RESEARCH REPORT
SWINE HEALTH

Title: Use of oral fluid (OF) samples to monitor virus shedding and antibody responses in pigs experimentally infected with high consequence swine viruses (foot and mouth disease, African swine fever, swine vesicular disease and classical swine fever viruses); **NPB #14-286.**

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Industry Summary:

Oral fluids (OF) obtained from pigs using cotton ropes are becoming a popular sample type for use in the diagnosis of diseases in pigs. The genetic material, antigens and antibodies to viruses that infect pigs can be measured in OF from pigs. Test methods exploiting this sample type are available for diseases frequently seen in North American pigs. Similar test methods need to be evaluated for diseases that are foreign or absent, but yet pose a threat to the North American pig industry. Examples include diseases caused by foot and mouth disease virus (FMDV), African swine fever virus (ASFV), swine vesicular disease virus (SVDV) and classical swine fever virus (CSFV). This project was designed to partially address this need.
Specific objectives were to create a repository of OF, swab and serum samples from FMDV, CSFV, ASFV and SVDV-infected pigs; use these samples to develop and/or validate existing test methods for detecting the genome of individual viruses (singleplex PCR) and multiple viruses simultaneous (multiplex assay) for FMDV, CSFV, ASFV and SVDV in OF; and develop and/or validate existing test methods for the detection of antibodies to FMDV, CSFV, ASFV and SVDV in OF and serum specimens collected during the convalescent phase of infection.
For FMDV, 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 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. Whole blood, serum and swabs of the mouth and nares were also collected from individual animals.

FMDV genome was detected in OF as early as one day after the animals were either injected with virus or exposed to infected animals and 21 days later FMDV could still be detected in OF when tested by a quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). Virus in these OF collected at 1-5 days post infection (DPI) grew in cell cultures, meaning infectious virus could be recovered from these samples. Slightly more virus was detected in OF compared to oral and nasal swabs. FMDV antigen was detected in OF by both a rapid penside test and an enzyme –linked immunosorbent assay (ELISA). Antibodies to FMDV were also detected in OF. The immunoglobulin (Ig) most commonly secreted at the mucosal surface is IgA. IgA was thus the most reliably detected antibody in OF in response to FMDV infection starting at 14 DPI and peaking at 21 – 28 DPI.

SVDV genome was also detected in OF as early as one day after the animals were inoculated with virus and was still detectable at 21 DPI when tested by qRT-PCR. Similar to FMDV, SVDV in the OF collected at 1-5 DPI grew in cell cultures, meaning infectious virus could be recovered from these samples. Similarly, slightly more virus was detected in OF compared to oral and nasal swabs. OF was also a better sample type for SVDV detection when compared to serum. With a modified competitive ELISA based on commercially available monoclonal antibodies, antibodies to SVDV were detected in OF starting at 6 DPI. Antibodies of the IgM and IgA isotype were also detected in OF with IgM response starting at 6 DPI, reaching a maximum at 7 or 14 DPI and dropping at 21 DPI. The IgA response started at 7 DPI and peaked at 14 DPI.

CSFV genome was detected in OF at 10 to 14 days after the animals were inoculated with virus. One group remained negative for virus genome in OF throughout the experiment. Virus was detected in sera earlier than in OF, starting at day 6 – 7 after inoculation of the pigs. Using a commercially available IDEXX HerdCheck CSFV Ab ELISA and a partially validated modification of the manufacturer's protocol, antibodies to CSFV were detected in OF starting at 14 - 21 DPI.

ASFV Malta '78 genome was detected in OF starting at 6 DPI to 21 DPI. Detection of virus in oral and nasal swabs mirrored detection in OF. However, whole blood was the best sample type for ASFV detection, becoming positive at 4 DPI and containing higher levels of virus genome in most pigs.

A fully integrated and automated assay for simultaneous detection and differentiation of FMDV, SVDV, CSFV and ASFV was developed. The fully integrated/automated assay was optimized, validated and used to successfully process and detect cell culture amplified viruses, as well as FMDV, SVDV, CSFV and ASFV in OF.

All these results demonstrate that OF can be used for the detection of genome and/or live virus of FMDV, SVDV, CSFV and ASFV. Additionally, FMDV antigen can be detected in OF. Furthermore, antibodies to these viruses can be detected in OF by a variety of serological assays, including competitive and isotype-specific (IgA and IgM) ELISAs. Likewise, detection of IgA in OF has potential use for detecting antibody response following vaccination. All these results point to the high potential for the use of OF for FMDV, SVDV, CSFV or ASFV surveillance employing both established and partially validated assays.

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Swine, Oral fluids, foreign animal diseases, virus detection, antibody detection, real-time polymerase chain reaction, enzyme-linked immunosorbent assays

Scientific Abstract:

The use of swine oral fluids (OF) for the detection of virus nucleic acids and antibody response to pathogens is becoming common practice. Consequently, assays have been developed for this purpose for endemic and foreign animal diseases of swine. Detection of foot-and-mouth disease virus (FMDV) genome in swine OF has previously been demonstrated. Confirmatory assays for FMDV diagnosis such as virus isolation and viral antigen detection have not been evaluated using swine OF. We have now validated molecular detection of FMDV in OF, evaluated antigen detection and FMDV isolation from swine OF and developed an IgA isotype ELISA for anti-FMDV antibody detection in OF. FMDV genome was detected in OF from experimentally infected pigs by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) from 1day post-infection (DPI) to 21 DPI. Live virus was isolated from OF at 1-5 DPI. Additionally, FMDV antigen was detected in OF from 1-6 DPI by a lateral flow immuno-chromatographic strip test and from 2-3 DPI using a double antibody sandwich ELISA. In addition, FMDV-specific IgA was detected in OF using an isotype-specific indirect ELISA starting at DPI 14.
These results further demonstrate the potential use of OF for FMDV genome, live virus, and viral antigen detection, as well as quantification of mucosal (IgA) antibody response.

For SVDV, viral RNA was detected in OF by qRT-PCR from 1 DPI to 21 DPI; and live virus isolated at 1-5 DPI. A competitive ELISA based on the monoclonal antibodies 5B7 was modified to detect antibodies to SVDV in OF starting at 6 DPI. Using isotype-specific indirect ELISAs, SVDV-specific IgM response was detected in OF starting at 6 DPI, peaking at 7 or 14 DPI and declining sharply at DPI 21 while IgA response started at DPI 7, peaked at DPI 14 and remained high until the end of the experiment.

In the first experiment, CSFV genome (at Ct considered positive) was detected in OF starting at 10 DPI and levels increased until the end of the experiment at 18 DPI. In experiment 2, CSFV genome was detected in OF starting at 14 DPI, coinciding with detection in oral swabs. However, CSFV detection in serum was earlier and stronger compared to OF and swabs. Using the commercially available IDEXX ELISA kit for anti-CSFV antibodies in swine OF, positive antibody response was detected at 9 and18 DPI in experiment 1 (n = 1 group) and at 14 to 21 DPI in experiment 2 (in 2 of 4 groups).

ASFV Malta genome was detected in OF starting at 6 DPI to 14 or 21 DPI. Detection of virus in oral and nasal swabs mirrored detection in OF. However, whole blood was the best sample type for ASFV detection, becoming positive at 4 DPI and containing higher levels of virus genome in most pigs.

A fully integrated and automated assay for simultaneous detection and differentiation of FMDV, SVDV, CSFV and ASFV was developed. The fully integrated/automated assay was optimized, validated and used to successfully process and detect cell culture amplified viruses, as well as FMDV, SVDV, CSFV and ASFV in OF.

Our data demonstrates that OF can be used for the detection of genome and live virus of FMDV, SVDV, CSFV and ASFV. Additionally, FMDV antigen can be detected in OF. Furthermore, antibodies to these viruses can be detected in OF by a variety of serological assays, including competitive and isotype-specific (IgA and IgM) ELISAs. Furthermore, detection of IgA in OF has the potential use for detecting antibody response following vaccination. All these results point to the high potential for the use of OF for FMDV, SVDV, CSFV or ASFV surveillance employing both established and partially validated assays. However, there is the possibility that virus detection in OF may vary for different virus species, between serotypes of the same virus, under different experimental settings, and/or sample handling and testing conditions.

