RESEARCH REPORT
SWINE HEALTH

Title: Development and validation of Singleplex and Multiplex Luminex Assays for Detection of antibodies to foot-and-mouth disease (FMD), swine vesicular disease (SVD), classical swine fever (CSF) and African swine fever (ASF) viruses in porcine oral fluids – NPB# 15-177

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Industry Summary:
Swine oral fluids (OF) are increasingly being used for the diagnosis of diseases in pigs. We have previously shown (NPB# 14-286) that the genome and antibodies to viruses that infect pigs can be measured in OF. The detection of antibodies was mainly by enzyme-linked immunosorbent assay (ELISA), which might be less sensitive for OF than for serum. Luminex assays could potentially improve this sensitivity. The objective of this project was to develop and validate Luminex assays for detecting antibodies to FMDV, CSFV, ASFV and SVDV in OF. Specific objectives were to generate swine OF for test method development and validation through experimental inoculations of pigs with FMDV, SVDV, CSFV and ASFV; obtain samples from other members of the FAD Oral Fluid Consortium and laboratories in endemic countries; develop individual Luminex assays for detection of antibodies to FMDV, SVDV, CSFV and ASFV in swine OF; validate and compare method for antibody detection for the 4 viruses in OF and serum by singleplex and multiplex Luminex assays.

Groups of pigs were either directly inoculated intradermally in the heel bulb of one hind limb with cell culture supernatants containing FMDV or were inoculated by contact with the directly inoculated pigs. For SVDV, each pig was inoculated intradermally in the heel bulb of one hind limb as well as through the mouth and the nares with cell culture supernatants containing SVDV (4 groups of 4pigs /group). CSFV and ASFV inoculations were performed by administering virus to each animal through the nares and the mouth. Oral fluids were collected from each group of pigs using cotton ropes. Serum and swabs of the mouth and nares were also collected from individual animals.

Luminex assays were developed using recombinant 3ABC (FMDV), 3D (SVDV), E^{rns} (CSFV) and p54 (ASFV) antigens produced in the baculovirus expression system. For multiplexing, the haemorrhagic diseases (ASF and CSF) and the vesicular diseases (FMD and SVD) were combined into duplex assays. At least 391 Samples from naïve animals as well as oral fluids from experimentally infected animals were tested to establish the specificity and sensitivity respectively for each assay. Oral fluids were also tested by previously optimized ELISAs to confirm the presence of relevant antibodies.

Singleplex Luminex assays for the detection of antibodies to FMDV, SVDV, ASFV and CSFV in OF were developed and partially validated. Positive antibody detection for FMDV started as early as 4 days post infection (DPI). Positive antibody responses to SVDV 3D started at 14 DPI. Similarly positive antibody detection for ASFV and CSFV started at 10 and 14 DPI respectively. Comparable results were obtained in the duplex and singleplex assays. These results also mirrored those for sera from corresponding groups of animals. The data demonstrates that Luminex assays can be used for the detection of antibodies to FMDV, SVDV, CSFV or ASFV in OF. There is therefore a high potential for the use of OF for FMDV, SVDV, CSFV or ASFV surveillance using both Luminex assays and ELISAs.

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Keywords:
Swine, Oral fluids, foreign animal diseases, antibody detection, Luminex, enzyme-linked immunosorbent assays

Scientific Abstract:
Swine oral fluids (OF) are increasingly being used for the diagnosis of diseases in pigs. We have previously shown (NPB# 14-286) that FMDV, SVDV, CSFV and ASFV nucleic acids and antibodies can be measured in OF. Antibodies were detected by enzyme-linked immunosorbent assay (ELISA), which might be of less sensitivitiviy for OF than for serum. Luminex assays are potentially more sensitivity and have multiplexing capability. The objective of this project was to develop and validate singleplex and multiplex Luminex assays for detecting antibodies to FMDV, CSFV, ASFV and SVDV in OF. Specific objectives were to generate swine OF for test method development and validation through experimental inoculations of pigs with FMDV, SVDV, CSFV and ASFV; obtain samples from other members of the FAD Oral Fluid Consortium and laboratories in endemic countries; develop individual Luminex assays for detection of antibodies to FMDV, SVDV, CSFV and ASFV in swine OF; validate and compare methods for antibody detection for the 4 viruses in OF and serum by singleplex and multiplex Luminex assays.

Groups of pigs were either directly inoculated intradermally in the heel bulb of one hind limb with cell culture supernatants containing FMDV or were inoculated by contact with the directly inoculated pigs. For SVDV, each pig was inoculated intradermally in the heel bulb of one hind limb and oronasally with cell culture supernatants containing SVDV (4 groups of 4pigs /group). CSFV and ASFV inoculations were performed by administering virus oronasally to each animal. Oral fluids were collected from each group of pigs using cotton ropes. Serum and swabs of the mouth and nares were also collected from individual animals.

Luminex assays were developed using recombinant 3ABC (FMDV), 3D (SVDV), E^{rns} (CSFV) and p54 (ASFV) antigens produced in the baculovirus expression system. Optimal amounts of antigen were coupled to fluorescent beads. The antigen-coupled beads were mixed with equal volume of OF in predetermined wells. Wells of negative and positive control sera were included on each plate. Plates were incubated in the dark at room temperature with shaking for 2 hours, washed 3 times with PBS (no tween)

and a cocktail of biotinylated goat anti-swine IgA, IgM and IgG at 1/400 in StabilGuard® Immunoassay Stabilizer (BSA-Free**,** Surmodics IVD, Inc., USA**)** was added to each well at 50 uL/well. Plates were further incubated for 30 min, washed, streptavidin- phycoerythrin diluted 1/100 in StabilGuard was added to all wells at 50 uL/well and plate incubated for 15 minutes followed by a final wash. Beads were resuspended in 150 uL of PBS and analyzed on the MAGPIX® instrument. Median fluorescence intensity (MFI) for each sample was recorded and the data expressed as a percentage of the ratio of the MFI for the test sample (S) to the MFI of the positive (serum) control (P) ie %S/P = MFI S/MFI P X 100.
For multiplexing, the haemorrhagic diseases (ASF and CSF) and the vesicular diseases (FMD and SVD) were combined into duplex assays. At least 391 samples from naïve animals as well as oral fluids from experimentally infected animals were tested to establish the specificity and sensitivity respectively for each assay. Oral fluids were also tested by ELISAs optimized for OF (Senthilkumaran et al, 2016, 2017; NPB #14-286 report) to confirm the presence of relevant antibodies.
Singleplex Luminex assays for the detection of antibodies to FMDV, SVDV, ASFV and CSFV in OF were successfully developed and partially validated. Positive antibody detection for FMDV 3ABC started as early as 4 DPI. Positive antibody responses to SVDV 3D started at 14 DPI. Similarly positive antibody detection for ASFV and CSFV started at 10 and 14 DPI respectively. Comparable results were obtained in the duplex and singleplex assays. These results also mirrored those for corresponding sera from groups of pigs. The data demonstrates that Luminex assays can be used for the detection of antibodies to FMDV, SVDV, CSFV or ASFV in OF. There is therefore a high potential for the use of OF for FMDV, SVDV, CSFV or ASFV surveillance using both Luminex assays and ELISAs.

Introduction:

Foot-and-mouth disease (FMD) caused by FMDV affects cloven-hoofed animals (Alexandersen et al, 2003). Swine vesicular disease (SVD) caused by the SVDV is known to affect pigs only (Lin and Kitching, 2000). The clinical signs and lesions seen in swine due to FMD and SVD are indistinguishable. Consequently, laboratory tests are an essential way to distinguish between the infections caused by these viruses.

ASFV typically causes an acute haemorrhagic disease in pigs with mortality approaching 100%, although some strains have been associated with milder chronic forms of the disease or nonapparent clinical signs (Penrith et al, 2013). CSFV also causes a haemorrhagic disease in swine with a similar clinical range as ASFV (Penrith et al, 2011). Therefore, timely laboratory tests are required to diagnose infections due to these viruses and/or distinguish them from viruses causing similar clinical manifestations.

