), and polymerase chain reaction (PCR) (Hansen et al., 1999, 2000; Pritchard et al., 1999). It is very important to select appropriate methods for detection of DEV antigen. The methods mentioned above are both time consuming and labour intensive; moreover, samples for virus isolation are contaminated easily, and the equipment or personnel required for PCR may not be available. However, the method most widely used in routine serological tests is virus neutralization (Thayer and Beard, 1998). Enzyme linked immune sorbent assays (ELISAs) are more specific and sensitive. Serological methods based on purified recombinant protein may have higher sensitivity and specificity as the target antigen is immuno-dominant and devoid of any non-specific moieties present in whole cell preparations (Dey et al., 2004; Mohan et al., 2006). The single serum dilution ELISA technique, with characterized sensitivity and specificity, has been applied in detection of many viruses, like infectious bursal disease (Snyder et al., 1984; Ramadass et al., 2008, Singh et al., 2010), Newcastle disease (Snyder et al., 1983, Mohan et al., 2006) and infectious bronchitis virus (Snyder et al., 1984). The genome of DEV is 158 kb in length and its G + C content is 44.91% (Li et al., 2009). Nucleotide sequence analysis indicated that genome of DEV is composed of two unique sequences, unique long (UL) and unique short (US), the latter being flanked by inverted repeat elements (IRS and TRS) of 13 kbp each. Like other herpesviruses, G + C content of the virus in repeat regions is 48.02%, higher than that in unique regions 44.05% (Li et al., 2009). There are many reports of prokaryotic expression of genes of the duck enteritis virus (Wu et al., 2011; Shen et al., 2010). An antigen capture ELISA was developed using UL24 gene of duck enteritis virus (Jia et al., 2009). Glycoprotein are major antigens recognized by infected host's immune system and play an important role in mediating target cell infection, cellular entry of free viruses and maturation or egress of the virus. DNA polymerase of DEV is a glycoprotein encoded by its UL30 gene, which is located in the

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unique long region of the DEV genome. UL30 gene is highly conserved between alphaherpes viruses and DEV. In order to curtail the economic impact of the disease in commercial duck industry, the development of newer diagnostic tools with better specificity and sensitivity is of importance. In this study, a recombinant UL30 antigen-based single serum dilution ELISA, a modification of conventional ELISA was developed for rapid measurement of specific antibodies from ailing or vaccinated birds.

2. Materials and methods

2.1. Cells and virus

Chicken embryo fibroblasts (CEF) were cultured at 37 °C with 5% CO_2 in minimal essential medium (MEM) (Sigma, USA) containing 10% foetal calf serum (FCS) (Thermo Scientific, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. DEV was isolated from the post-mortem samples viz., liver and spleen of six ducks reared in backyard from the State of Kerala, India. The samples were transferred to laboratory, placed in sterile PBS. The material was centrifuged at 1200 × *g* for 15 min. The supernatant was used as viral inoculum to infect monolayer of CEF cells. After DEV inoculation, the CEF cells were incubated in MEM containing 3% FCS. Usually, maximum virus titres could be obtained 72 h post infection (hpi) when cytopathic effect (CPE) was over 75%. DEV from CEF was harvested by three freeze–thaw cycles and clarified by centrifuging for at 10,000 × *g* for 10 min in a F2402H rotor (Beckman, USA). DEV viral DNA was extracted as described by Hansen et al. (2000).