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 et al. 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 samples 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 et al. 2010; Prickett et al. 2008; Romagosa et al. 2012). Similarly, OFs have been used to diagnose FMD and ASF in pigs (Mur et al. 2013; Vosloo et al. 2013). However, assays based on OFs haven't been developed or fully validated for routine surveillance of most exotic swine diseases. Development and/or validation of OFs-based assays for FADs are therefore critical to ensure potential use of this sample type for FAD diagnostic assays.

The United States and Canada are two of the largest pig producers and exporters in the world. The estimated direct and indirect loss to the US industries resulting from a FMD outbreak is approximately $120B over a 10 year period (Hayes et al. 2011). In addition, the value of protection against FAD to the US pork industry is estimated at $137M per year (Hayes et al. 2011). The reagents and assays validated in this project will provide new tools for rapid detection and characterization of multiple FADs that affect swine. The utilization of automated and integrated technology for simultaneous detection of multiple porcine FADs will significantly reduce the cost, labour and time required for pathogen detection and surveillance, allow more efficient use of samples and enhance biosecurity for the swine industry. Furthermore, the automation and integration increases the consistency of results, reduces operator induced errors and variations, reduces the chance for cross contamination due to less handling, and improves turn-around time so that preventive measures at the farm level can be implemented in a timely manner to contain the spread of the disease.

Objectives:

a) Generate a repository of OFs, swab and serum samples from FMDV, CSFV, ASFV and SVDV-infected pigs. Aliquots of OF samples will be preserved in MTM for downstream testing.

b) Validation/methods comparison studies on existing singleplex PCR for detection of ASFV, CSFV, SVDV and FMDV in OFs.

c) Integration and validation of a fully automated multiplex assay for simultaneous detection of FMDV, CSFV, ASFV and SVDV.

d) Validation/methods comparison studies for detection of antibodies to FMDV, CSFV, ASFV and SVDV in OF and serum specimens collected during the convalescent phase of infection.
e) Once methods have been established, perform a feasibility evaluation of whether PrimeStore® Molecular Transport Medium (MTM) will support antibody recovery for downstream serological testing on archived samples.

Materials & Methods:

Animals:

Pigs aged 4 to 5 weeks were obtained from local suppliers in Manitoba, Canada. These animals were examined upon arrival and moved into cubicles. In most experiments, at least 4 cubicles with 4 pigs/cubicle (except 6 pigs/cubicle for FMDV) were used for each virus studied. Samples were also obtained from vaccine studies and as such groups were modified as shown below in the virus-specific description. 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:

FMDV (serotypes O and A), ASFV (Malta '78 strain), CSFV (Diepholz, genotype 2.3) and SVDV (UKG 27/72 strain) were used in experimental inoculations in this project. Viruses were produced in appropriate cell cultures and the passage number kept as low as possible. Virus titres were determined in corresponding cell cultures.

Animal inoculation and sampling:

Foot-and-mouth disease virus

Experiment1: FMDV O UKG 2001

Two pigs for each of 4 cubicles (8 pigs total) were inoculated intradermally with cell culture supernatants containing 10^6 TCID50 (50% tissue culture infectious dose) in the heel bulb of one hind limb. These served as donors in a contact challenge for the remaining 4 pigs/cubicle for 4 cubicles (16 pigs in total).

Sampling was done daily for the first 7 days post inoculation (DPI) and then once every week until the end of the experiment. Collection of OF samples was performed as previously described (Prickett et al. 2008). Briefly, a 5/8-inch cotton rope was suspended at shoulder height within the cubicle for 20 to 30 minutes. Since pigs are naturally attracted to the rope, they chewed on the rope, depositing OF in the process. Oral fluids were then 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 were then spun at 2000g for 20 minutes to separate any debris and aliquots used for virus and antibody detection. Blood for serum was collected from the anterior vena cava using a 20 gauge needle. Nasal and oral swabs were taken with Dacron tipped swabs.

Experiment 2: FMDV serotype A/TAI/2014 (part of a vaccine efficacy studies)

This consisted of 4 vaccine groups and 1 unvaccinated control group as shown in the table 1 below. Vaccinated groups were challenged at either 7 or 21 days post vaccination (DPV), and the unvaccinated control group was challenged on the same day as the vaccinated pigs with a pig adapted FMDV A/TAI/2014 virus.

Designation	Vaccine	Day of vaccination	Challenged
Group A	A Malaysia 97	21	Yes
Group B	A22 Iraq 64	21	Yes
Group C	A Malaysia 97	7	Yes

Group D	A22 Iraq 64	7	Yes
Group E	Unvaccinated	None	Yes

Table 1: Groups of pigs vaccinated with FMD serotype A vaccines and challenged with FMDV A/TAI/2014

For FMDV challenge, pigs were inoculated with 0.2 mL of 10,000 $TCID_{50}$ of FMDV A/TAI/2014 virus into the heel bulb of the left hind limb (in two sites with 0.1 mL per site). The animals were monitored for clinical signs consistent with FMD. Oral fluids, serum and swabs were collected before vaccination, weekly post vaccination and then daily for 14 days post challenge (DPC) as described above.

Experiment 3: FMDV serotype A/TAI/2014 (part of a bivalent vaccine efficacy studies)

This was a repeat of experiment 2 using a combination of the two vaccines in one immunization and the same challenge virus. The groups are shown in the table 2 below. Similarly, vaccinated groups were challenged at either 7 or 21 days post vaccination (DPV), concurrently with the unvaccinated control group.

Designation	**Vaccine**	**Day of vaccination**	**Challenged**
Group A	A Malaysia 97/A22 Iraq 64	21	Yes
Group B	A Malaysia 97/A22 Iraq 64	7	Yes
Group C	Unvaccinated	None	Yes

Table 2: Groups of pigs vaccinated with bivalent FMD serotype A vaccine and challenged with FMDV A/TAI/2014

Challenge with FMDV A/TAI/2014 virus and sample collection was similar to FMDV experiment 2 above.

Swine vesicular disease virus

Experiment 1 and 2: both with SVDV UK27/72

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 (4pigs /cubicle in 4 cubicles for a total of 16 pigs) for each experiment.

In both experiments, sampling was done daily for the first 7 DPI and then once every week until the end of the experiment. Oral fluids, serum and swabs were collected as described above.

Classical swine fever virus

Experiment 1: CSFV Diepholz

This experiment comprised of a single group of 4 pigs. Each pig was inoculated orally with 10^6 TCID50 of virus in a total volume of 6 mL per pig. After 6 days all pigs were re-inoculated with the same amount of virus. Sampling was done daily for 18 days. Oral fluids, serum and swabs were collected as described above.

Experiment 2: CSFV Diepholz

Each pig was inoculated oronasally with 10^6 TCID50 of virus (4pigs /cubicle in 4 cubicles for a total of 16 pigs), 3 mL/ animal (1 mL per nostril and 1 mL orally). Sampling was done daily for the first 7 DPI and then once every week until the end of the experiment. Oral fluids, serum and swabs were collected as described above.

African swine fever virus

ASFV Malta '78 strain

The pigs in this experiment were 5-6 weeks old at the time of inoculation. Each pig was inoculated oronasally with 10^5 HAD_{50} in 4 mL (1 mL per nostril and 2 mL orally), 4pigs /cubicle in 4 cubicles for a total of 16 pigs). Oral fluids, serum, whole blood and swabs were collected every other day for the first 14 DPI and then once every week until the end of the experiment. All samples were stored at -70˚C.

Molecular detection of virus

Nucleic acid extraction

The MagMax™ Viral RNA Isolation kit (Ambion) was used for RNA extraction from 55µl 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 was also used for purification using a Deep Well Magnetic Particle Processor. The nucleic acid was eluted into 50µl of Elution buffer.