Disease surveillance in populations is commonly done by using individual samples. In most cases, such as diseases with low prevalence, a high number of samples could be required. Additionally, collection of individual samples requires restraining of animals and use of special equipment. These factors render this approach to disease surveillance costly. Furthermore, the handling and blood collection from animals may pose a risk of further spreading a disease. A more cost-effective approach to disease surveillance is the use of OF samples in the assessment of group/herd health status (Mur et al, 2013). Oral fluid collection requires significantly less invasive methods rendering this easy for almost anyone to perform in the field. Therefore, this is becoming a more attractive sampling option for most swine production systems.

Oral fluid samples have been used to detect porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV2) and influenza A viruses in pigs (Prickett and Zimmerman, 2010; Prickett et al, 2008; Romagosaet al, 2012). Similarly, OF have been used to diagnose FMD and ASF in pigs (Mur et al, 2013; Vosloo et al, 2013). However, assays based on OF haven't been developed or fully validated for routine surveillance of most exotic swine diseases. Development and/or validation of OF-based assays for FADs are therefore critical to ensure potential use of this sample type for FAD diagnostic assays.

ELISAs have been adapted for detection of antibodies in OF (Mur et al, 2013). However, these assays are of low sensitivity because antibody levels in OF are lower than in sera. Consequently, assays with greater sensitivity are required for efficient detection of antibodies in OF. Luminex assays are rapid, simple, convenient, more sensitive, and multiplexing allows them to be cost effective and less labor intensive. The assays utilize small volumes of samples. Langenhorst et al (2012), successfully used a fluorescent microsphere immunoassay (FMIA) to detect anti-PRRSV antibodies in OF. The aim of this project was to develop singleplex assays for detection of antibodies to FMDV, SVDV, CSFV and ASFV in swine oral fluids. Once the singleplex assays were validated, we combined FMDV and SVDV; and CSFV and ASFV into duplex formats.

Objectives:

A. Generate swine OF for test method development and validation through experimental inoculations of pigs with FMDV, SVDV, CSFV and ASFV; obtain samples from other members of the FAD Oral Fluid Consortium and laboratories in endemic countries.
B. Develop individual Luminex assays for detection of antibodies to FMDV, SVDV, CSFV and ASFV in swine OF.
C. Validation/methods comparison studies for antibody detection for the 4 viruses in OF and serum by Luminex assays.
D. Develop a multiplex format of the Luminex assays for simultaneous detection of antibodies to FMDV, SVDV, CSFV and ASFV in swine OF.
E. Validation/methods comparison of the multiplex Luminex assay on swine OF and serum.

Materials & Methods:

Animals:

Pigs aged 6 to 7 weeks were obtained from a local supplier. These animals were examined upon arrival and moved into cubicles. At least 4 cubicles with a minimum of 4 pigs/cubicle were used for each virus studied. Food and water were provided *ad libitum* and the pigs allowed a minimum of 7 days to acclimatise to their new surroundings before virus inoculation. The animals were visually monitored and their rectal temperatures measured daily.

Viruses:

The viruses used in experimental inoculations in this study were FMDV (serotypes O, A and Asia 1 Shamir), ASFV (Malta '78 strain), CSFV (Diepholz, genotype 2.3) and SVDV (UKG 27/72 strain). These were produced in appropriate cell cultures, passage numbers kept as low as possible and virus titres determined in corresponding cell cultures.

Animal inoculation and sampling:

Animal inoculation and sampling was previously described in the final report for NPB #14-286. The same procedure was followed for additional inoculations in this report. Briefly, pigs were inoculated intradermally in the heel bulb of the left hind limb with cell culture supernatants containing 10^5 TCID50 (50% tissue culture infectious dose) for FMDV. Similarly, for SVDV, pigs were inoculated intradermally in the heel bulb of the left hind limb as well as oronasally with cell culture supernatants containing 10^7 TCID50 of virus. For ASFV and CSFV, pigs were inoculated oronasally with 10^6 HAD50 or 10^6 TCID50 of virus respectively.

Sampling was done daily for the first 7-10 days post inoculation (DPI) and then once every week until the end of the experiment (DPI 28). Blood for serum and/or plasma were collected from the anterior vena cava using a 20 gauge needle. Nasal and oral swabs were taken with Dacron tipped swabs.

Collection of OF samples was performed as previously described (Prickett et al, 2008). Briefly, a

5/8-inch cotton ropes were suspended at shoulder height within the cubicle for 20 to 30 minutes. Since pigs are naturally attracted to the rope, they would chew on the rope, depositing OFs in the process. Oral fluids will then be obtained by wringing the moistened rope in a zip lock plastic bag and cutting one corner of the bag to drain the fluid into a 50-mL falcon tube. Samples will then be spun to separate any debris and aliquots were used for virus and antibody detection. All samples were stored at -70˚C.

PCR assay:

The MagMax™ Viral RNA Isolation kit (Ambion) were used for RNA extraction from 55 µl of each sample according to manufacturer's protocol [Manual: MagMax-96 Viral RNA Isolation Kit (AM1836), Version: 2013-October-08]. The MagMAX™ Express-96 Instrument were used for purification using a Deep Well Magnetic Particle Processor. The RNA was eluted into 90µl of Elution buffer. DNA (for ASFV) was extracted using the QIAamp® DNA Mini Kit (Qiagen) according to manufacturer's protocol.

FMDV and SVDV RNA detection by rRT-PCR were carried out according to published protocols (Moniwa et al, 2007; Senthlikumaran et al, 2016, 2017).

The rRT-PCR for detection of CSFV RNA was performed using QuantiTect Probe RT-PCR Kit (Qiagen) following a previously published protocol (Araigna et al, 2010). Similarly real-time PCR for the detection of ASFV DNA was carried out using previously described primers, probes and protocol (Tignon et, 2011).

Development of the Luminex assays:

Luminex assays were developed using recombinant 3ABC (FMDV), 3D (SVDV), E^{rns} (CSFV) and p54 (ASFV) antigens. These antigens were produced in the baculovirus expression system. Codon optimized genes for each antigen were cloned into the AB baculovirus transfer vector, followed by co-transfection with linearized baculovirus DNA into *Spodofera frugiperda* (Sf-9) cells (AB Vector, California, USA). Expression of individual protein in the transfected cells was monitored for up to 120 hrs. Amplification of baculovirus, infection of Sf-9 cells for protein expression and recovery of recombinant proteins from cell pellets were performed as previously described (Pinette et al, 2014) and purified by batch procedure using Ni-NTA agarose according to manufacturer's protocol (Qiagen, Maryland, USA). Protein expression was confirmed by Western blot using anti Histag antibody showing recombinant proteins of the expected size.

The Luminex assay for FMDV, SVDV, CSFV and ASFV were initially validated using known positive and negative serum samples in singleplex formats. Similarly, OF were tested by previously optimized ELISAs (Senthilkumaran et al, 2016, 2017; NPB #14-286 report) to confirm the presence of relevant antibodies. The optimized conditions for Luminex for serum from FMDV, SVDV, CSFV and ASFV-infected animals were then adapted for use with oral fluids.

The optimal amount of purified protein was used for bead coupling for FMDV 3ABC (Bead region 44), SVDV 3D (Bead region 47), CSFV E^{rns} (Bead region 36) and ASFV p54 (bead region 54). Beads were coupled according to manufacturer's recommendations.

