

## 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<sup>®</sup>, 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

\*Corresponding author: Mohammad M Hossain, Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, Kansas State University, 1800 Denison Ave, N-211 Mosier Hall, Manhattan, Kansas 66506 USA, Tel: 7855324855; Fax: 7855324481; E-mail: [mofazzal@vet.k-state.edu](mailto:mofazzal@vet.k-state.edu)

Received February 22, 2017; Accepted February 26, 2017; Published February 28, 2017

Citation: Hossain MM, Rowland RRR (2017) Evaluation of Classical Swine Fever Virus (CSFV)-Specific IgA, IgG, and IgM Antibody response in Swine Vaccinated with Alphavirus Replicon Particles-Expressed Antigens. Adv Tech Biol Med 5: 213. doi: [10.4172/2379-1764.1000213](https://doi.org/10.4172/2379-1764.1000213)

Copyright: © 2017 Hossain MM, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

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

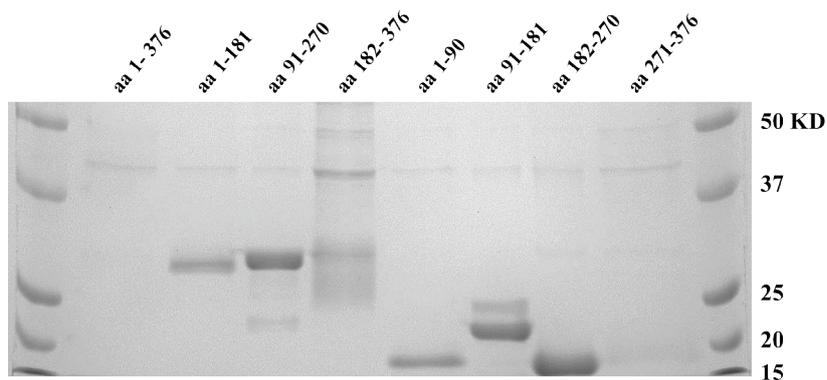
Ten 3 to 4-week-old weanling Large White and Landrace crossbred domestic pigs were immunized with alphavirus-based vaccine constructs. Alphavirus-based vaccine constructs were created by cloning genes for CSFV E2 into Venezuelan equine encephalitis (VEE) vector. The VEE virus expressing the gene of interest 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 ( $2.5 \times 10^8$  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  $-80^\circ\text{C}$  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

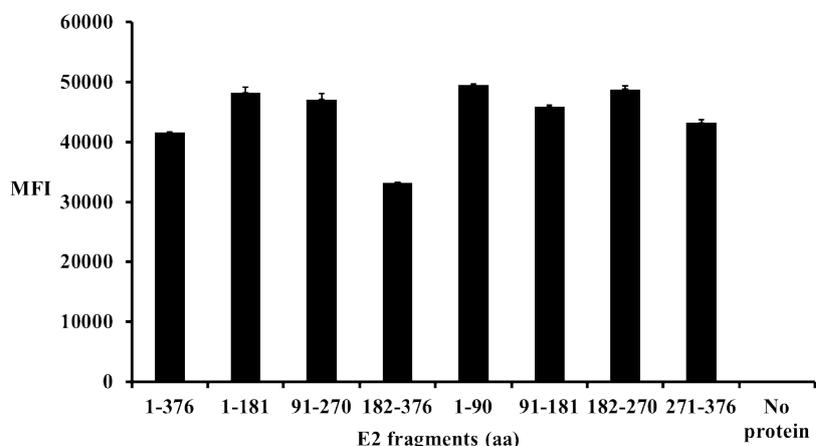
Several recombinant antigen fragments have been generated from

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].