Singleplex PCR assay:

FMDV RNA detection targeted the 3D gene in a TaqMan qRT-PCR carried out according to a published protocol (Moniwa et al. 2007). This qRT-PCR uses primers and probe that specifically target a conserved region of the FMDV 3D gene resulting in the amplification of an 88 bp product. The forward primer FMDV 1186F (5'ACTGGGTTTTAYAAACCTGTGATG3'), reverse primer FMDV 1237R (5'TCAACTTCTCCTKGATGGTCCCA3') and TaqMan probe (5'ATCCTCTCCTTTGCACGC3') labelled on the 5' end with 6'FAM and the 3' end with minor groove binder (MGB) were used. AgPath-ID™ One-Step RT-PCR reagents (Life Technologies) and 96-well plates on ABI SDS7900 were for all tests.

SVDV qRT-PCR also amplifies a 154 bp conserved region of the 3D gene. The primers and probe are SVDV6875 forward primer 5' TTCAGAATGATTGCATATGGGG 3', SVDV7028 reverse primer 5' TCACGTTTGTCCAGGTTACC 3' and SVDV Probe, 5' 6FAM-TTCATACCCGTGGCCYATC-NFQ-MGB 3'. AgPath-ID™ One-Step RT-PCR reagents (Life Technologies) and 96-well plates on ABI SDS7900 were for all tests.

The qRT-PCR for CSFV RNA detection targeted the 5'-UTR using the forward primer CSF-Taq 5'-TCA GTA GTT CGA CGT RRG CAG AA-3', reverse primer CSF-Taq 5'-GCA TGC CCT CGT CCA CRT-3' and probe CSF-5'UTR-MGB-2 5'-6FAM-CYY RCC TCG AGA TGC-MGBNFQ-3'. The assay was performed using the FasTaq TaqMan kit.

Real-time PCR for the detection of ASFV DNA will be carried out using previously described primers, probes and protocol (528 Tignon,M. 2011). The primers (forward: 5′-TGCTCATGGTATCAATCTTATCG and reverse: 5′-CCACTGGGTTGGTATTCCTC-3′) were designed from conserved regions of the p72 gene such that they could amplify this segment for all 22 known genotypes of ASFV (528 Tignon,M. 2011). The TaqMan® probe (5′ FAM-TTCCATCAAAGTTCTGCAGCTCTT-TAMRA 3′) is labelled with the 5′ reporter dye 6-carboxy-fluorescein (FAM) and the 3′ quencher dye 6-carboxy-tetramethylrhodamin (TAMRA).

Primers and probes for all 4 viruses were custom made by Integrated DNA Technologies (IDT) and ABI respectively.

Integration and validation of a fully-automated multiplex assay for simultaneous detection of FMDV, CSFV, ASFV and SVDV:

An in-house developed 3-step multiplex PCR/RT-PCR and probe hybridization-based assay for simultaneous detection of FMDV, CSFV, ASFV and SVDV and an internal control were converted into a one-step computer controlled CARD (chemical and reagent device) assay that integrated sample preparation/nucleic acid extraction, PCR amplification and probe-based

detection so that user handling was not required after sample introduction. The PCR primers for the multiplex PCR/RT-PCR and virus-specific capture probes were designed using comprehensive sequence databases of the four HC viruses (e.g. over 2800 FMDV and 800 CSFV sequences were assembled for the FMDV and CSFV databases). The multiplex PCR/RT-PCR and capture probes have been validated *in silico* and also with over 45 strains of the four viruses including multiple strains that represent each of the seven FMDV serotypes and the three CSFV genotypes, a panel of non-target viruses and bacteria that are associated with livestock, as well as clinical material from healthy and experimentally infected swine. All four viruses were detected by the 3-step multiplex assay in clinical samples from experimentally infected swine (e.g. as early as DPI 1 for FMDV and SVDV).

The current 3-step multiplex assay requiring separate nucleic acid extraction, PCR amplification and hybridization-based detection steps were integrated into a one-step fully automated CARD assay in two phases. Integration of the PCR/RT-PCR and detection modules occurred first, using viral RNA from a panel of diverse viruses as input material to validate the PCR amplification and detection steps of the integrated assay. Sample extraction was subsequently integrated with the RT-PCR and detection modules into a fully-automated computer controlled assay that did not require user intervention after sample introduction.

The PCR/RT-PCR component of the integrated assays was migrated to the PCR module of the CARD and used optimized reaction buffer composition previously identified to be optimal for CARD assays during the development of an 8-plex swine respiratory disease complex assay. When these optimizations were applied to the single-step CARD assay, the sensitivity and specificity was comparable to PCR assays that currently require separate nucleic acid extraction and PCR amplification steps. For optimization and integration of the detection component of the integrated assay, optimized hybridization and washing conditions previously identified to be optimal for CARD assays were incorporated. Multiple highly specific probes for each of the four viruses and an internal armoured RNA control were designed and tested with a panel of target and non-target bacteria and viruses affecting livestock on-CARD. The optimized and integrated PCR/RT-PCR amplification and detection components of the assay were further integrated with the sample preparation/nucleic acid extraction component using scripts designed for the fully-integrated swine respiratory disease complex assay.

The fully integrated assay was tested with a panel of target viruses representing the genetic diversity of each target virus to validate the ability of the assay to detect genetically diverse variants of the four viruses (FMDV, SVDV, CSFV and ASFV). Panels of samples containing non-target bacteria and viruses associated with livestock, as well as environmental samples and OF samples from healthy swine were used to evaluate the specificity of the assay. Paired OF and other clinical samples from experimentally infected swine collected as part of this project were used to determine the diagnostic sensitivity of the assay. Results from the new fully automated multiplex CARD assay were compared with results from currently used assays.

Rapid FMDV antigen detection by lateral flow test:

An in-house lateral flow immunochromatographic (LFI) strip test for rapid detection of FMDV serotypes O, A and Asia 1 was recently developed at the NCFAD (Yang et al. 2013). Oral fluid samples from FMDV-infected pigs were tested on LFI as previously described (Yang et al. 2013).

Serological assays:

Detection of antibodies to FMDV in oral fluids

FMDV isotype specific IgA:

This assay was performed as described in the publication of the data derived from this project (Senthilkumaran et al. 2017). Briefly, 96-well ELISA plates (NUNC- Maxisorb, Thermo Fisher Scientific Inc, USA) were coated with (50 µl/well) of an optimal dilution of a rabbit anti-FMDV O or anti-FMDV A antibody in carbonate buffer, pH 9.6 for FMDV serotype O and A respectively and incubated overnight at 4°C. The contents of each well were emptied and the plates were blocked with 10% horse serum in PBST (phosphate - buffered saline, 0.05% tween-20, pH 7.2) or PBST containing 5% skim milk for FMDV O or FMDV A respectively for 1h at 37°C with shaking. After washing five times with PBST, FMDV serotype O or FMDV serotype A antigen in the respective blocking buffer (50 µl/well) was added to the plates and incubated for 1h at 37°C with shaking. The plates were washed five times and OF diluted 1/2 in the respective blocking buffer were added to the plates at 50 µl/well. After 1h incubation, plates were washed and mouse anti-pig IgA (AbD Serotec, Raleigh, NC, USA,) at approximately 0.17 µg/mL or 0.06 µg/mL in blocking buffer for FMDV O and FMDV A respectively was added to the plates (50 µl/well) and incubated for 1h at 37°C with shaking. The plates were washed five times, and tetramethylbenzidine (TMB, Thermo Scientific, Rockford, USA) substrate was added to the plates (50 µl/well). The plates were incubated in the dark at room temperature for 10 min. After adding the stop solution (2 M H_2SO_4), the optical density was read at 450 nm (OD_{450}) using an ELISA plate reader (SpectraMax Plus 384 Microplate Reader, Sunnyvale, USA). For the determination of diagnostic specificity, 299 OF samples collected from groups of clinically healthy and FMDV-free pigs in Canada and the USA were tested on the same assay In addition, for the determination of analytical specificity, OF from pigs infected with swine vesicular disease virus (SVDV) and vesicular stomatitis virus (VSV) were tested.