For oral fluids, 25 µL of coupled beads (2000 beads) in StabilGuard were dispensed per well of 96-well Luminex plate followed by 25 µL of OF (undiluted) to predetermined wells. Duplicate wells of negative and positive control sera at 1/2500 for FMDV and SVDV, 1/4000 for CSFV and 1/400 for ASFV were included on each plate. Plates were incubated in the dark at room temperature with shaking for 2 hours, washed 3 times with PBS (no tween) using a preprogrammed BioRad plate washer with magnet placed under the plate to avoid losing beads. A cocktail of biotinylated goat anti-swine IgA, IgM and IgG at 1/400 in StabilGuard was added to each well at 50uL/well. Plates were further incubated in the dark at room temperature with shaking for 30min and washed as above. Streptavidin- phycoerythrin diluted 1/100 in StabilGuard was added to all wells at 50uL/well and plate incubated in the dark at room temperature for 15 minutes with shaking followed by a final wash as described above. Beads were resuspended in 150 uL of PBS and analyzed on the MagPlex Luminex machine. Median fluorescence intensity (MFI) for each

sample was recorded and the data expressed as a percentage of the ratio of the MFI for the test sample (S) to the MFI of the positive (serum) control (P) ie %S/P = MFI S/MFI P X 100.

For sera, 25 µL of coupled beads (2000 beads) in StabilGuard were dispensed per well of 96-well Luminex plate followed by 25 µL of serum (diluted 1/160) in StabilGuard to predetermined wells. Duplicate wells of negative and positive control sera at 1/160 were included on each plate. Plates were incubated in the dark at room temperature with shaking for 1 hour, washed as described for OF and goat anti-swine IgG at 1/1000 in StabilGuard was added to each well at 50 µL/well. The rest of the procedure was as described for OF except streptavidin- phycoerythrin was used at 1/500.

For multiplexing, the haemorrhagic diseases (ASF and CSF) and the vesicular diseases (FMD and SVD) were combined into a duplex assays. For the ASF/CSF duplex, 25 µL volume of beads contained 2000 ASFV and 2000 CSFV antigen coated beads, for a total of 4000 beads. Similarly, the FMD/SVD duplex contained 4000 beads at equal proportions. The rest of the assays were as described above.

Approximately 391 Samples from naïve animals as well as OF from experimentally infected animals were tested to establish the specificity for each assay.

Statistical analysis:

Assay performance by specimen (OF and serum) collected over time post-inoculation were summarized using descriptive statistics.

Results:

Generation of swine oral fluids for test method development and validation through experimental inoculations of pigs with FMDV, SVDV, CSFV and ASFV; obtaining samples from other members of the FAD Oral Fluid Consortium

For each experiment, at least 4 x 1 mL and 1 x 5mL aliquots of OF were collected from each group for most sampling time points. However, during acute disease, pigs were less interested in the ropes and thus lower volumes were obtained. At least one set of samples has been left untouched and stored at -70°C.

Oral, nasal swabs and sera were also aliquoted in duplicates and one set has been preserved at -70°C for future use. Oral fluids from naïve pigs were obtained from farms in Canada to use as negatives for test validation. Additional samples negative for the viruses of interest were obtained from Veterinary Diagnostic Laboratory, Iowa State University.

Development and validation of individual Luminex assays for detection of antibodies to FMDV, SVDV, CSFV and ASFV in swine OF

Luminex assays were successfully developed and optimized for the detection of antibodies to FMDV, SVDV, CSFV and ASFV in swine OF. The optimal amount of protein for bead coupling was determined as 12 µg for FMDV 3ABC, 36 µg for SVDV 3D, 12 µg for CSFV E^{rns} and 6 µg for ASFV p54. These assays detected antibodies in OF sequentially collected from pigs experimentally infected with FMDV (Figure 1 A, B, C), SVDV (Figure 2 A, B), CSFV (Figure 3) and ASFV (Figure 4). 391 negative OF samples were tested to establish the cut off and diagnostic specificity of each assay. The cut off %S/P ratios were 38, 28, 10 and 7 for FMDV, SVDV, CSFV and ASFV respectively. At these cut offs, the specificities of the assays were 98.7%, 93.9%, 97.4% and 99.2% for FMDV, SVDV, CSFV and ASFV respectively.