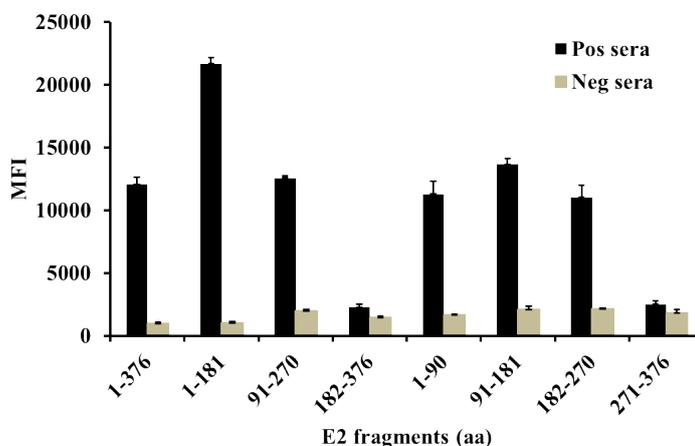
In brief, for the production of recombinant proteins, bacteria were cultured in LB medium with ampicillin at  $37^\circ\text{C}$  until the  $\text{OD}_{600}$  reached at 0.4-0.6. Gene expression was induced by adding isopropyl  $\beta$ -D-1-thiogalacto-pyranoside (IPTG) to a final concentration of  $1.0 \mu\text{g/ml}$  and incubation continued for an additional 4 hours with shaking. Bacteria were pelleted by centrifugation at  $4,000 \times g$  for 10 min at  $4^\circ\text{C}$ , 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  $50 \mu\text{L}$  of EDTA free protease inhibitor cocktail (Thermo Scientific, Massachusetts, USA) and lysozyme to a final concentration of  $1\text{mg/ml}$ . 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  $20,000 \times g$ . The supernatant was recovered and filtered through a  $0.45 \mu\text{m}$  PVDF filter (Fisher Scientific, Pennsylvania, USA).



**Figure 1:** SDS-PAGE of recombinant CSFV E2 proteins incorporated into the FMIA. Lane M contains the protein markers.



**Figure 2:** Binding activity of anti-5xhistidine antibody against recombinant CSFV E2 proteins. Anti-His™ mouse monoclonal antibody was diluted 1:100 in sample buffer and FMIA was performed in the presence of CSFV E2 proteins conjugated microspheres. Results are reported as mean fluorescent intensity (MFI) and standard errors.



**Figure 3:** Multiplex detection of CSFV E2 recombinant fragments specific IgG responses in serum samples from animals vaccinated with alpha virus replicon particles. FMIA was performed in the presence of 1:200 diluted serum samples. Data represent mean fluorescence intensity (MFI) and  $\pm$  standard deviation of triplicate determinations.

The 6x His-tagged protein was affinity purified using a PrepEase His-Tagged Protein Purification high yield Kit (Affymetrix, USB, California, USA). The protein concentration was measured using BIO-RAD protein assay reagent (BioRad, California, USA) on a precision microplate reader (Molecular Devices, California, USA) using bovine serum albumin (Sigma-Aldrich, Missouri, 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.

### Coupling of recombinant antigens to microsphere beads

Recombinant proteins were covalently coupled to Luminex MagPlex<sup>®</sup> polystyrene, carboxylated magnetic microsphere beads (Luminex Corporation, Texas, USA) using the method described in the commercial kit. Briefly, 500  $\mu$ l of beads at a concentration of  $1.25 \times 10^7$  beads/ml were placed into a low protein-binding microfuge tube (USA Scientific, Inc., Florida, USA). For all subsequent procedures, the beads were protected from prolonged exposure to light. The beads were removed from suspension by attachment to a magnetic separator and washed in 100  $\mu$ l of sterile water followed by gentle vortexing and sonication for 20 sec to disperse any aggregated beads. The beads were magnetically separated and re-suspended in 80  $\mu$ l of activation buffer (0.1 M sodium dihydrogen phosphate pH 6.2). After vortexing and sonication, 10  $\mu$ l of 50 mg/ml Sulfo-NHS (N-Hydroxysulfosuccinimide), and 10  $\mu$ l of 50 mg/ml 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, EDC (Thermo Scientific) were added. Beads were incubated for 20 min at room temperature. The activated beads were magnetically separated and washed twice with 250  $\mu$ l of PBS, re-suspended in 100  $\mu$ l coupling buffer followed by the addition of 25  $\mu$ g of recombinant protein. The final volume was brought up to 500  $\mu$ l in PBS and the reaction mixture gently rotated for two hours at room temperature. The coupled beads were washed three times in PBS with 0.05% Tween-20 and 0.05% w/v sodium azide (PBST), and re-suspended in 1 ml of PBST with 1% fish gelatin (Sigma-Aldrich) and stored at 4°C in the dark until use.

