Evaluation of Classical Swine Fever Virus (CSFV)-Specific IgA, IgG, and IgM Antibody response in Swine Vaccinated with Alphavirus Replicon Particles-Expressed Antigens

Mohammad M Hossain* and Raymond RR Rowland

Department of Diagnostic Medicine/Pathobiology, Center of Excellence for Emerging and Zoonotic Animal Diseases (CEEZAD), College of Veterinary Medicine, Kansas State University, Manhattan, KS 66506, USA

Abstract

Classical swine fever virus (CSFV) E2 glycoprotein was expressed in an alphavirus based replicon particles (RP) expression system. A fluorescent microsphere immunoassay (FMIA) has been developed for the detection of CSFV E2-specific antibody in swine vaccinated with alphavirus RP. CSFV full length E2 (aa 1-376) was fragmented into several pieces and recombinant proteins were expressed in *Escherichia coli*. Purified proteins were conjugated to microsphere beads, the target antigens were assembled into a single multiplex, and tested against sera vaccinated with alphavirus-expressed antigens. The results reported as mean fluorescence intensity (MFI) obtained from the median value for at least 100 microspheres and the MFI values converted to positive per sample (S/P) ratio. Of the eight recombinant E2 proteins evaluated in this study, the highest MFI values were obtained for E2 (aa 1-181). CSFV E2 glycoprotein was expressed in alphavirus based replicon expression system. The results show that vaccinated animals had CSFV-specific IgA, IgG and IgM in serum and oral fluids. The MFI values for the negative serum sample showed 20-70-fold reduction compared to positive serum sample. Antibody response to CSFV antigens were IgG>IgM>IgA. The results demonstrated that the simultaneous detection of IgG, IgM and IgA antibodies could provide an improved diagnostic tool.

Keywords: Alphavirus replicon particles; Classical swine fever virus; Fluorescent microsphere immunoassay; Luminex

Introduction

Classical swine fever virus (CSFV) is an enveloped positive-stranded RNA virus and member of the Pestivirus genus of the family Flaviviridae [1]. It has a worldwide distribution and is endemic in Central and South America, Europe, and Asia and parts of Africa [2]. Classical swine fever (CSF), a highly contagious and an economically damaging disease of swine. CSF can occur in several forms of varying severity, including highly lethal, acute, chronic, or subclinical. Acute infection can cause fever, skin hemorrhages, and death after 10 to 20 days. CSFV is closely related to the ruminant pestiviruses that cause bovine viral diarrhea (BVD) in cattle and border disease (BD) in sheep [3]. BVDV infection of swine is more common in feral pigs and on farms where pigs may come into contact with cattle. The BVD and BD viruses can naturally infect swine and antibodies generated during infections cross-react with CSFV, thus making a CSF diagnosis problematic. A swine herd positive for CSFV-specific antibodies is considered indicative of a CSF outbreak. Early detection and removal of infected animal is important for CSFV control.

Various immunological diagnostic assays for CSFV are currently available but substantial improvements in the sensitivity, speed, and affordability of these assays are required. Several serological tests for routine diagnosis of CSFV have been developed including indirect fluorescence antibody (IFA) test, serum virus neutralization (SVN) test, immunoperoxidase monolayer assay (IPMA), and enzyme-linked immunosorbent assay (ELISA) [4,5]. Among all available techniques, ELISA has long since been the most common technique for the detection of antibody against E2 and Erns of CSFV [6,7]. These assays have proved to be safe alternatives to gold standard techniques, such as the virus neutralization test (VNT) and virus isolation. Additionally, antibody detection techniques have advantages for the early detection of CSFV infection [8].

A recent study described the development of a fluorescence microsphere-based immunoassay (FMIA) or Luminex™, which can be used for the detection of multiple targets simultaneously with high sensitivity and specificity [9]. The most recent FMIA has also been proven to perform for the detection of porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) specific IgG antibodies by incorporation of non-species-specific conjugates Protein A, G, and A/G in place of the secondary antibody [10]. The detection of CSFV E2-specific antibody for the Differentiation of Infected from Vaccinated Animals (DIVA) target by FMIA has previously been developed [11]. FMIA is a high-throughput multiplex platform for the detection of antibodies to at least 100 target antigens in a small volume. In previous studies, a multiplex serological platform has been used for the detection of antibody against multiple targets including antigenic domains of CSFV E2 and Erns [12].