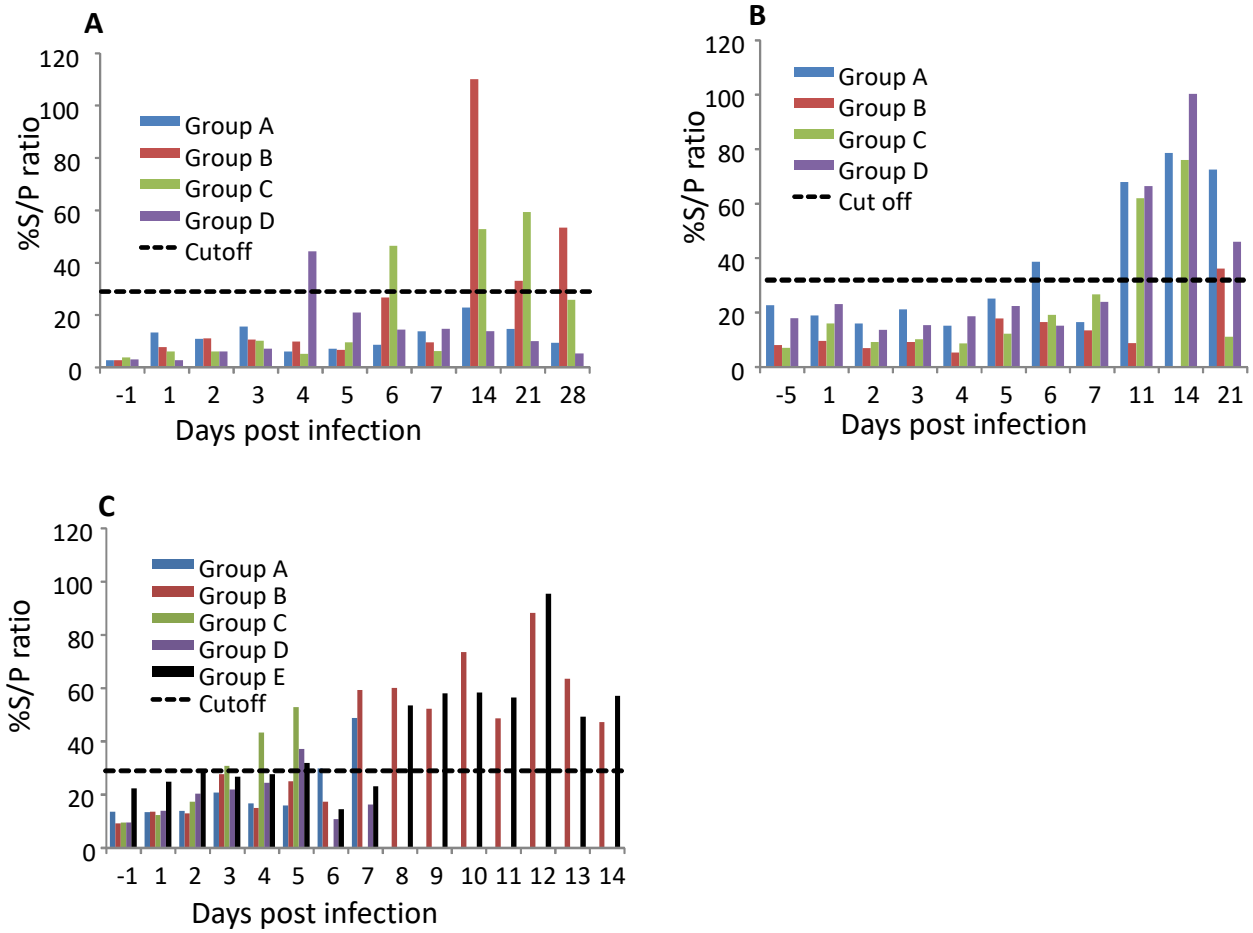

Figure 1: Antibody detection by Luminex assay in oral fluids from FMDV-inoculated pigs. Four groups of 6 pigs per group were housed in separate cubicles. Two pigs per cubicle (8 pigs total) were inoculated intradermally with cell culture supernatants containing 10^5 TCID50 (50% tissue culture infectious dose) of FMDV O UKG/2001 **(A)** or Asia1 Shamir **(B)**in the heel bulb of one hind limb to serve as donors in a contact challenge for the remaining 4 pigs/cubicle. **C:** 5 groups of pigs were vaccinated with FMD vaccine and challenged after 7 or 21 days post vaccination (DPV) with 10^4 TCID50 of FMDV A/TAI/2014 in the heel bulb of the left hind limb: **Group A**: vaccinated with FMD A Malaysia 97 and challenged at 21 DPV; **Group B**: vaccinated with FMD A Iraq 64 and challenged at 21 DPV; **Group C**: vaccinated with FMD A Malaysia 97 and challenged at 7 DPV; **Group D**: vaccinated with FMD A Iraq 64 and challenged at 7 DPV; **Group E**: vaccinated with a combination of FMD A Malaysia 97/ A Iraq 64 and challenged at 21 DPV. Oral fluids were collected from each group and tested for antibodies to FMDV 3ABC by a Luminex assay. Antibody response measured as percent sample to positive ratio (%S/P), Oral fluids with %S/P $\geq$ 38 are considered positive for antibodies to FMDV.

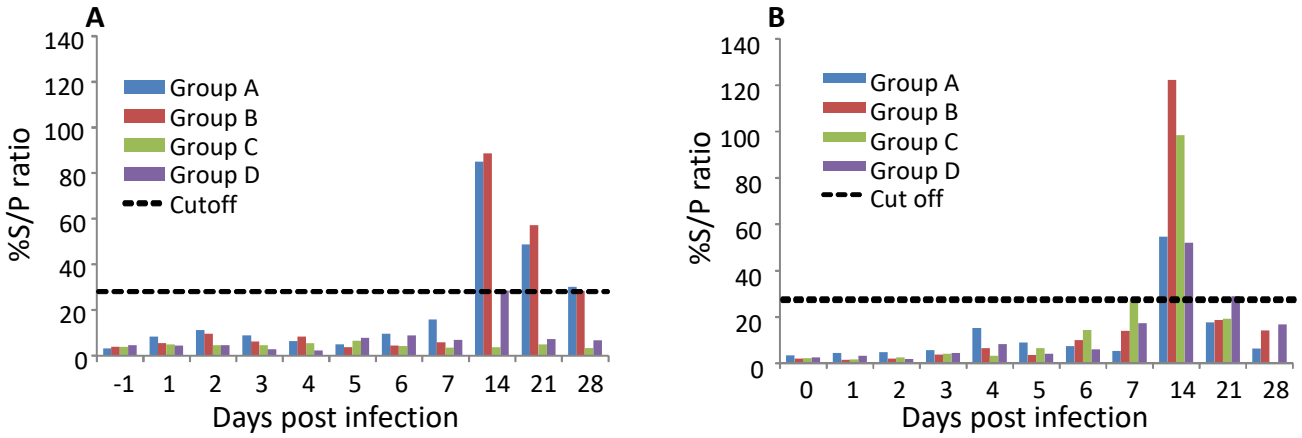

Figure 2: Antibody detection by Luminex assay in oral fluids from SVDV-inoculated pigs. In both **A and B**, 4 groups of 4 pigs per group were housed in separate cubicles. All pigs were inoculated intradermal (200 µL) in the heel bulb of one hind limb, intranasal (800 µL) and

oral (1mL) with a total of 2mL of cell culture supernatants containing a 10^7 TCID50. Oral fluids were collected from each group and tested for antibodies to SVDV 3D by a Luminex assay. Antibody response measured as percent sample to positive ratio (%S/P), Oral fluids with %S/P ≥ 28 are considered positive for antibodies to SVDV.

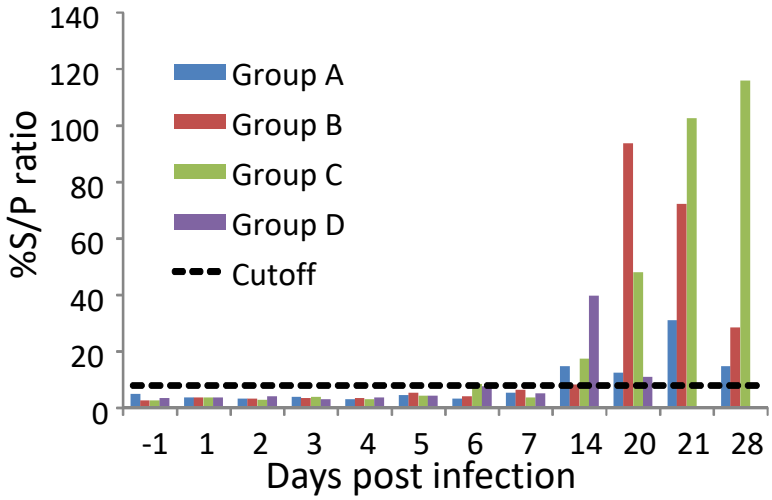

Figure 3: Antibody detection by Luminex assay in oral fluids from CSFV-inoculated pigs. 4 groups of 4 pigs per group were housed in separate cubicles. Each pig was inoculated oronasally with 10^6 TCID50 of CSFV Diepholz, 3 mL/ animal (1 mL per nostril and 1 mL orally). Oral fluids were collected from each group and tested for antibodies to CSFV E^{rns} by a Luminex assay. Antibody response measured as percent sample to positive ratio (%S/P), Oral fluids with %S/P ≥ 10 are considered positive for antibodies to CSFV.

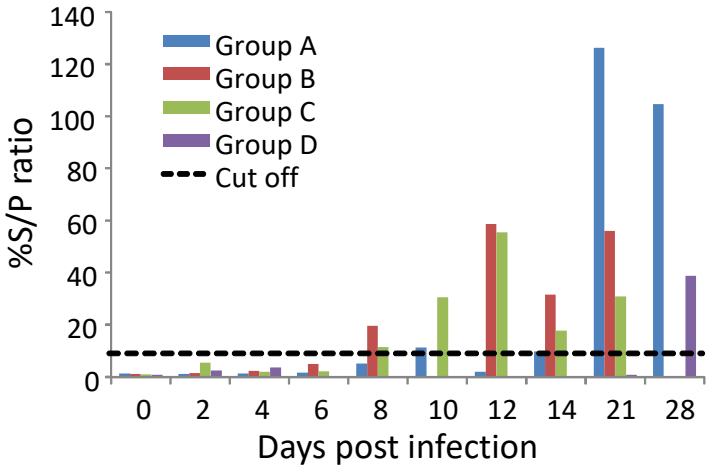

Figure 4: Antibody detection by Luminex assay in oral fluids from ASFV-inoculated pigs. 4 groups of 4 pigs per group were housed in separate cubicles. Each pig was inoculated oronasally with 10^5 HAD50 of ASFV Malta '78, 4 mL/ animal (1 mL per nostril and 2 mL orally). Oral fluids were collected from each group and tested for antibodies to ASFV p54 by a Luminex assay. Antibody response measured as percent sample to positive ratio (%S/P), Oral fluids with %S/P ≥ 7 are considered positive for antibodies to ASFV.

Comparison of antibody detection for the FMDV, SVDV, CSFV and ASFV in oral fluids and serum by Luminex assays

Positive antibody response to FMDV 3ABC was low and transient in OF compared to sera (Figure 5). Group D OF (FMDV O UKG experiment) was positive at 4 DPI. Interestingly, serum from one animal in this group was also positive at 4 DPI, suggesting that a single animal might have accounted for the antibody positivity in OF. At 14 DPI, antibody responses were high in both OF and sera. At 21 - 28 DPI, antibody levels in OF from most groups dropped while sera from all groups remained positive.