Antibodies to FMDV 3B non-structural protein

Antibodies against the 3B non-structural protein of FMDV were detected by a competitive ELISA (cELISA). This ELISA uses a recombinant 3ABC protein as antigen and antibodies in serum compete with an anti-3B monoclonal antibody for specific epitopes on the recombinant protein (Clavijo et al. 2004; Moniwa et al. 2012).

Detection of antibodies to SVDV in oral fluids

Competitive ELISA for the detection of antibodies to SVDV in oral fluids

Presence of antibodies against SVDV in OF was tested by a solid phase competitive ELISA (C-ELISA), using capture and detection monoclonal antibodies 5B7 from IZSLER, Brescia, Italy, as previously described (Brocchi et al. 1995). The protocol was optimised for detecting SVDV-specific antibodies in OF as described by (Senthilkumaran et al. 2016). Briefly, the same conditions for capture antibody and antigen steps used for sera (Brocchi et al. 1995) were maintained for OF, but SVDV antibody-positive/negative OF and detection antibody were titrated in a checkerboard to determine the optimal conditions. Oral fluid samples (n = 210) collected from groups of clinically healthy and SVDV-free pigs in Canada and the USA were tested in duplicate to establish the cutoff for this C-ELISA for OF. All of the OF collected from groups of SVDV-inoculated pigs were then tested by the optimised C-ELISA.

SVDV isotype specific IgA and IgM:

Detection of isotype-specific anti-SVDV (IgA, and IgM) antibodies in OF was carried out by indirect ELISA using a modification of the SVDV C-ELISA (Prickett et al. 2011; De Clercq et al. 1998; Brocchi et al. 1995). To determine the optimal conditions for the indirect ELISAs, titrations of SVDV antibody-positive/negative OF and detection antibodies were performed using the optimal capture and antigen conditions for SVDV C-ELISA as described by (Senthilkumaran et al. 2016). The cutoff OD and subsequently the diagnostic specificity for each assay were determined using 220 and 199 OF samples collected from groups of clinically healthy and SVDV-free pigs in Canada and the USA for IgM and IgA respectively. The analytical specificity and cross

reactivity of the isotype specific ELISAs (SVDV IgM and SVDV IgA) were tested with the oral fluids collected from pigs experimentally infected with foot and mouth disease virus (FMDV O UKG 11/2001) and vesicular stomatitis virus (VSV NJ Mexico 95).

Detection of antibodies to CSFV in oral fluids

The antibody ELISA for CSFV used a modification of the IDEXX HerdCheck CSFV Ab ELISA. In this assay, antibodies in test samples block the binding of the CSFV-specific MAb to immobilized CSFV antigen. Briefly, OF samples were applied to wells of an antigen coated microtitre plate. Following incubation and washing, CSFV MAb conjugated to horseraddish peroxidase was added to each well. After incubation and washing, TMB substrate was added to each well. In the absence of antibodies to CSFV, the MAb was bound to CSFV antigen as indicated by colour development. If the sample had antibodies to CSFV, these antibodies blocked or reduced binding of the MAb to CSFV antigen hence preventing or reducing colour development. A percent blocking of Mab binding to antigen was then calculated. Oral fluid samples (n = 303) collected from groups of clinically healthy and CSFV-free pigs in Canada and the USA were tested in duplicate to establish the cutoff for this ELISA for OF. All of the OF collected from groups of CSFV-inoculated pigs were then tested by the optimised ELISA.

Detection of antibodies to ASFV in oral fluids

Anti-ASFV antibodies in serum and OF were quantified by a validated in-house indirect ELISA. For this ELISA, ASFV is grown in Vero cells and viral antigen is harvested by solubilization with Triton X-114. The detection system is based on a commercially available, polyclonal rabbit anti-porcine IgG (H&L) conjugated with horseradish peroxidase. When porcine antibody has reacted with ASFV antigen and polyclonal rabbit anti-porcine IgG (H&L) conjugated with horseradish peroxidase, in turn, reacted with the porcine antibody, detection will occur after the addition of an enzyme substrate system. Hydrogen peroxide is the substrate and 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) is the hydrogen donor. The enzyme conversion of substrate results in the generation of a chromogenic product which is then measured photometrically. Intensity of colour is directly related to the amount of porcine anti-ASFV antibody present in the serum or oral fluids being tested. Plates are coated with optimal concentration of antigen in carbonate coating buffer (100 µl into each well of the microplate) and incubated overnight at 25 ± 2°C. Test samples and controls are diluted in diluent buffer (sera at 1/40 and oral fluids at 1/5). Prior to sample application, antigen-coated plates are aspirated using a microplate washing system. After five consecutive washes residual wash buffer is discarded by tapping onto a lint-free absorbent material and 100 µl test and control samples are dispensed into appropriate wells of the microplate. Plates are incubated for 2 hours +/- 5 minutes at 25 ± 2°C. Prior to conjugate application, wash plates are washed as described above, 100 µl of conjugate immediately dispensed into all wells and incubated for 1 hour +/- 3 minutes at 25 ± 2°C. Plates are washed as previously described, 100 µl of substrate immediately dispensed into all the wells and incubated in a microplate shaker at RT. The optical density (OD) is monitored between 7.30 to 16.30 minutes and results recorded when control values fall within predetermined specifications for the antigen batch. A sample is considered positive if its corrected OD is ≥ 0.320 for serum and 0.2 for oral fluids (based on the testing of 160 negative OF).

Statistical analysis:

For FMDV IgA ELISA, Receiver operating characteristic (ROC) analysis was performed using easyROC (version 1.2, http://www.biosoft.hacettepe.edu.tr/easyROC/). Results for known negative OF samples and from inoculated animals starting at DPI 14, 21, and 28 (allowing for the development of the humoral response) were used for the ROC analysis (Senthilkumaran et al. 2017). The optimal cutoff was determined by the Youden method.

For SVDV serology, ROC analyses were performed using MedCalc Statistical Software version 16.2.0. Ostend, Belgium (https://www.medcalc.org; 2016) as previously described (Senthilkumaran et al. 2016). The C-ELISA and IgA ROC analyses used the results from inoculated animals at DPI 14, 21, and 28 and all negative animals. Results for OF samples from DPI 0 - 7 (32 results) from the inoculated groups were excluded from the C-ELISA and IgA ROC analysis to allow for the development of the humoral response. The IgM ROC analysis used the results from inoculated animals at DPI 6, 7, and 14. Results for OF samples from DPI 0 -4, 21, and 28 (35 results) from the inoculated groups were excluded from the IgM ROC analysis.

For initial assay development for the fully-automated multiplex assay for simultaneous detection of FMDV, CSFV, ASFV and SVDV, fluorescent intensity values captured by the instrument were imported and analyzed quantitatively in Excel. Fluorescent intensity ≥ 30 was considered positive. Raw images for all samples were also inspected to ensure no air bubbles or other image artifacts could skew the data or cause false positives. Samples with suspected false positive results were either reimaged or reprocessed.

Results: Reported by objectives.

a) Generation of a repository of OF, swab and serum samples from FMDV, CSFV, ASFV and SVDV-infected pigs. Aliquots of OF samples will be preserved in MTM for downstream testing.

For each experiment, at least 4 aliquots of OF (1 mL each) 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.

b) **Validation/methods comparison studies on existing singleplex PCR for detection of FMDV, SVDV, CSFV and ASFV in OF.**

Foot-and-mouth disease virus

Experiment 1:

The results of this experiment have been published (Senthilkumaran C, Yang M, Bittner H, Ambagala A, Lung O, Zimmerman J, Giménez-Lirola LG, Nfon C. Detection of genome, antigen, and antibodies in oral fluids from pigs infected with foot-and-mouth disease virus. *Can J Vet Res. 2017 81(2):82-90.*)

Experiment 2:

All infected pigs developed fever starting at 2 – 3 days post challenge (DPC). All pigs in unvaccinated control group and most pigs in the rest of the groups were euthanized at 4 and 7 DPC respectively due to severe disease. However, in both group A and B, one pig each was completely protected from systemic disease. All pigs in groups C and D developed clinical signs. The disease was less severe in group B compared to group A.