In the present study, we established a multiplex fluorescent microsphere immunoassay for the detection of CSFV specific IgA, IgG, and IgM antibodies in swine vaccinated with alphavirus expressed E2 antigens. It can be used for rapid, sensitive, and specific detection of CSFV antibody and includes a simple method with high-throughput
capability. Multiplex detection of CSFV E2-specific IgA, IgG, and IgM would be a novel confirmatory diagnostic approach.

**Materials and Methods**

**Vaccination and serum sample collection**

Ten 3 to 4-week-old weanling Large White and Landrace crossbred domestic pigs were immunized with alphavirus-based vaccine constructs. Alphavirus-based vaccine constructs were created by cloning genes for CSFV E2 into Venezuelan equine encephalitis (VEE) vector. The VEE virus expressing the gene of interest was grown in Vero cells and the culture fluid was used to prepare the vaccines as previously described [13]. After acclimation, animals were vaccinated with 2 ml/animal (2.5 × 10^8 RP/ml) dose of replicon particle (RP) intramuscularly. Animals were boosted with same doses of vector construct at days 21 and 42 post vaccination. Blood samples were collected at 0, 7, 14, 21, 28, 35, 42, 49, and 57 days post-immunization (dpi). Serum was separated by centrifugation and stored at -80ºC until use. Oral fluids were collected from group of pig (pens) at 0, 18, 21, 28, 32, 47, 49, and 57 days post immunization.

**Recombinant CSFV E2 protein expression and purification**

Several recombinant antigen fragments have been generated from Chinese lapinized vaccine strain (C-strain) of CSFV E2 glycoprotein for the antigen targets in FMIA. Full length E2 (1-376 aa) has been fragmented into 7 small pieces (aa 1-181; aa 91-270; aa 182-376; aa 1-90; aa 91-181; aa 182-270; aa 271-376). The total fragments of E2 (8 including full length) were cloned into expression vector pHUE [14], expressed in *Escherichia coli*, and purified using the methods as described previously [9].

In brief, for the production of recombinant proteins, bacteria were cultured in LB medium with ampicillin at 37°C until the OD₆₀₀ reached 0.4-0.6. Gene expression was induced by adding isopropyl β-D-1-thiogalacto-pyranoside (IPTG) to a final concentration of 1.0 µg/ml and incubation continued for an additional 4 hours with shaking. Bacteria were pelleted by centrifugation at 4,000 x g for 10 min at 4°C, subjected to three freeze-thaw cycles and re-suspend in 5 ml of 1X LEW buffer (50 mM sodium phosphate monobasic, 300 mM sodium chloride, pH 7.0). Bacteria were lysed by the addition of 50 µL of EDTA free protease inhibitor cocktail (Thermo Scientific, Massachusetts, USA) and lysozyme to a final concentration of 1mg/ml. The mixture was incubated on ice for 30 min with shaking followed by sonication for 30 sec on ice then centrifugation for 30 min at 20,000 x g. The supernatant was recovered and filtered through a 0.45 µm PVDF filter (Fisher Scientific, Pennsylvania, USA).
Multiplex detection of CSFV E2 recombinant fragments specific IgG responses in serum samples from animals vaccinated with alphavirus replicon particles. FMIA was performed in the presence of 1:200 diluted serum samples. Data represent mean fluorescence intensity (MFI) and ± standard deviation of triplicate determinations.

Coupling of recombinant antigens to microsphere beads

Recombinant proteins were covalently coupled to Luminex MagPlex® polystyrene, carboxylated magnetic microsphere beads (Luminex Corporation, Texas, USA) using the method described in the commercial kit. Briefly, 500 µl of beads at a concentration of 1.25 × 10^7 beads/ml were placed into a low protein-binding microfuge tube (USA Scientific, Inc., Florida, USA) as the protein standard. Purified protein was analyzed by SDS-polyacrylamide gel (10%) electrophoresis (PAGE) as described previously [15] and gels stained with Coomassie Blue.