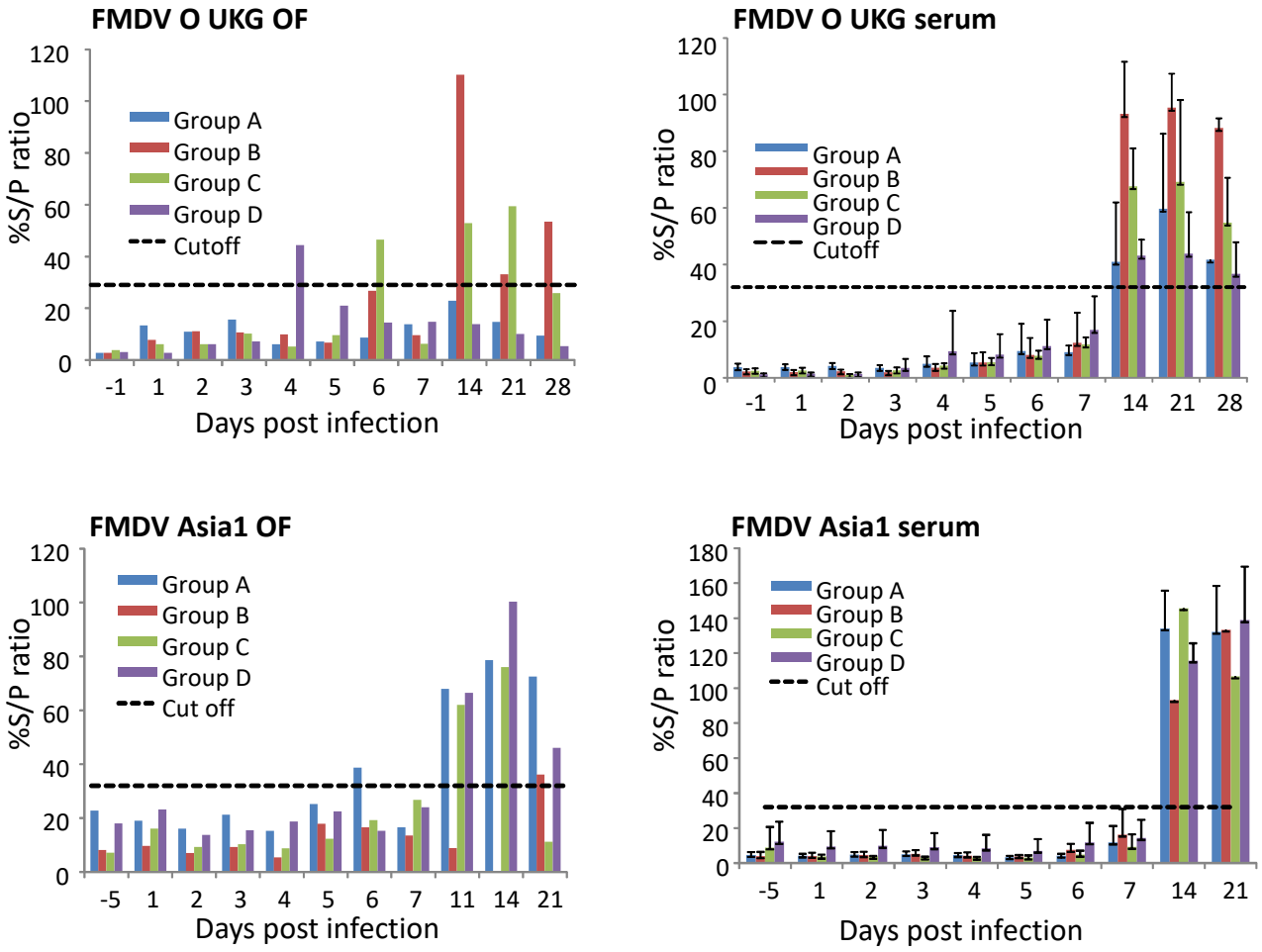

Figure 5: Antibody detection by Luminex assay in oral fluids and sera from FMDV-inoculated pigs. Four groups of 6 pigs per group were housed in separate cubicles. Two pigs per cubicle (8 pigs total) were inoculated intradermally with cell culture supernatants containing 10^5 TCID50 (50% tissue culture infectious dose) of either FMDV O UKG/2001 or Asia1 Shamir in the heel bulb of one hind limb to serve as donors in a contact challenge for the remaining 4 pigs/cubicle. Oral fluids from each group and sera from each pig were collected and tested for antibodies to FMDV 3ABC by a Luminex assay. Antibody response measured as percent sample to positive ratio (%S/P). %S/P $\geq$ 38 and $\geq$ 32 are considered positive for antibodies to FMDV in oral fluids and sera respectively. The histograms for sera represent mean and the error bars represent standard deviation of means. OF: oral fluids

Positive detection of antibodies to SVDV 3D started at 14 dpi in both OF and sera (Figure 6). Groups that lacked antibodies in OF were also negative for sera (group average). Positive response lasted longer in sera than in OF, OF becoming negative at 21 or 28 DPI.