FMDV RNA was detected in OF from all groups starting at 1 DPC. At 7 DPC, groups with surviving animals were positive for FMDV genome in OF. Virus was isolated from OF at 1-4 DPC irrespective of vaccination status (figure 1). Virus was also isolated from OF from groups A and B at 5-7 DPC. Virus detection in OF mirrored the average detection in oral swabs for most groups. Interestingly, contrary to OF, viremia was reduced or absent in most pigs in the vaccinated groups. Transient viremia in the unvaccinated group lasted between 1-3 DPC but OF were positive up to 4 DPC.

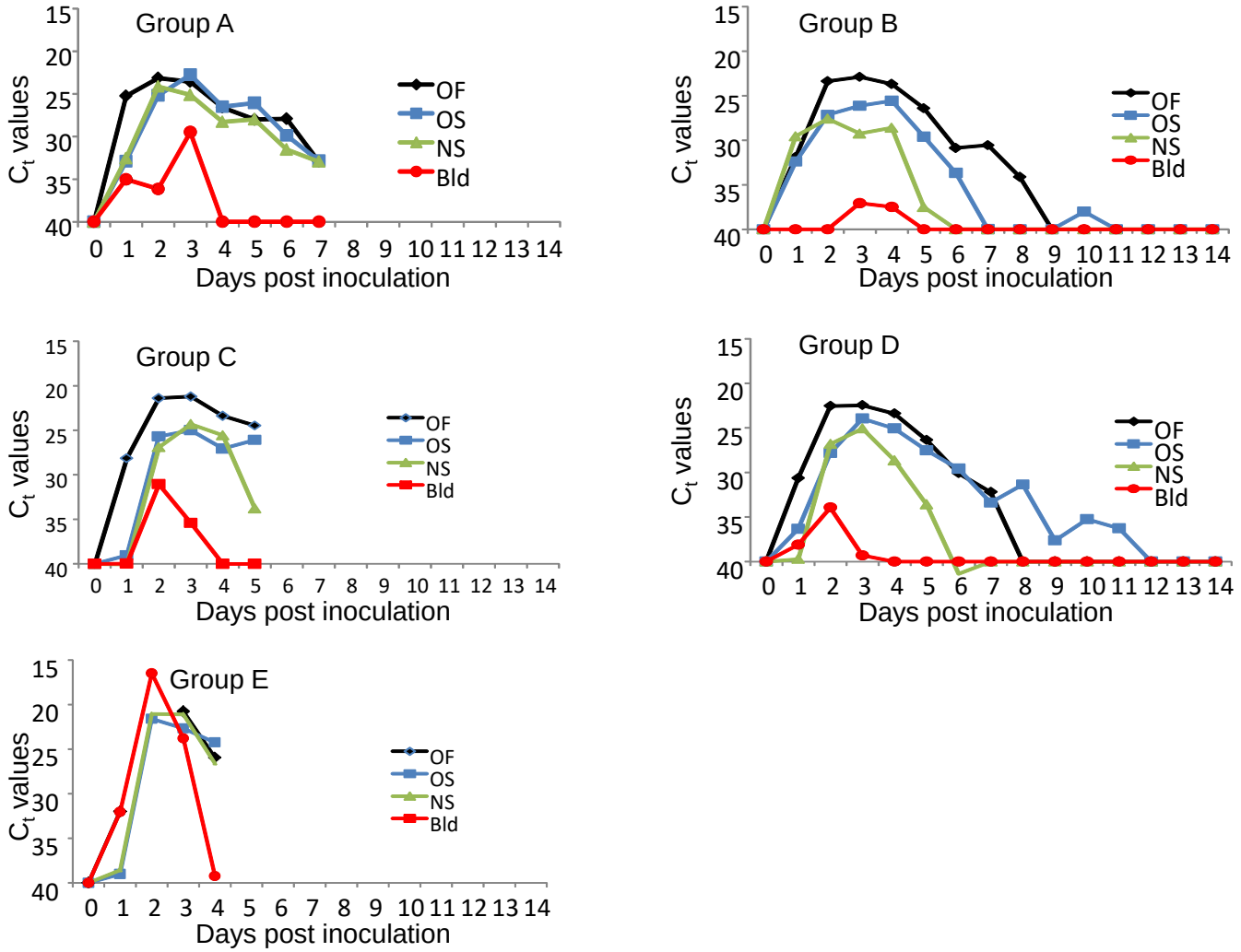

Figure 1: Detection of FMDV in various sample types by real-time reverse transcription polymerase chain reaction. OF, oral fluid; OS, oral swab; NS, nasal swab, Bld, blood (serum). Clinical scores are given for individual animals per group

Experiment 3:

Pigs developed variable degrees of fever between 1 and 6 DPC. The disease was severe resulting in either mortality or euthanasia of all Group B and unvaccinated control (group C) pigs between 3 and 5 DPC. In group A, 80% of the pigs were protected.

FMDV genome was detected in OF starting at 1 DPC for groups B and C (challenged 7 days after vaccination and the unvaccinated groups respectively) but was delayed for 24 h in group A (challenged 21 days after vaccination). Positive FMDV genome detection lasted until 8 DPC in group A (figure 2). In vaccinated groups there was reduced viremia but no apparent effect of vaccination on the detection of viral genome in OF and oral swabs.

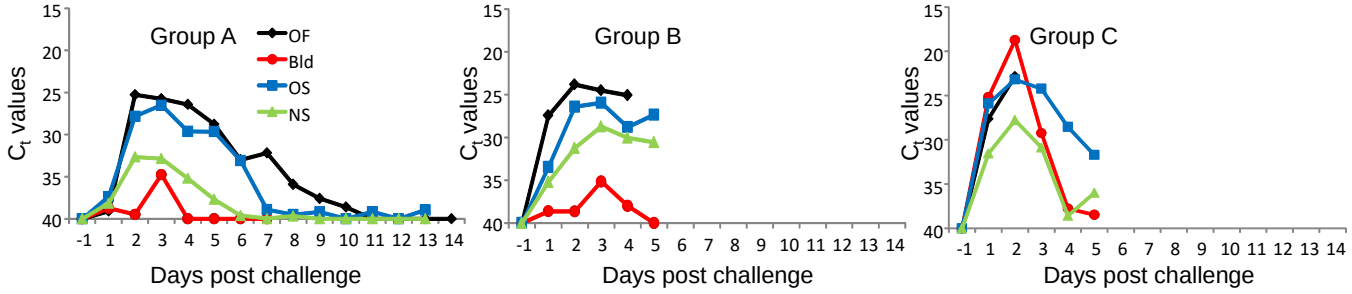

Figure 2: Detection of FMDV in various sample types by real-time reverse transcription polymerase chain reaction. OF, oral fluid; OS, oral swab; NS, nasal swab, Bld, blood (serum). Clinical scores are given for individual animals per group

Swine vesicular disease virus

Experiment 1:

The results of this experiment have been published (Senthilkumaran C, Bittner H, Ambagala A, Lung O, Babiuk S, Yang M, Zimmerman J, Giménez-Lirola LG, Nfon C. Use of Oral Fluids for Detection of Virus and Antibodies in Pigs Infected with Swine Vesicular Disease Virus. *Transbound Emerg Dis.* 2016 Sep 15. doi: 10.1111/tbed.12563).

Experiment 2:

Pigs developed clinical signs by 3 DPI. These included lameness, shivering, mild fever and vesicles in the interdigital space, around the coronary bands and on the hocks. By 15 DPI 56% of pigs had died or been euthanized because of severe clinical signs including hoof loss.

SVDV genome was detected in OF from DPI 1-14 for all groups (Figure 3). SVDV genome detection in OF coincided with detection in oral swabs and sera. Contrary to SVDV experiment 1, viremia was detected in 100% of inoculated pigs at DPI 1 and 3 (Table 1).