### 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  $\mu$ l of PBS with 4% goat serum (PBS-GS) and 50  $\mu$ l serum samples or serial 2-fold diluted penta-His<sup>™</sup> mouse monoclonal IgG (Qiagen GmbH, California, USA) were placed in each well of a Corning<sup>™</sup>Costar<sup>™</sup> 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  $\mu$ l PBS-GS. Biotin labeled goat anti-swine IgM ( $\mu$ ) (KPL, Maryland, USA), Goat anti-pig IgA H&L (Biotin), (Abcam Inc., Massachusetts, USA), biotin-SP-conjugated goat anti-swine IgG (2  $\mu$ g/ml) (Jackson Immuno Research Laboratories, Inc., Pennsylvania, USA), and biotinylated anti-mouse IgG (H+L) (2  $\mu$ g/ml) (Vector Laboratories, Inc., California, USA) were diluted in PBS-GS. Beads were re-suspended in 50  $\mu$ l of diluted conjugates. After 30 min of incubation, wells were washed and 50  $\mu$ l 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  $\mu$ l 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  $S/P = (\text{Mean MFI of test sample} - \text{Mean MFI of negative control}) / (\text{Mean MFI of positive control} - \text{Mean MFI of negative control})$

## 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

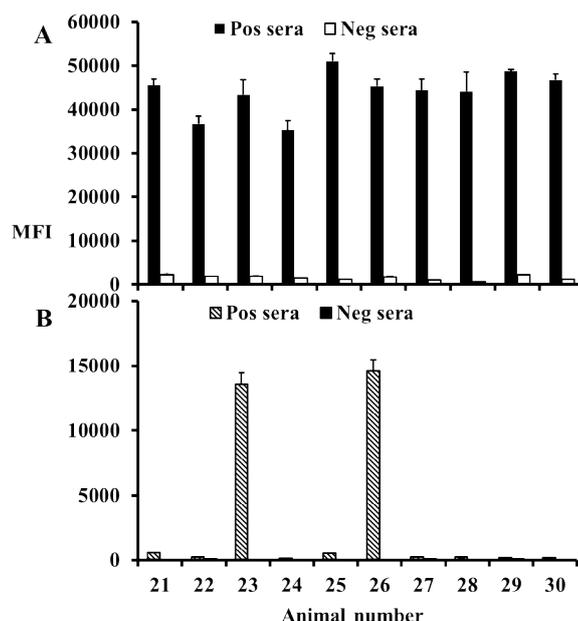
collected at 0, 7, 14, 21, 28, 35, 42, 49, and 57 days post-immunization. IgM response against CSFV E2 (aa 1-181) was determined by FMIA and the reactivity of antibody was followed over time, as shown in Figure 5. CSFV E2 antigen specific IgM was detected in 3 out of 10 animals at 7 days post immunization (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 remained 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

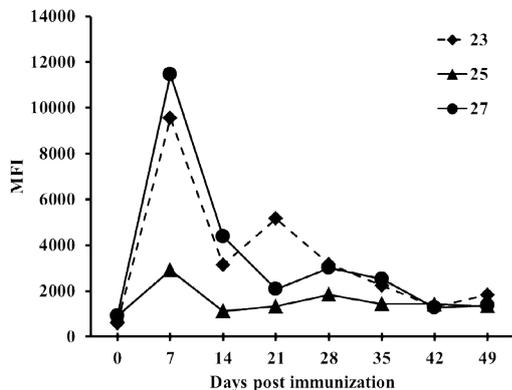
For the detection of CSFV E2 (aa 1-181) specific IgA, IgG, and IgM, a FMIA was performed using CSFV E2 (aa 1-181) in the presence of serum and oral fluids then antibody responses were followed over the study period (Figure 6). CSFV IgG antibodies were highly reactive to E2 antigen compared to IgA and 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