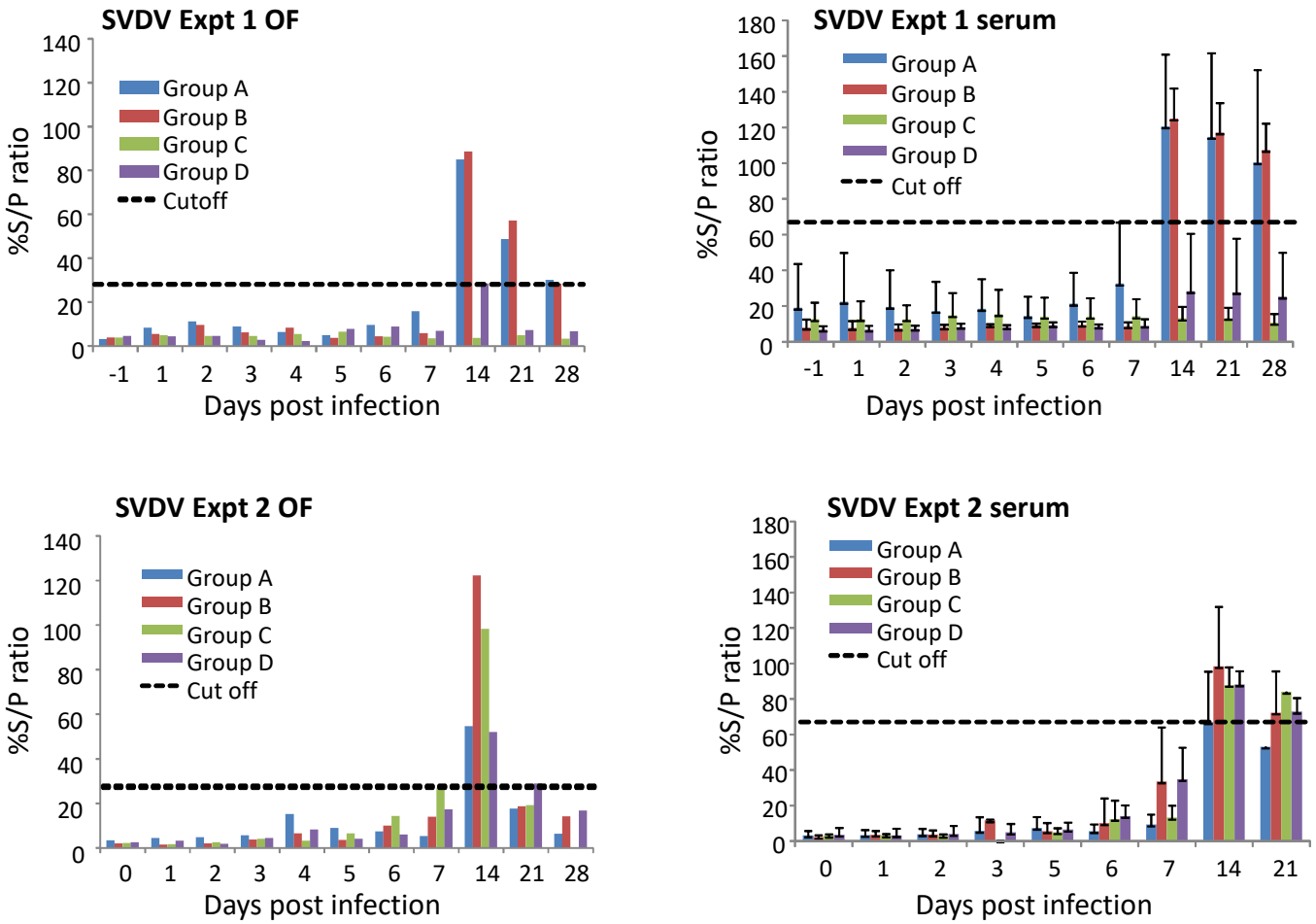

Figure 6: Antibody detection by Luminex assay in oral fluids and sera from SVDV-inoculated pigs. In both **A and B**, 4 groups of 4 pigs per group were housed in separate cubicles. All pigs were inoculated intradermal (200 µL) in the heel bulb of one hind limb, intranasal (800 µL) and oral (1mL) with a total of 2mL of cell culture supernatants containing a 10^7 TCID50. Oral fluids from each group and sera from each pig were collected and tested for antibodies to SVDV 3D by a Luminex assay. Antibody response measured as percent sample to positive ratio (%S/P). %S/P ≥ 28 and ≥ 67 are considered positive for antibodies to SVDV in oral fluids and sera respectively. The histograms for sera represent mean and the error bars represent standard deviation of means. OF: oral fluids

Positive antibody response to CSFV E^{rns} started at 14 dpi in OF and 21 DPI in group average for sera (Figure 7). However, sera from some animals were positive for antibodies to CSFV at 14 DPI. Unlike for SVDV, the magnitude and duration of antibody response to CSFV in OF and sera mirrored each other.

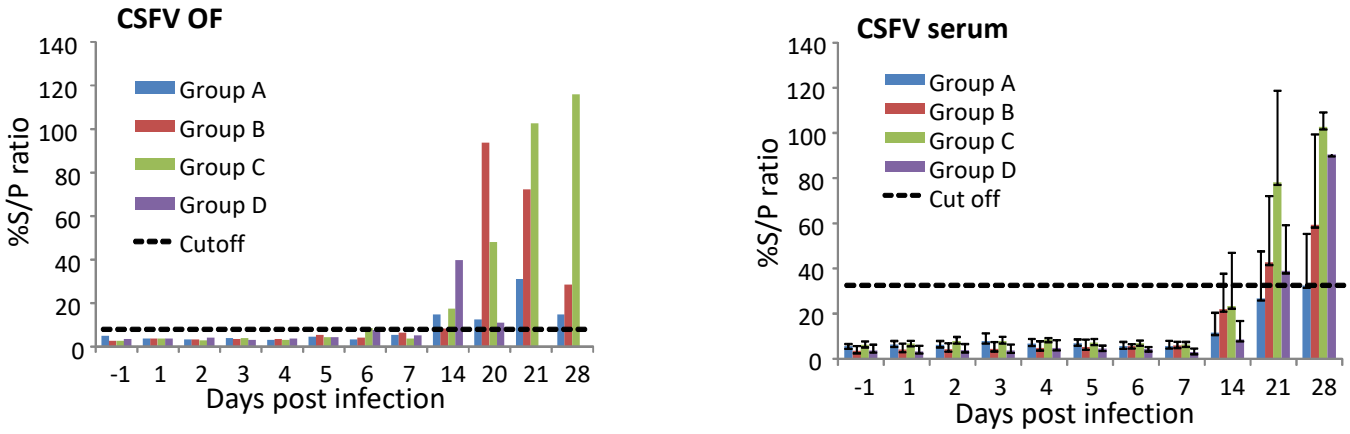

Figure 7: Antibody detection by Luminex assay in oral fluids and sera from CSFV-inoculated pigs. 4 groups of 4 pigs per group were housed in separate cubicles. Each pig was inoculated oronasally with 10^6 TCID50 of CSFV Diepholz, 3 mL/ animal (1 mL per nostril and 1 mL orally). Oral fluids collected from each group and sera from each pig were tested for antibodies to CSFV E^{rns} by a Luminex assay. Antibody response measured as percent sample to positive ratio (%S/P). %S/P ≥ 10 and 32.5 are considered positive for antibodies to CSFV in oral fluids and sera respectively. OF: oral fluids

Positive antibody response to ASFV p54 started at 8 dpi in OF and 12 DPI in group average for sera (Figure 8). Nevertheless, sera from some animals were positive for antibodies to ASFV at 10 DPI. Groups that lacked antibodies in OF were also negative or weak responders for sera (group average). Similar to CSFV, the magnitude and duration of antibody response to ASFV in OF and sera mirrored each other.

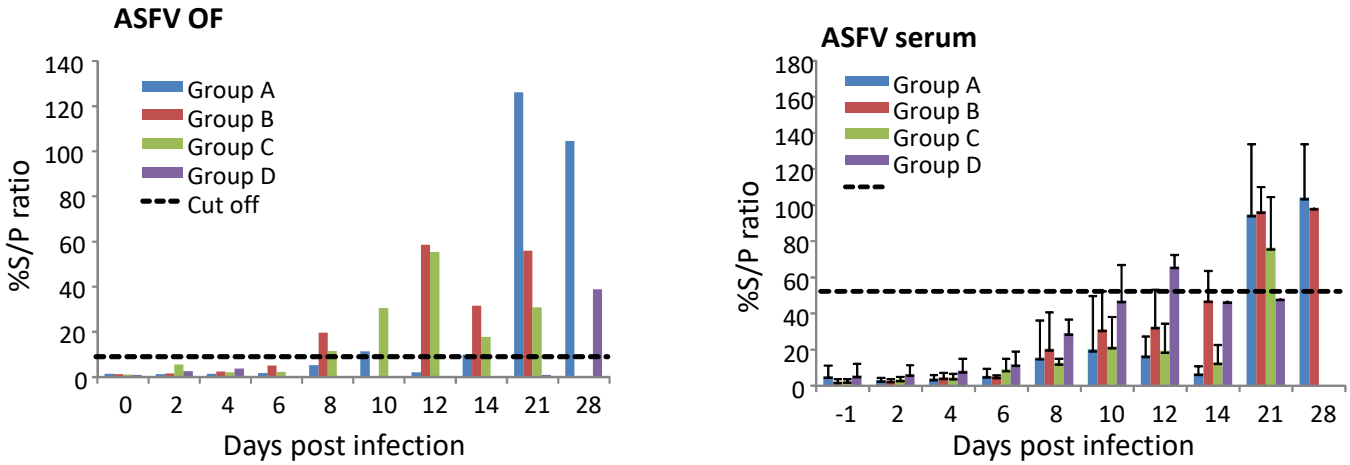

Figure 8: Antibody detection by Luminex assay in oral fluids and sera from ASFV-inoculated pigs. 4 groups of 4 pigs per group were housed in separate cubicles. Each pig was inoculated oronasally with 10^5 HAD50 of ASFV Malta '78, 4 mL/ animal (1 mL per nostril and 2 mL orally). Oral fluids collected from each group and sera from each pig were tested for antibodies to ASFV p54 by a Luminex assay. Antibody response measured as percent sample to positive ratio (%S/P). %S/P ≥ 7 and 52 are considered positive for antibodies to ASFV in oral fluids and sera respectively. OF: oral fluids

Development and validation of multiplex format of the Luminex assays for simultaneous detection of antibodies to FMDV and SVDV; CSFV and ASFV in swine OF

For practical purposes, multiplexing was done by grouping diseases with similar clinical manifestation. Therefore duplex Luminex assays were developed for detection of antibodies to FMDV and SVDV (vesicular disease agents), and CSFV and ASFV (hemorrhagic diseases). The singleplex and duplex assays produced mostly identical results (Figures 9 -12).