Group A		0 DPI	1 DPI	2 DPI	3 DPI	4 DPI	5 DPI	6 DPI	7 DPI	14 DPI	21 DPI	28 DPI
OF	**OF**	40.0	23.3	18.0	17.0	18.6	18.9	21.8	20.8	25.1	33.8	40.0
Oral swabs	**109**	40.0	28.9	19.2	23.1	21.9	22.3	27.7	40.0	32.4	40.0	40.0
	110	40.0	35.6	21.2	17.8	18.7	23.5					
	111	40.0	29.9	18.7	17.8	23.3	29.5	21.6	30.9	31.9		
	112	40.0	30.3	22.2	18.4	22.8	22.3	25.9	30.4	40.0		
Nasal swabs	**109**	40.0	40.0	30.3	28.8	26.4	28.6	30.9	40.0	38.8	40.0	38.9
	110	40.0	40.0	31.0	20.5	19.6	21.3					
	111	40.0	36.2	31.1	20.4	25.6	31.3	30.1	31.1	40.0		
	112	40.0	35.2	26.1	18.0	21.9	23.0	22.6	29.4	40.0		
Serum	**109**	40.0	29.7	26.9	20.8	28.5	34.3	40.0	40.0			
	110	40.0	29.6	22.4	20.2	30.9	33.5					
	111	40.0	23.6	21.0	23.1	40.0	40.0	40.0	40.0			
	112	40.0	27.2	20.9	18.7	30.7	35.2	40.0	40.0			
Group B		**0 DPI**	**1 DPI**	**2 DPI**	**3 DPI**	**4 DPI**	**5 DPI**	**6 DPI**	**7 DPI**	**14 DPI**	**21 DPI**	**28 DPI**
OF	**OF**	40.0	28.4	20.0	17.0	18.4	19.3	19.4	20.6	21.4	34.9	39.5
Oral swabs	**113**	40.0	31.6	30.2	26.9	22.6	26.3	21.8	28.9	31.9	39.9	40.0
	114	40.0	33.0	20.3	20.2	21.5	19.7	26.0	30.3	33.1	40.0	37.9
	115	40.0	35.9	29.5	25.0	26.6	25.8	31.2	29.3	31.8	40.0	38.4
	116	40.0	33.6	24.2	18.7	21.4	29.4	30.4	30.6	33.9		
Nasal swabs	**113**	40.0	40.0	34.7	31.7	26.6	30.8	32.0	27.3	33.3	40.0	40.0
	114	40.0	36.2	29.4	31.8	25.9	33.4	32.5	27.1	34.5	40.0	40.0
	115	40.0	40.0	30.2	27.8	29.0	30.8	31.0	30.7	40.0	40.0	40.0
	116	40.0	34.8	28.2	24.4	28.8	31.5	26.9	27.8	40.0		
Serum	**113**	40.0	31.6	31.1	32.7	36.3	32.0	40.0	40.0			
	114	40.0	28.9	25.8	21.9	34.7	34.2	40.0	37.7			
	115	40.0	27.1	28.4	26.6	40.0	38.1	35.9	40.0			
	116	40.0	23.8	23.3	19.8	35.8	40.0	40.0	40.0			
Group C		**0 DPI**	**1 DPI**	**2 DPI**	**3 DPI**	**4 DPI**	**5 DPI**	**6 DPI**	**7 DPI**	**14 DPI**	**21 DPI**	**28 DPI**
OF	**OF**	40.0	26.6	19.9	16.9	17.2	18.6	21.2	23.4	24.6	38.1	
Oral swabs	**117**	40.0	28.5	27.7	23.3	21.6	20.9					
	118	40.0	32.0	30.1	18.8	22.0	26.7	23.8	29.6	32.4	32.7	
	119	40.0	39.5	22.0	21.5	22.9	28.6	27.6	40.0	29.0		
	120	40.0	31.9	23.1	17.0	20.7	23.3	23.8	27.6			
Nasal swabs	**117**	40.0	40.0	34.7	27.4	30.0	30.1					
	118	40.0	40.0	27.9	18.6	17.6	27.3	28.0	24.9	30.6	40.0	
	119	40.0	31.2	25.9	20.7	26.6	23.9	29.7	30.4	40.0		
	120	40.0	34.8	33.6	30.5	28.5	30.2	30.6	29.0			
Serum	**117**	40.0	29.1	24.2	21.6	27.8	33.2					
	118	40.0	26.0	22.8	24.2	40.0	33.8	40.0	40.0	ND	ND	
	119	40.0	23.7	19.5	23.0	40.0	34.6	40.0	40.0	ND		
	120	40.0	26.1	24.2	28.3	40.0	40.0	40.0	39.5			
Group D		**0 DPI**	**1 DPI**	**2 DPI**	**3 DPI**	**4 DPI**	**5 DPI**	**6 DPI**	**7 DPI**	**14 DPI**	**21 DPI**	**28 DPI**
OF	**OF**	40.0	28.5	21.1	16.9	17.2	20.8	21.9	24.7	24.4	40.0	35.8
Oral swabs	**121**	40.0	34.5	27.7	20.9	24.3	26.3	27.2	30.6	30.1		
	122	40.0	32.5	25.8	19.6	21.1	23.3	28.8	28.1	32.4	32.3	40.0
	123	40.0	35.6	28.8	17.5	25.2	28.4	29.0	30.1	34.5	37.5	32.0
	124	40.0	36.0	30.6	19.5	22.4	27.9	29.5	28.3	31.4		
Nasal swabs	**121**	40.0	39.7	30.3	27.0	22.2	28.7	28.0	31.0	32.2		
	122	40.0	40.0	22.3	22.6	22.2	30.4	31.0	33.9	40.0	40.0	37.2
	123	40.0	38.5	33.9	21.9	23.5	27.3	31.0	27.6	34.9	40.0	40.0
	124	40.0	40.0	33.0	23.8	23.6	29.2	28.6	30.7	33.7		
Serum	**121**	40.0	27.6	24.2	23.8	34.6	35.6	40.0	40.0	ND		
	122	40.0	27.1	25.0	23.9	40.0	36.1	40.0	40.0	ND	ND	ND
	123	40.0	25.5	24.2	20.6	40.0	29.7	40.0	40.0	ND	ND	ND
	124	40.0	25.9	22.4	18.5	40.0	36.5	40.0	40.0	ND		

Table 1: Swine vesicular disease virus detection in oral fluids and other samples by real-time reverse transcription polymerase chain reaction. OF, oral fluid; DPI, days post infection; green cells represent positive results. Crossing threshold (Ct) < 35.99 was considered positive for SVDV genome. The lower the Ct value, the higher the amount of SVDV RNA. Any sample with undetermined Ct was given a Ct of 40.