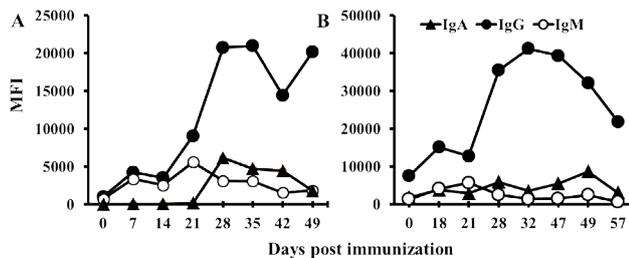
The production of antibodies following immunization is important to suppress viral infections. The aim of this study was to detect changes in the serum antibody responses following vaccination against CSFV with an alphavirus expressed E2 subunit vaccine. E2 and Erns are known to induce virus-neutralizing 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



**Figure 4:** Detection of IgG (A) and IgA (B) in CSFV-E2 alpha virus replicon particles vaccinated pigs. FMIA was performed in the presence of twelve sera samples (1:100) from immunized animals and CSFV E2 antigen fragment (aa 1-181). Results are reported as mean fluorescent intensity (MFI) and standard errors.



**Figure 5:** Kinetics of CSFV-E2-specific IgM in alpha virus replicon particle immunized pigs. FMIA was performed in the presence of three sera samples (1:200) and CSFV E2 antigen fragment (aa 1-181). Data represent mean fluorescent intensity (MFI) and  $\pm$  standard deviation of triplicate determinations.



**Figure 6:** Kinetics of CSFV E2 antibody isotypes (IgA, IgG and IgM) in serum (A) and oral fluids (B). CSFV E2-specific antibodies were detected using E2 fragment (aa 1-181) in FMIA. Results are reported as mean fluorescent intensity (MFI) and standard errors.

specific IgA, IgG, and IgM antibodies response in swine vaccinated with alphavirus expressed antigen. Of the eight E2 protein fragments evaluated in this study, the highest MFI values were obtained for E2 (aa 1-181) (Figure 3). In representative positive samples after immunization with CSFV E2 antigen, anti-CSFV E2 antibody responses were detected in all animals and E2 (aa 1-181) was observed to showed the highest IgG response. Based on these results, E2 (aa 1-181) was identified as a superior target for these studies. It has been reported that the E2 protein possesses an immunogenic domain located in the region about 120 residues [16].

ELISA-based test for the detection of antibodies against CSFV E2 have been previously reported [16-18]. However, limited research has been reported on the detection of IgM antibody responses against CSFV E2. Our results demonstrated that IgM antibody response was detected 7 days after vaccination with alphavirus expressed antigen (Figure 5). The data also suggested that seroconversion of IgM is 2 weeks faster than IgG. This conclusion is supported by previous work by others using an indirect ELISA test for the detection of IgG and IgM antibody responses after vaccination; IgM was detected one week earlier than IgG [18]. Compared to previously reported CSFV ELISA results, FMIA based detection of CSFV-specific IgM might potentially be a rapid and useful tool for determination of immune status of animals after vaccination.

CSFV E2 specific IgA, IgG, and IgM were detected in oral fluid samples collected from a group of pigs (pens) under experimental

conditions. Secretory IgA (SIgA) produced by plasma cells in the salivary glands and duct-associated lymphoid tissue (DALT) are the largest proportion of locally produced antibody [19]. The CSFV antibody isotype (IgA, IgG, and IgM) responses in serum and oral fluid samples are shown in Figure 6. The purpose of this study was to contrast the kinetics of CSFV E2-specific antibody in oral fluids and serum after vaccination with alphavirus replicon particles. 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