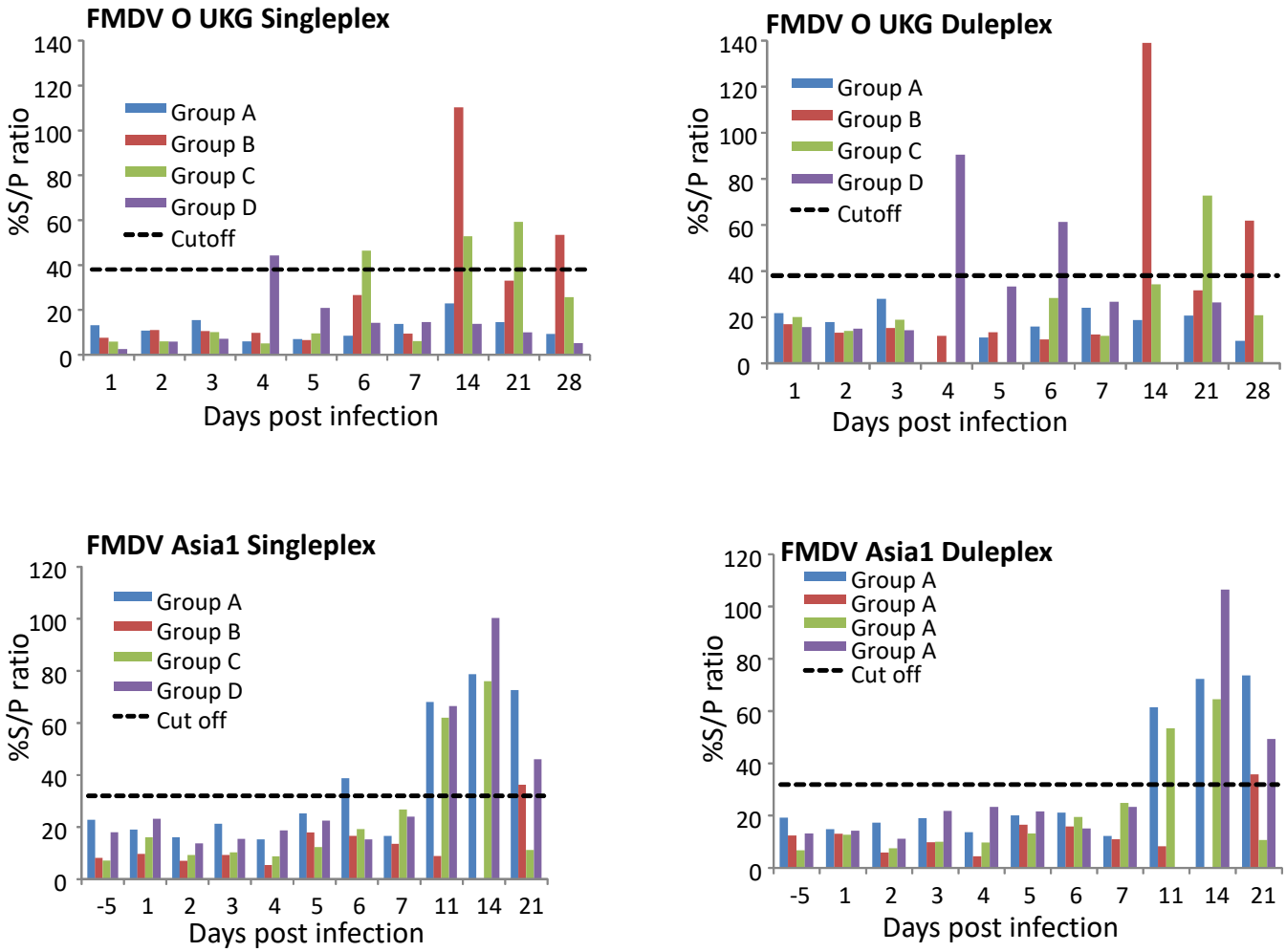

Figure 9-: Antibody detection by singleplex and duplex Luminex assays in oral fluids from FMDV-inoculated pigs. 4 groups of 6 pigs per group were housed in separate cubicles. Two pigs per cubicle (8 pigs total) were inoculated intradermally with cell culture supernatants containing 10^5 TCID50 (50% tissue culture infectious dose) of FMDV O UKG/2001 or Asia1 Shamir in the heel bulb of one hind limb to serve as donors in a contact challenge for the remaining 4 pigs/cubicle. Oral fluids were collected from each group and tested for antibodies to FMDV by a singleplex (FMDV 3ABC only) and duplex (combination of FMDV 3ABC and SVDV 3D) Luminex assays. Antibody response measured as percent sample to positive ratio (%S/P), Oral fluids with %S/P $\geq$ 38 are considered positive for antibodies to FMDV.

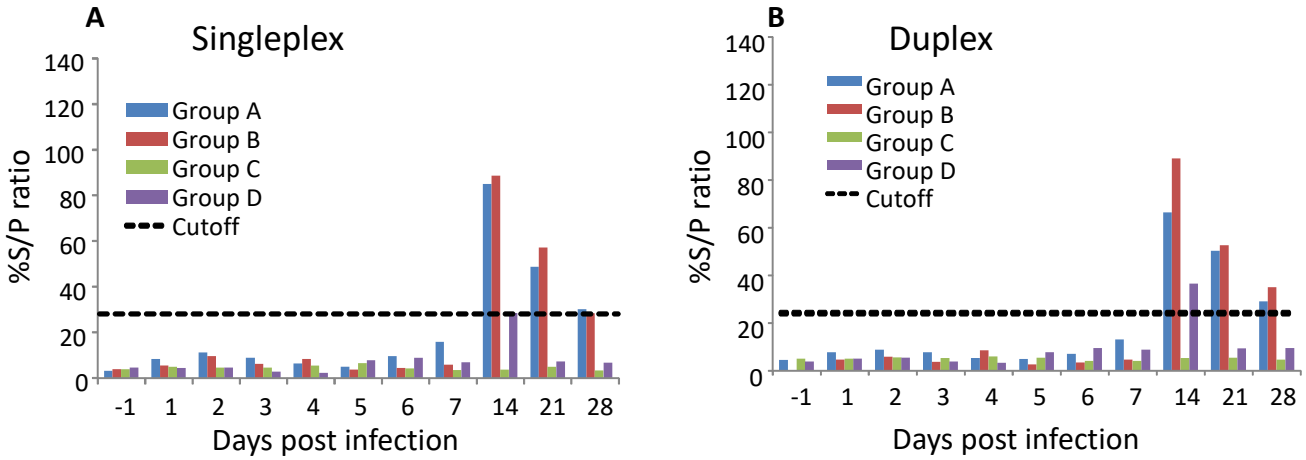

Figure 10: Antibody detection by singleplex and duplex Luminex assays in oral fluids from SVDV-inoculated pigs. Four groups of 4 pigs per group were housed in separate cubicles. All pigs were inoculated intradermal (200 µL) in the heel bulb of one hind limb, intranasal (800 µL) and oral (1mL) with a total of 2mL of cell culture supernatants containing a 10^7 TCID50. Oral fluids were collected from each group and

tested for antibodies to SVDV by a singleplex (SVDV 3D only) and duplex (combination of FMDV 3ABC and SVDV 3D) Luminex assays. Antibody response measured as percent sample to positive ratio (%S/P), Oral fluids with %S/P ≥ 28 are considered positive for antibodies to SVDV.