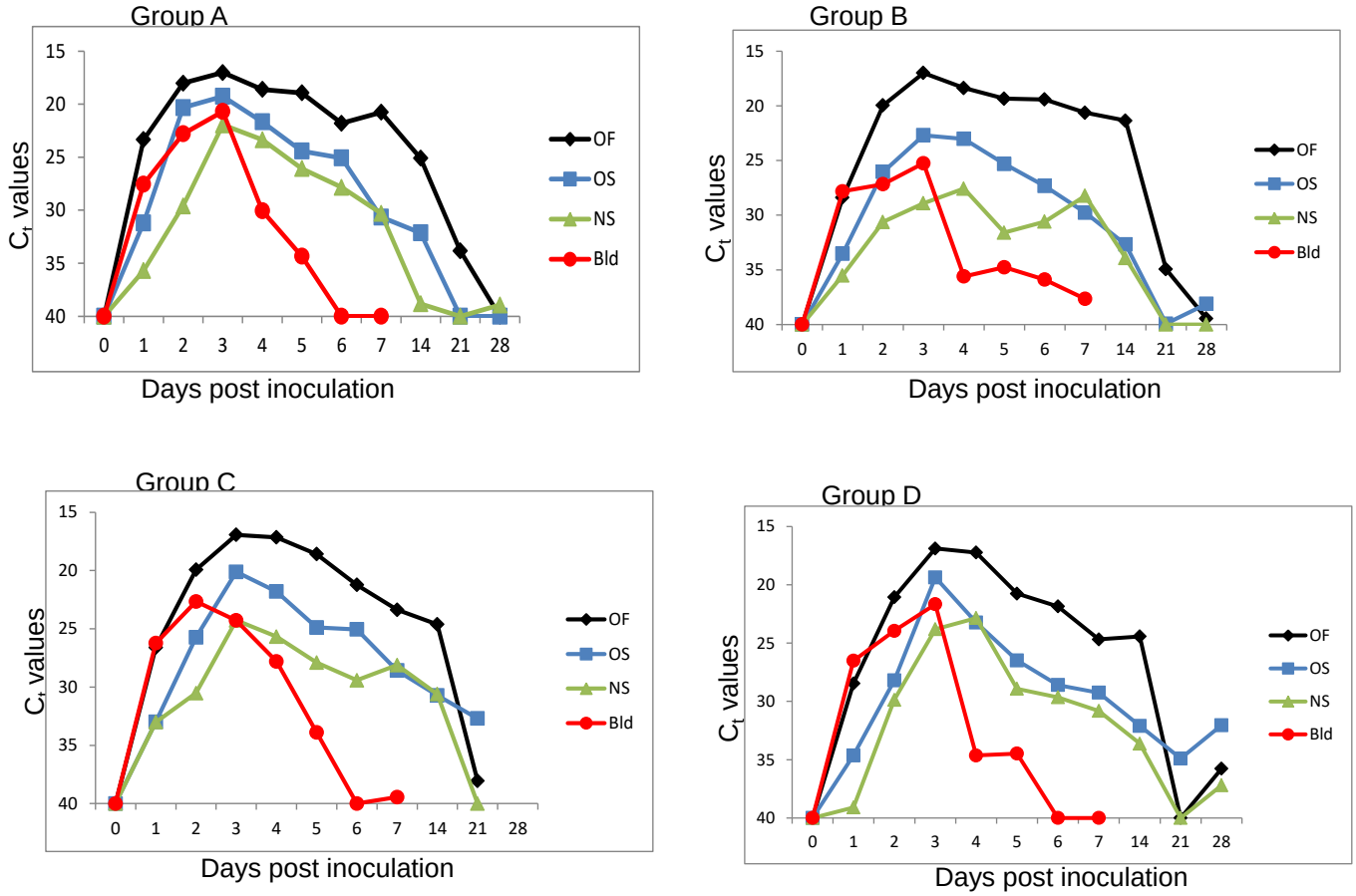

Figure 3: Detection of SVDV in various sample types by real-time reverse transcription polymerase chain reaction. This figure is a graphic representation of table 1. OF, oral fluid; OS, oral swab; NS, nasal swab, Bld, blood (serum).

Classical swine fever virus

Experiment 1:

All pigs had pyrexia (rectal temperatures of 40.2 to 41.8°C) by 6 DPI. By 9 DPI the pigs were depressed, slow to rise and anorexic. They also had piloerection at 13 DPI and dry faeces. Three of four pigs had either skin hemorrhages or erythema at 15-16 DPI. At 18 DPI, one pig died suddenly and the rest of the pigs were euthanized.

CSFV genome (at a level considered positive) was detected in OF starting at 10 DPI and levels increased until the end of the experiment at 18 DPI (figure 4).

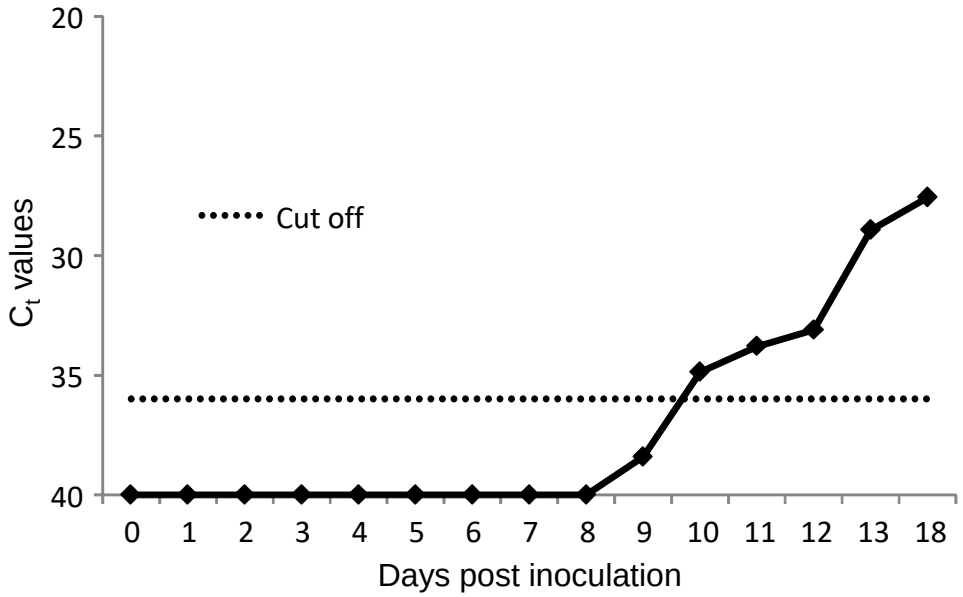

Figure 4: Detection of CSFV in oral fluids by real-time reverse transcription polymerase chain reaction.

Experiment 2:

Pigs started showing either skin hemorrhages or erythema at 3 DPI. All pigs had mild pyrexia (rectal temperatures of 40.3 to 41.0°C) by 8 DPI. By 9 DPI the pigs were depressed, slow to rise and passed dark dry faeces. Some pigs had severe clinical signs including severe diarrhea, generalized skin haemorrhages and were euthanized for humane reasons.

CSFV genome was detected in OF starting at 14 DPI, coinciding with detection in oral swabs. However, CSFV detection in serum was earlier and stronger compared to OF and swabs (Table 2).

	Group A	-1	1	2	3	4	5	6	7	14	21	28
OF	OF	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	35.2		
Oral swabs	p61	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	36.2	40.0
	p62	40.0	40.0	40.0	40.0	40.0	40.0	40.0	39.7	40.0	34.7	33.5
	p63	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	37.6	40.0	
	p64	40.0	40.0	40.0	40.0	40.0	40.0	40.0	38.3	40.0		
Serum	p61	40.0	40.0	40.0	40.0	40.0	40.0	39.7	34.0	32.6	38.4	40.0
	p62	40.0	40.0	40.0	40.0	40.0	40.0	38.0	32.7	27.5	33.1	31.6
	p63	40.0	40.0	40.0	40.0	40.0	40.0	40.0	35.8	27.7	29.9	
	p64	40.0	40.0	40.0	40.0	40.0	40.0	36.9	34.6	26.8		
	Group B	**-1**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**14**	**21**	**28**
OF	OF	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	36.2	24.9	25.5
Oral swabs	p65	40.0	40.0	40.0	40.0	40.0	40.0	40.0	35.7	39.0	35.8	37.6
	p66	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	36.3		
	p67	40.0	40.0	40.0	40.0	40.0	40.0	40.0	35.7	37.0	25.7	
	p68	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	36.9	33.2	40.0
Serum	p65	40.0	40.0	40.0	40.0	40.0	40.0	36.9	34.3	27.4	32.5	30.7
	p66	40.0	40.0	40.0	40.0	40.0	40.0	34.7	32.7	27.5		
	p67	40.0	40.0	40.0	40.0	40.0	40.0	36.2	33.1	25.3	30.1	
	p68	40.0	40.0	40.0	40.0	40.0	40.0	36.6	33.1	27.8	33.6	35.1
	Group C	**-1**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**14**	**21**	**28**
OF	OF	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	37.0	37.4	40.0
Oral swabs	p69	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	36.8
	p70	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	39.6	34.9	
	p71	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	35.9	40.0
	p72	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0
Serum	p69	40.0	40.0	40.0	40.0	40.0	40.0	40.0	38.6	39.5	40.0	40.0
	p70	40.0	40.0	40.0	40.0	40.0	40.0	37.8	34.1	31.7	40.0	
	p71	40.0	40.0	40.0	40.0	40.0	40.0	37.3	36.4	40.0	40.0	40.0
	p72	40.0	40.0	40.0	40.0	40.0	40.0	37.7	34.4	36.3	40.0	40.0
	Group D	**-1**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**14**	**21**	**28**
OF	OF	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	34.5		
Oral swabs	p73	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	36.8	40.0	
	p74	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	
	p75	40.0	40.0	40.0	40.0	40.0	40.0	40.0	36.1	32.8	36.9	
	p76	40.0	40.0	40.0	40.0	40.0	40.0	40.0	38.9	40.0	34.2	
Serum	p73	40.0	40.0	40.0	40.0	40.0	40.0	37.4	35.8	27.3	32.8	
	p74	40.0	40.0	40.0	40.0	40.0	40.0	40.0	36.3	30.1	30.9	
	p75	40.0	40.0	40.0	40.0	40.0	40.0	40.0	36.0	28.9	34.2	
	p76	40.0	40.0	40.0	40.0	40.0	40.0	38.0	35.9	28.6	29.8	