Fluorescence microsphere immunoassay procedure

The eight CSFV E2 recombinant antigen bead sets were combined with an unconjugated bead set as a background control. Since all of the recombinant proteins possessed a His tag, a penta-His monoclonal antibody was used to estimate the relative amount of recombinant protein attached to each bead set as described previously [9]. Prior to making the bead mixture, the number of beads was counted under a microscope using a hemacytometer. Approximately 1,250 beads in 50 µl of PBS with 4% goat serum (PBS-GS) and 50 µl serum samples or serial 2-fold diluted penta-His™ mouse monoclonal IgG (Qiagen GmbH, California, USA) were placed in each well of a Corning™ Costar™ polystyrene white round-bottom 96-well plate (Corning, Inc., New York, USA). Plates were incubated for 30 min at room temperature with gentle agitation then washed three times with 190 µl PBS-GS. Biotin labeled goat anti-swine IgM (µ) (KPL, Maryland, USA), Goat anti-pig IgA H&L (Biotin), (Abcam Inc., Massachusetts, USA), biotin-SP-conjugated goat anti-swine IgG (2 µg/ml) (Jackson Immuno Research Laboratories, Inc., Pennsylvania, USA), and biotinylated anti-mouse IgG (H+L) (2 µg/ml) (Vector Laboratories, Inc., California, USA) were diluted in PBS-GS. Beads were re-suspended in 50 µl of diluted conjugates. After 30 min of incubation, wells were washed and 50 µl of streptavidin-conjugated phycoerythrin (SA-PE) (Moss, Inc., Maryland, USA), diluted 1:500 in PBS-GS was added and incubated for 30 minutes at room temperature. The beads were washed three times and re-suspended in 100 µl of PBS-GS and read the plate. Data was analyzed using xPONENT 4.2 software, and the results reported as mean fluorescence intensity (MFI) obtained from the median value for at least 100 beads. Results were reported as MFI of the test sample minus MFI of the background bead set [9]. All antigen targets were assembled into a single multiplex and tested against sera. Signal/positive (S/P) ratio was calculated using the following equation S/P=(Mean MFI of test sample-Mean MFI of negative control)/(Mean MFI of positive control-Mean MFI of negative control)

Results

SDS-PAGE of recombinant CSFV E2 proteins incorporated into the FMIA.

As shown in Figure 1, Coomassie Blue staining of recombinant proteins resolved by SDS-PAGE identified a major band for each protein. All proteins possessed the predicted size, including molecular masses of 50 KD for full length (aa 1-376), 28 KD for aa 1-181, 28 KD for aa 91-270, 32 KD for aa 182-376, 18 KD for aa 1-90, 17.8 KD for aa 91-181, 18 KD for aa 182-270, and 22 KD for aa 271-376. In addition to protein purity, penta-His mAb was used to evaluate the relative amount of protein attached to each bead set. As shown in Figure 2, all bead sets were positive for binding.
Even though full length (aa 1-376) and aa 271-376 protein bands are weak, the penta-His mAb revealed similar binding activities with all eight recombinant proteins on the surface of the beads, whereas recombinant proteins without penta-His mAb did not show binding activity. Overall, the results show that the use of the anti-His mAb provides a convenient means for assessing antigen binding to the beads.