1. Simmonds P, Becher P, Collett MS, Gould EA, Heinz FX, et al. (2012) Family flaviviridae. In: *Virus taxonomy*. Ninth report of the International committee on taxonomy of viruses. Elsevier, San Diego.
2. Edwards S, Fukusho A, Lefevre PC, Lipowski A, Pejsak Z, et al. (2000) Classical swine fever: The global situation. *Vet Microbiol* 73: 103-119.
3. Postel A, Schmeiser S, Oguzoglu TC, Indenbirken D, Alawi M, et al. (2015) Close relationship of ruminant pestiviruses and classical swine fever virus. *Emerg Infect Dis* 21: 668-672.
4. Greiser-Wilke I, Blome S, Moennig V (2007) Diagnostic methods for detection of classical swine fever virus-status quo and new developments. *Vaccine* 25: 5524-5530.
5. Blome S, Meindl-Böhmer A, Loeffen W, Thuer B, Moennig V (2006) Assessment of classical swine fever diagnostics and vaccine performance. *Rev Sci Tech* 25:1025-1038.
6. Kumar R, Barman NN, Khatoon E, Kumar S (2016) Development of single dilution immunoassay to detect E2 protein specific classical swine fever virus antibody. *Vet Immunol Immunopathol* 172: 50-54.
7. Moser C, Ruggli N, Tratschin JD, Hofmann MA (1996) Detection of antibodies against classical swine fever virus in swine sera by indirect ELISA using recombinant envelope glycoprotein E2. *Vet Microbiol* 51: 41-53.
8. Dewulf J, Koenen F, Mintiens K, Denis P, Ribbens S, et al. (2004) Analytical performance of several classical swine fever laboratory diagnostic techniques on live animals for detection of infection. *J Virol Methods* 119:137-143.
9. Hossain MM, Wilson WC, Faburay B, Richt J, McVey DS, et al. (2016) Multiplex detection of IgG and IgM to Rift Valley fever virus nucleoprotein, nonstructural proteins and glycoprotein in ovine and bovine. *Vector Borne Zoonotic Dis* 16: 550-557.
10. Hossain MM, Rowland RR (2017) Fluorescence microsphere immunoassay for detection of antibodies to porcine reproductive and respiratory syndrome virus and porcine circovirus type 2 using protein A, protein G, and protein A/G. *J Immunol Tech Infect Dis* 6: 1-6.
11. Xia H, Harimoorthy R, Vijayaraghavan B, Blome S, Widén F, et al. (2015) Differentiation of classical swine fever virus infection from CP7\_E2alf marker vaccination by a multiplex microsphere immunoassay. *Clin Vaccine Immunol* 22: 65-71.

12. Hossain MM, Rowland RR (2016) Multiplex detection of classical swine fever virus (CSFV) specific IgG, IgM and IgA antibodies in swine immunized with alphavirus expressed antigens. *J Antivir Antiretrovir* 8: 55.
13. Bosworth B, Erdman MM, Stine DL, Harris I, Irwin C, et al. (2010) Replicon particle vaccine protects swine against influenza. *Comp Immunol Microbiol Infect Dis* 33: e99-e103.
14. Catanzariti AM, Soboleva TA, Jans DA, Board PG, Baker RT (2004) An efficient system for high-level expression and easy purification of authentic recombinant proteins. *Protein Sci* 13:1331-1339.
15. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.
16. Lin M, Trottier E, Mallory M (2005) Enzyme-linked immunosorbent assay based on a chimeric antigen bearing antigenic regions of structural proteins Erns and E2 for serodiagnosis of classical swine fever virus infection. *Clin Diagn Lab Immunol* 12: 877-881.
17. Sakoda Y, Wakamoto H, Tamura T, Nomura T, Naito M, et al. (2012) Development and evaluation of indirect enzyme-linked immunosorbent assay for a screening test to detect antibodies against classical swine fever virus. *Jpn J Vet Res* 60: 85-94.
18. Li W, Mao L, Yang L, Zhou B, Jiang J (2013) Development and partial validation of a recombinant E2 based indirect ELISA for detection of specific IgM antibody responses against classical swine fever virus. *J Virol Methods* 191: 63-68.
19. Brandtzaeg P (2007) Do salivary antibodies reliably reflect both mucosal and systemic immunity? *Ann N Y Acad Sci* 1098: 288-311.
20. Kittawornrat A, Engle M, Panyasing Y, Olsen C, Schwartz K, et al. (2013) Kinetics of the porcine reproductive and respiratory syndrome virus (PRRSV) humoral immune response in swine serum and oral fluids collected from individual boars. *BMC Vet Res* 9: 61.
21. Challacombe SJ, Russell MW, Hawkes J (1978) Passage of intact IgG from plasma to the oral cavity via crevicular fluid. *Clin Exp Immunol* 34: 417-422.