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Short communication

# Development of single dilution immunoassay to detect E2 protein specific classical swine fever virus antibody

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ABSTRACT

Classical swine fever virus (CSFV) is the causative agent of a highly contagious disease in swine. The disease is endemic in different parts of the world and vaccination is the only way to protect pigs from CSFV infection. The virus surface protein E2 is the major immunogenic protein eliciting protective immunity against CSFV infection in swine. The whole virus antigen cannot differentiate CSFV from other pestiviruses as it cross reacts with border disease and bovine viral diarrhoea viruses. Commercial available ELISA is based on the whole CSFV particle and can lead to false positive results. Moreover, the available commercial ELISA is not cost effective. In the present study, a recombinant E2 protein based single serum dilution ELISA was developed which showed enhanced sensitivity, specificity and accuracy as compared to commercial CSFV detection ELISA. The recombinant E2 protein based ELISA could be an alternate to existing diagnostics against CSFV infection in pigs.



Classical swine fever virus (CSFV) is the causative agent of a highly infectious disease in swine. The classical swine fever (CSF) or hog cholera can cause huge economic impact to the pig industry worldwide (Moennig, 2000). It affects both domestic and wild pigs with varying degrees of pathogenicity. Although CSF is distributed throughout the world, it has mainly been reported from Asia, parts of Africa, Central and South America and Europe (Artois et al., 2002; Barman et al., 2014; Flores-Gutierrez and Infante, 2008). Frequent outbreaks of CSF have been reported from different parts of the world, including Belgium (1990–94), Germany (1993–2000), Italy (1995–97) and the Netherlands (1997). Recently, CSF outbreaks have been reported from Madagascar, Singapore, Laos, Lithuania, Myanmar, Colombia, and Republic of Korea (Ji et al., 2015). However, the disease has been eradicated from Australia, North America, and New Zealand (Anonymous, 2008).

CSFV belongs to the family *Flaviviridae* under genus *Pestivirus*. CSFV is an enveloped virus having a positive strand RNA with an approximately size of 12.5 kb and comprises of a single large open reading frame (ORF) (Meyers and Thiel, 1996). The genome of CSFV is flanked by two untranslated regions (UTRs) flanking the entire ORF which encodes a polypeptide of approximately 3900 amino acids (Meyers et al., 1996). This polyprotein gives four structural (C, Erns, E1, E2) and eight nonstructural proteins after processing by the cellular and viral proteases (Npro, P70, NS2, NS3, NS4A, NS4B, NS5A, NS5B) (Lowings et al., 1996; Meyers and Thiel, 1996). Npro protein is a non-structural protein, which functions as a cysteine proteinase (Bauhofer et al., 2005). Erns is a glycoprotein secreted from the cells infected by CSFV and is known to inhibit the interferon induction by the host cell (Matzener et al., 2009; Weiland et al., 1999). E1 is a type I trans-membrane protein involved in the adsorption of virus to the host cells (Fernandez-Sainz et al., 2009). CSFV encodes another small hydrophobic protein P7, which is flanked by sequences that are recognized by signal peptidase and is essential for the production of infectious virus (Moser et al., 1999).

E2 is an envelope glycoprotein present on the surface of CSFV and is important to induce host immune response during infection (Qi et al., 2009; Zhang et al., 2006). The E2 protein contains conserved antigenic determinant regions and it is the major immunogenic protein eliciting protective immunity against CSFV infection in swine (Greiser-Wilke et al., 1990; Rumenapf et al., 1991; van Rijn et al., 1996). E2 protein contains two linear B cell epitopes YYEP and TAVSPTTLR spanning towards its carboxy and amino terminus, respectively. The amino acid sequence motif YYEP is specific to pestivirus while TAVSPTTLR is specific to CSFV (Lin et al., 2000; Yu et al., 1996). In addition, E2 protein is accompanied with four relatively independent antigenic domains (A, B, C and D). The domain A has three subdomains (A1, A2 and A3). The E2

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is frequently used to design DNA vaccines against CSFV by different research groups (Beer et al., 2007; Bouma et al., 1999; Qi et al., 2008). Structurally, the E2 protein forms a homodimer during entry and heterodimer with E1 while attachment of the viral particles to the cell (Zhang et al., 2006).

In epidemiological surveys, detection of virus specific antibodies in serum samples is important in order to monitor the circulation of wild CSFV in population. Neutralizing assay is the test of choice to detect CSFV infection in the laboratory. However, detection of neutralizing antibody is time consuming, needs skill and well set up cell culture laboratory. Development of single dilution indirect ELISA can be a convenient alternative tool to detect CSFV specific antibody in pig sera (Li et al., 2013; Yang et al., 2012) using complete E2 protein as detecting antigen. Single serum dilution could be better than serial dilution method because it requires less time, chemical and plasticware making it a cost effective assay. Here we are reporting the expression of complete E2 protein using the bacterial expression system. The bacterial expressed E2 protein showed efficient binding with monoclonal antibody and with the serum collected from the field outbreaks. The study will be useful in designing an efficient diagnostics against CSFV infection.

The porcine kidney cells (PK-15) were procured from ATCC (Manassas, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and essential antibiotics at 37 °C in 5% $CO_2$. The CSFV vaccine strains were procured from college of veterinary science, Guwahati, India. The virus was grown in PK-15 cells using standard protocols (Hulst et al., 2000). The infectious virus particles were recovered by repeated freeze and thaw followed by filtering the extracted supernatant through 0.22 μm membrane filter. The stock of the virus was stored at −80 °C for subsequent use. Titration of the virus was done in 96-wells microtitre plate containing PK-15 cells. Vaccine virus was diluted ten-fold in DMEM containing 10% calf serum into each of the five wells in a 6-well plate. The titer of virus stock was calculated by immunoperoxidase assay using CSFV monoclonal antibody after 72 h post-infection as described earlier (Mittelholzer et al., 1998).