Multiplex detection of CSFV E2 IgA and IgG antibodies in alphavirus RP vaccinated animals

A multiplex FMIA was performed using CSFV E2 recombinant antigens in the presence of serum sample. Of the eight E2 proteins, fragment (aa 1-181) showed the highest MFI values (Figure 3). In this study, IgA and IgG responses to CSFV E2 (aa 1-181) in 10 animals immunized with alphavirus RP are illustrated in Figure 4. The positive serum sample used for this study was obtained from a pig at 28 days after alphavirus RP immunization. The results for the positive serum showed 35,000-50,000 MFI values when biotinylated anti-porcine IgG was used as the secondary antibody in the assay. All the sera samples were highly reactive to CSFV E2 (aa 1-181). The MFI values for the negative serum sample showed 20-70 fold reduction, when biotin-conjugated anti-swine IgG used for the detection of porcine Ig (Figure 4A). Similarly, IgA response to CSFV E2 antigen in 10 animals immunized with alphavirus RP was tested using FMIA. The results for the 10 positive sera sample showed that two out of 10 sera (animal 23 and 26) were reactive to E2 (aa 1-181) antigen. The results for the positive sera showed 15,000 MFI values. The negative sera sample for animal 23 and 26 showed 300 fold reduction when biotin conjugated anti-swine IgA was used as the secondary antibody in the assay (Figure 4B).

Early detection of CSFV E2-specific IgM in alphavirus RP vaccinated animals

To determine changes in CSFV E2-specific IgM over time, 10 animals were immunized with alphavirus RP and serum samples were collected at 0, 7, 14, 21, 28, 35, 42, 49, and 57 days post-immunization. IgM response against CSFV E2 (aa 1-181) was determined by FMIA and the reactivity of antibody was followed over time, as shown in Figure 5. CSFV E2 antigen specific IgM was detected in 3 out of 10 animals at 7 days post immunization (dpi). The results for the serum samples 23 and 27 showed 10,000-12,000 MFI values when biotinylated anti-porcine IgM was used as the secondary antibody in the assay. Animal 25 showed 3-fold reduced MFI value compared to other two animals. IgM in animal 23 was first detected at 7 day after immunization and was observed to remain elevated at 21 days and, by 28 days, had decreased to near background MFI values.

Detection of CSFV-E2-specific IgA, IgG, and IgM in alphavirus RP vaccinated animals

For the detection of CSFV E2 (aa 1-181) specific IgA, IgG, and IgM, a FMIA was performed using CSFV E2 (aa 1-181) in the presence of serum and oral fluids then antibody responses were followed over the study period (Figure 6). CSFV IgG antibodies were highly reactive to E2 antigen compared to IgA and IgM. IgG was first detected 18 dpi in oral fluids and 21 dpi in serum and peaked at 28 dpi in both serum and oral fluids. IgA response was recorded at 28 dpi in both serum and oral fluid. CSFV E2-specific IgM was detected at 28 dpi in both serum and oral fluids. Collectively, the results show that antibody response pattern in serum and oral fluids were almost similar (Figures 6A and 6B).

Discussion

The production of antibodies following immunization is important to suppress viral infections. The aim of this study was to detect changes in the serum antibody responses following vaccination against CSF with an alphavirus expressed E2 subunit vaccine. E2 and Erns are known to induce virus-neutralizing antibodies and play an important role in protective immunity in the natural host. A multiplex fluorescent microsphere immunoassay has been used to evaluate CSFV E2...
Kinetics of CSFV E2-specific IgM in alpha virus replicon particles immunized animals after vaccination. Our results showed that a comparison of the quantitative antibody response in serum vs. oral fluid, tested using FMIA, found no significant difference (Figure 6). The data in current studies were supported by previous finding in PRRSV-specific ELISA [20]. Also, our study showed that the highest MFI values were obtained for IgG in oral fluid, suggested that IgG in oral fluid is derived from serum via the gingival crevicular fluid [21].

Conclusion

A fluorescent microsphere immunoassay has been developed that can detect CSFV E2-specific IgA, IgG and IgM antibody in a single serum sample. Multiplex FMIA based detection of CSFV antigen specific IgA and IgM in alphavirus-derived replicon particles immunized swine is a new development in CSFV diagnosis. Development of a reliable sensitive FMIA method to detect CSFV-specific antibodies in swine could expedite the progress of control programs that focus on achieving the goal of CSFV control and management of CSF disease.

Acknowledgement

This work was supported by USDA NIFA Award 2013-68004-20362.

References