2.2. Cloning, expression and purification of recombinant DEV UL30 protein

A partial UL30 gene was amplified by PCR from the Kerala strain of DEV using self designed primers. The forward primer carried a restriction site for Bam HI: 5′CCGGATCCATGGCAGAGTCGGGTAGAAAC3′ (position 98950 and 98970 bases) and the reverse primer carried a restriction site for Sal I: 5′CCGTCGACTCACCGTTTATCTTAACCTTAC3′ (position 100439 and 100460 bases) based on NCBI Gen Bank sequence (JF999965.1). PCR amplification was carried out using the following cycling conditions: 94 °C for 5 min followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1.5 min, with a final extension of 72 °C for 10 min. The PCR amplified gene fragment was cloned into a T/A cloning vector pTZ57R/T (MBI Fermentas, Germany). The size of amplicon was 1510 bp. PCR product was visualised by agar gel electrophoresis and confirmed by restriction enzyme digestion and sequencing. The sequence encoding partial of UL30 gene was submitted to Gen Bank under the accession number JN208148. The partial UL30 gene was subcloned in frame into Bam HI and Sal I sites of a His tagged prokaryotic expression vector, pET 32a (+) (Novagen, Germany). The recombinant plasmid (pET-UL30) was confirmed by restriction enzyme digestion and DNA sequencing. The construct was transformed into *Escherichia coli* BL21 (DE_3) Rosetta cells (Novagen, Germany) and protein production was induced with isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM final concentration, MBI Fermentas, Germany) at 37 °C for 6 h in a 100 ml Erlenmeyer flask. The culture pellet containing the 6× histidine tagged UL30 fusion protein was solubilised with 8 M urea and purified by affinity chromatography containing Ni-NTA (Invitrogen, USA). The recombinant protein was further purified using the Amicon ultra-4 centrifuge filter device (Millipore, USA) with a membrane cut-off of 50 kDa and further dialyzed using 10% glycerol in PBS. The concentration of purified protein was determined by Modified Lowry's Protein assay kit according to the manufacturer's protocol (Pierce, USA). The confirmation and purity of the recombinant UL30 protein was analyzed by SDS-PAGE and Western blotting using anti-His antibody (Qiagen, Germany) and sera raised against the recombinant UL30 protein.

2.3. Generation of monospecific antisera in rabbits

For preparation of monospecific antibodies, two rabbits, one male and one female were first immunized subcutaneously with 0.5 mg of purified recombinant UL30 protein emulsified in 0.5 ml Freund's complete adjuvant (Santa Cruz, USA). Equal volumes of Freund's incomplete adjuvant (Santa Cruz, USA) and 1 mg of purified recombinant UL30 protein were used for subsequent booster doses. Three booster injections at 15 days interval were given after the primary injection. Seven days after last boost, blood was collected from the ear vein from which sera were collected and stored at −80 °C.

2.4. ELISA procedure

2.4.1. Recombinant antigen coating

Flat-bottom polystyrene microtitre plates (Greiner bio-one, USA) were coated with recombinant UL30 antigen and plates were stored at 4 °C overnight. By checkerboard titration, the optimal antigen concentration (0.5 ng to 1.5 μg/well) was determined. Plates were then incubated with blocking solution (PBS with 2% [w/v] bovine serum albumin) for 1 h at 37 °C, and after three washes with PBS supplemented with 0.05% Tween 20 (PBST) stored at −20 °C until use.

2.4.2. Test sera

A total of 226 duck sera samples were diluted initially 1:100 in PBS just prior to use. One hundred micro litres of serum samples were diluted serially (1:100–1:12,800) in duplicates. Following incubation at 37 °C for 1 h, the wells were washed with PBST.

2.4.3. Peroxidase conjugate

A volume of 100 μl of peroxidase-conjugated IgG fraction of goat anti-duck conjugate (KPL, USA) diluted 1:3000, was added to each test well and control wells. The concentration of anti-duck IgG peroxidise conjugate was determined by checker board titration.

2.4.4. Substrate

O-Phenylenediamine dihydrochloride (OPD) (Sigma, USA), 1 mg/ml in 0.1 M citrate phosphate buffer (pH 5.0) mixed with 1 μl/ml of 35% hydrogen peroxide was used. The substrate, 100 μl/well was dispensed immediately into wells and incubated until the colour reaction developed. The plates were read at 490 nm in an ELISA reader (Bio-Rad, USA).

2.5. Virus neutralization test

The serum samples were inactivated at 56 °C for 30 min. The sera were then diluted two fold, starting at 1:2 dilution, in MEM and mixed with 50 μl of 100 $TCID_{50}$ viral isolate per well (final serum dilution, 1:4096). After 1 h incubation at 37 °C, the antibody–virus mixture was added to 1×10^4 CEF cells plated on a 96 well culture plate (Costar, Corning, USA). On incubation at 37 °C for 4 days, the cell monolayer were washed with PBS and stained with haematoxylin and eosin stain. The virus neutralization titre of antibodies in a serum sample was determined as reciprocal of the highest serum dilution that causes a 50% reduction of cell monolayer.

2.6. Determination of ELISA antibody titres

ELISA antibody titres were determined by standard serial dilution and end-points were calculated by a subtraction method as