Viral RNA was extracted using TRIzol (Invitrogen, USA) according to the standard protocol. The cDNA was synthesized from extracted RNA using superscript II RT (Invitrogen, USA) and gene specific reverse primer (5′ CCG*CTCGAG*TCAACCAGCGGCGAGTTGTTCTG 3′) designed from available GenBank sequence (Accession number NC_002657). The E2 gene sequence was PCR amplified using E2 forward (5′ CG*GAATTC*ATGCGGCTAGCCTGCAAGGAAGATTAC 3′) and E2 reverse (5′ CCG*CTCGAG*TCAACCAGCGGCGAGTTGTTCTG 3′) primers. Underline sequences are complementary to the CSFV genome and italics sequences are the restriction site *Eco*RI and *Xho*I, respectively. The amplified PCR products were purified by QIAquick gel extraction kit (QIAGEN, Germany) and sequenced by BigDye terminator v 3.1 matrix standard kit and 3130xl Genetic Analyzer Data Collection software v3.0 (Applied Biosystems, USA). The data extracted from analyzer was analyzed by DNAstar software (DNASTAR Inc, USA).

The complete E2 protein gene was cloned into pET28a prokaryotic expression vector (Novagen, Germany) flanking *Eco*RI and *Xho*I restriction sites. The pET28a has His tagged at the N terminal which was used to purify the E2 protein by affinity chromatography. The integrity of the E2 gene after cloning was confirmed by sequencing. The pET28a containing complete E2 gene was transformed into *Escherichia coli* BL21 (DE3) pLysS cells (Novagen, Germany) and induced by 1 mM isopropyl-β-*D*-thiogalactoside (MBI Fermentas, Germany). The fusion protein was extracted after induction of transformed BL21 cells for 4 h at 37 °C 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 10 kDa and further dialyzed using 10% glycerol in PBS. The concentration of the purified protein was determined by modifying Lowry's Protein assay kit according to the manufacturer's protocol (Pierce, USA). The expression of the recombinant E2 protein was further confirmed by SDS-PAGE and western blot using an anti E2 monoclonal antibody WH 303 (Wensvoort et al., 1986).

Two rabbits were immunized subcutaneously with 0.5 mg of purified recombinant E2 protein emulsified in Freund's complete adjuvant (Sigma, USA) for the preparation of polyclonal antibodies. Subsequent booster was given by purified recombinant E2 protein with Freund's incomplete adjuvant (Sigma, USA) for 2 occasions at 14 days interval. The sera sample was collected 14 days after final injection and stored at −80 °C. The antibody against recombinant E2 protein was confirmed by western blot using goat anti-rabbit IgG conjugated to horseradish peroxidase (Pierce, USA) as well as by commercial CSFV kit (HerdChek, IDEXX, USA). A total of 210 pig serum samples were screened for the CSF infection. Blood samples of suspected pigs were collected under aseptic condition and serum were separated after centrifugation at 1500 × *g* for 20 min, and stored at −20 °C until use. Additionally serum samples were screened negative for swine flu and porcine reproductive and respiratory syndrome using a commercial kit (PRRS X3 and influenza A kit from IDEXX, USA).

96-well flat bottom polystyrene plates (Greiner, USA) were coated with recombinant E2 protein (3 μg/well) using nitrate buffer (pH = 9.6) and incubated at 4 °C overnight. Plates were washed with phosphate buffer saline containing Tween-20 (PBST) and blocked with 5% lactalbumin hydrolysate for 1 h at 37 °C. Optimal concentration of E2 protein was determined by the checkerboard titration method (Robinson et al., 1985). Pig serum samples were initially diluted 10 times and then diluted serially and incubated at 37 °C for 1 h. The plates were then washed with PBST, and incubated with 100 μl of the HRP-conjugated anti-pig antibody raised in rabbit (Pierce, USA) for another 1 h at 37 °C temperature. The plates were washed and the E2 protein binding with serum samples was detected with 100 μl of TMB (Invitrogen, USA) for 15 min at 37 °C temperature. The enzymatic reaction was stopped by 100 μl of 2 M $H_2SO_4$, and plates were read at 450 nm in a microtitre plate reader (Biotek, USA). Any serum sample showing an OD above the mean +3 standard deviation of the negative wells was considered positive. The negative serum samples were used to construct positive-negative threshold (PNT) baseline as shown earlier (Snyder et al., 1983). Similarly the positive curve was also plotted. The absorbance of the test sample dilutions was calculated using the formula:

$$\text{SPratio} = (\text{ODofsample} - \text{ODofnegative})/(\text{ODofpositive} - \text{ODofnegative})$$

The PNT line was calculated using negative serum samples which were screened negative by commercial kit. The collected negative serum samples were diluted and resultant OD values were plotted against dilution. The resultant PNT line was used to find out the titer for the known positive serum samples by the subtraction method as described earlier (Snyder et al., 1983). The OD values obtained for every logarithmic dilution was compared with observed titer and the highest correlation coefficient was selected to calculate the titer from that dilution. The constants like slope and intercept were calculated by the scatter plot as described earlier (Snyder et al., 1983).

The sensitivity, specificity and accuracy of the single dilution sera in comparison to the commercial CSFV diagnostic are determined using following formulae.

$$\text{Sensitivity} = (x/x + y) \times 100$$

where, 'x' is the number of sera positive by commercial CSFV diagnostic ELISA and single dilution ELISA; 'y' is the number of sera