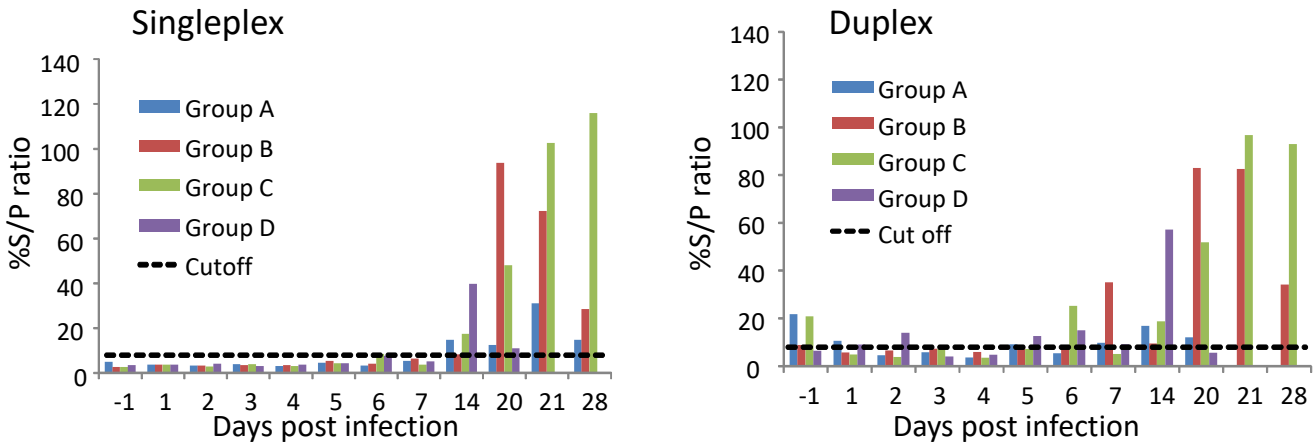

Figure 11: Antibody detection by singleplex and duplex Luminex assays in oral fluids from CSFV-inoculated pigs. 4 groups of 4 pigs per group were housed in separate cubicles. Each pig was inoculated oronasally with 10^6 TCID50 of CSFV Diepholz, 3 mL/ animal (1 mL per nostril and 1 mL orally). Oral fluids were collected from each group and tested for antibodies to CSFV by a singleplex (CSFV E^{rns} only) and duplex (combination of CSFV E^{rns} and ASFV p54). Antibody response measured as percent sample to positive ratio (%S/P), Oral fluids with %S/P ≥ 10 are considered positive for antibodies to CSFV.

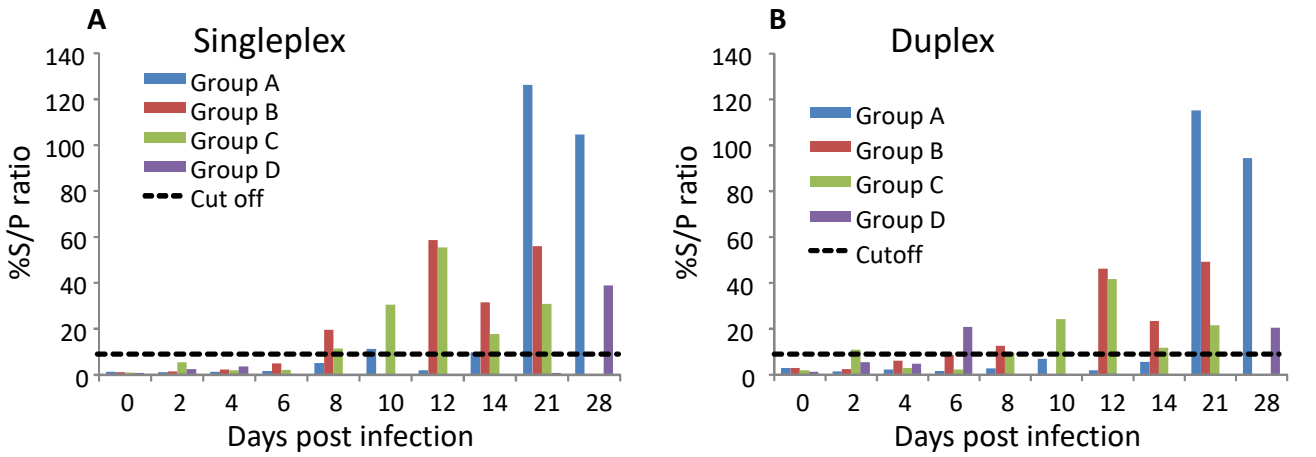

Figure 12: Antibody detection by singleplex and duplex Luminex assays in oral fluids from ASFV-inoculated pigs. 4 groups of 4 pigs per group were housed in separate cubicles. Each pig was inoculated oronasally with 10^5 HAD50 of ASFV Malta '78, 4 mL/ animal (1 mL per nostril and 2 mL orally). Oral fluids were collected from each group and tested for antibodies to ASFV by a singleplex (ASFV p54 only) and duplex (combination of CSFV E^{rns} and ASFV p54). Antibody response measured as percent sample to positive ratio (%S/P), Oral fluids with %S/P ≥ 7 are considered positive for antibodies to ASFV.

Discussion:

Oral fluids provide an opportunity for filling the knowledge gap on mucosal immunity to FMDV, SVDV, CSFV and ASFV in swine. Antibodies in OF can be secreted locally or derived from blood through leakage. Salivary glands and duct-associated lymphoid tissue (DALT) contain plasma cells that secrete IgA into OF. In addition, IgM and IgG are produced in plasma cells located in salivary glands, tonsils, and other DALT (Decorte et al. 2014). We have previously reported (NPB #14-286, Senthilkumaran et al. 2016 and 2017) the detection of antibodies in OF by C-ELISA (SVDV), blocking

ELISA (CSFV) and indirect ELISA (ASFV) as well by isotype ELISAs for IgM (SVDV) and IgA (FMDV and SVDV). ELISAs are supposedly less sensitive for antibody detection in OF because of comparatively lower levels of antibodies than in sera. Therefore Luminex assays which are relatively more rapid, simple, convenient and, perhaps more sensitive, were evaluated for antibody detection in OF. Our data shows antibody detection in OF by Luminex assays. The antibody response trend in OF parallels sera for ASFV and CSFV-infected pigs. Antibody response to FMDV 3ABC and SVDV 3D in OF drops to background levels at either 21 or 28 DPI while persisting in corresponding sera. Both FMDV 3ABC and SVDV 3D are non-structural proteins requiring significant replication of the virus for sufficient antigen to stimulate the immune system. Therefore the transient antibody response may reflect low levels and short duration of antigenic stimulation of the local immune response. Similarly, the late onset of anti-SVDV 3D antibody detection in sera from SVDV infected pigs could be explained by the low and transient viremia implying low systemic replication of the virus. Alternative antigens for FMDV and SVDV such as recombinant capsid polyproteins or virus like particles might improve this Luminex assay. On the other hand, the simultaneous rise and/or equal persistence of levels of antibodies to CSFV and ASFV in OF and sera suggests that Luminex assays for OF in place of sera can be used for serosurveillance for these viruses. However, contrary to virus genome detection, apparently for FMDV and SVDV, a high percentage of antibody positive animals per group may be required for detectable levels of antibodies in OF. In other words negative animals in the group may dilute antibodies in OF contributed by the seropositive animals.

An advantage of Luminex assays over ELISAs is the potential for multiplexing. We adopted strategic multiplexing by grouping viruses that cause similar clinical signs. Combining the assays for SVDV and FMDV; and ASFV and CSFV produced similar results as the singleplex assays for each agent. These combinations will allow for diagnosing a disease while simultaneously ruling out the most probable differential diagnosis.

Our data supports the potential of swine OF being used in place of serum for routine serological surveillance of swine herds for some FADs using Luminex assays. Furthermore, the Luminex assays can be used for sera when necessary. Further validation is required before these assays can be put into full use. In addition, other antigens, especially for SVDV and FMDV, can be evaluated to find the most immunogenic targets for these assays. At the same time, confirmatory assays for antibody detection in OF will have to be considered.

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