Table 2: Classical swine fever virus detection in oral fluids and other samples by real-time reverse transcription polymerase chain reaction. OF, oral fluid; DPI, days post infection; green cells represent positive results. Crossing threshold (Ct) < 35.99 was considered positive for CSFV genome. The lower the Ct value, the higher the amount of SVDV RNA. Any sample with undetermined Ct was given a Ct of 40.

African swine fever virus

ASFV Malta – infected pigs had fever (rectal temperatures of 40.1 to 42.2) by 7 DPI. One pig was euthanized at 9 DPI for humane reasons. By 21 DPI 56% of the pigs had either died or been euthanized for humane reasons because of severe clinical signs including bloody faeces, blood dripping from the rectum, paleness and loss of appetite.

ASFV genome was detected in OF starting at 6 DPI to 14 and 21 DPI. Detection of virus in oral and nasal swabs mirrored detection in OF. However, whole blood was the best sample type for ASFV detection, becoming positive at 4 DPI and containing higher levels of virus genome in most pigs (Table 3 and figure 5).

	Group A	0	2	4	6	8	10	12	14	21	28
OF	OF	40.0	40.0	40.0	29.8	32.4	37.3	32.4	28.0	34.5	36.8
Oral swabs	125	40.0	40.0	40.0	40.0	37.0	30.6	29.9	28.8	32.2	
	126	40.0	40.0	37.1	22.7	21.2					
	127	40.0	40.0	40.0	34.6	37.1	30.5	25.6	33.7	40.0	34.0
	128	40.0	40.0	40.0	36.9	35.1	30.2	27.6	33.3	36.4	40.0
Nasal swabs	125	40.0	40.0	40.0	36.3	33.5	35.5	30.2	28.2	31.7	
	126	40.0	40.0	34.0	27.9	25.2					
	127	40.0	40.0	40.0	35.8	32.5	31.5	31.5	29.4	35.9	34.9
	128	40.0	40.0	40.0	37.0	29.9	36.2	40.0	32.4	32.9	32.1
Blood	125	40.0	40.0	40.0	40.0	40.0	40.0	20.3	23.6	19.6	
	126	40.0	40.0	19.7	19.2	22.3					
	127	40.0	40.0	40.0	40.0	40.0	30.6	22.1	23.5	20.7	23.3
	128	40.0	40.0	40.0	40.0	40.0	40.0	23.3	23.6	21.7	25.2
	Group B	0	2	4	6	8	10	12	14	21	28
OF	OF	40.0	40.0	40.0	0.0	25.0	26.1	27.3	28.2	28.4	36.2
Oral swabs	129	40.0	40.0	40.0	34.0	33.1	31.3	24.7	40.0	31.8	
	130	40.0	40.0	40.0	22.0	27.0	29.4				
	131	40.0	40.0	40.0	24.5	26.4	25.8	28.2	40.0		
	132	40.0	40.0	40.0	24.2	22.4	28.2	31.2			
Nasal swabs	129	40.0	40.0	40.0	35.6	31.6	32.7	26.8	29.7	33.1	
	130	40.0	40.0	37.5	29.5	32.5	29.4				
	131	40.0	40.0	40.0	28.6	24.2	28.4	32.0	35.7		
	132	40.0	40.0	37.6	27.1	23.3	28.2	29.9			
Blood	129	40.0	40.0	40.0	40.0	40.0	26.3	24.7	22.7	22.4	20.6
	130	40.0	40.0	23.3	18.1	21.8	23.5				
	131	40.0	40.0	22.7	19.0	23.4	24.2	26.7	26.7		
	132	40.0	40.0	20.2	18.5	20.5	23.4	20.3			
	Group C	0	2	4	6	8	10	12	14	21	28
OF	OF	40.0	40.0	40.0	30.2	28.1	32.6	34.5	27.2	36.4	32.3
Oral swabs	133	40.0	40.0	40.0	40.0	33.3	33.1	27.0	30.3		
	134	40.0	40.0	40.0	30.6	27.0	32.4	33.6	37.6	36.5	33.7
	135	40.0	40.0	40.0	25.7	26.2	27.8	31.5			
	136	40.0	40.0	40.0	36.8	31.3	33.2	35.1	40.0	40.0	40.0
Nasal swabs	133	40.0	40.0	40.0	35.5	34.2	35.3	24.9	28.9		
	134	40.0	40.0	40.0	27.6	28.5	31.4	35.3	37.6	36.6	34.8
	135	40.0	40.0	40.0	27.9	27.2	28.6	32.3			
	136	40.0	40.0	40.0	36.9	35.3	36.7	30.6	33.1	30.8	33.6
Blood	133	40.0	40.0	40.0	40.0	40.0	29.3	23.9	20.4		
	134	40.0	40.0	28.9	19.6	21.0	25.8	26.7	28.4	20.9	24.6
	135	40.0	40.0	23.3	18.5	21.2	25.0	21.5			
	136	40.0	40.0	40.0	40.0	40.0	40.0	21.3	24.8	24.8	24.0
	Group D	0	2	4	6	8	10	12	14	21	28
OF	OF	40.0	40.0	40.0	26.8	24.8	29.2	34.7	34.0	36.5	36.2
Oral swabs	137	40.0	40.0	40.0	30.6	29.2	30.7	28.9			
	138	40.0	40.0	40.0	28.8	31.8	30.5	34.1	32.1	33.9	32.8
	139	40.0	40.0	36.5	27.1	26.2	28.6				
	140	40.0	40.0	40.0	22.4	26.4	28.8				
Nasal swabs	137	40.0	40.0	40.0	30.5	29.4	25.6	30.4			
	138	40.0	40.0	40.0	27.9	29.9	28.9	31.5	32.1	36.4	37.2
	139	40.0	40.0	33.8	26.8	25.7	25.9				
	140	40.0	40.0	36.0	26.5	27.0	28.3				
Blood	137	40.0	40.0	29.4	17.7	22.7	24.6	20.9			
	138	40.0	40.0	21.9	21.7	22.4	26.2	23.4	25.4	23.4	23.4
	139	40.0	40.0	21.6	19.3	22.6	22.5				
	140	40.0	40.0	20.6	19.2	21.6	25.9	23.9			

Table 4: African swine fever virus detection in oral fluids and other samples by real-time reverse transcription polymerase chain reaction. OF, oral fluid; DPI, days post infection; green cells represent positive results. Crossing threshold (Ct) < 35.99 was considered positive for CSFV genome. The lower the Ct value, the higher the amount of SVDV RNA. Any sample with undetermined Ct was given a Ct of 40.

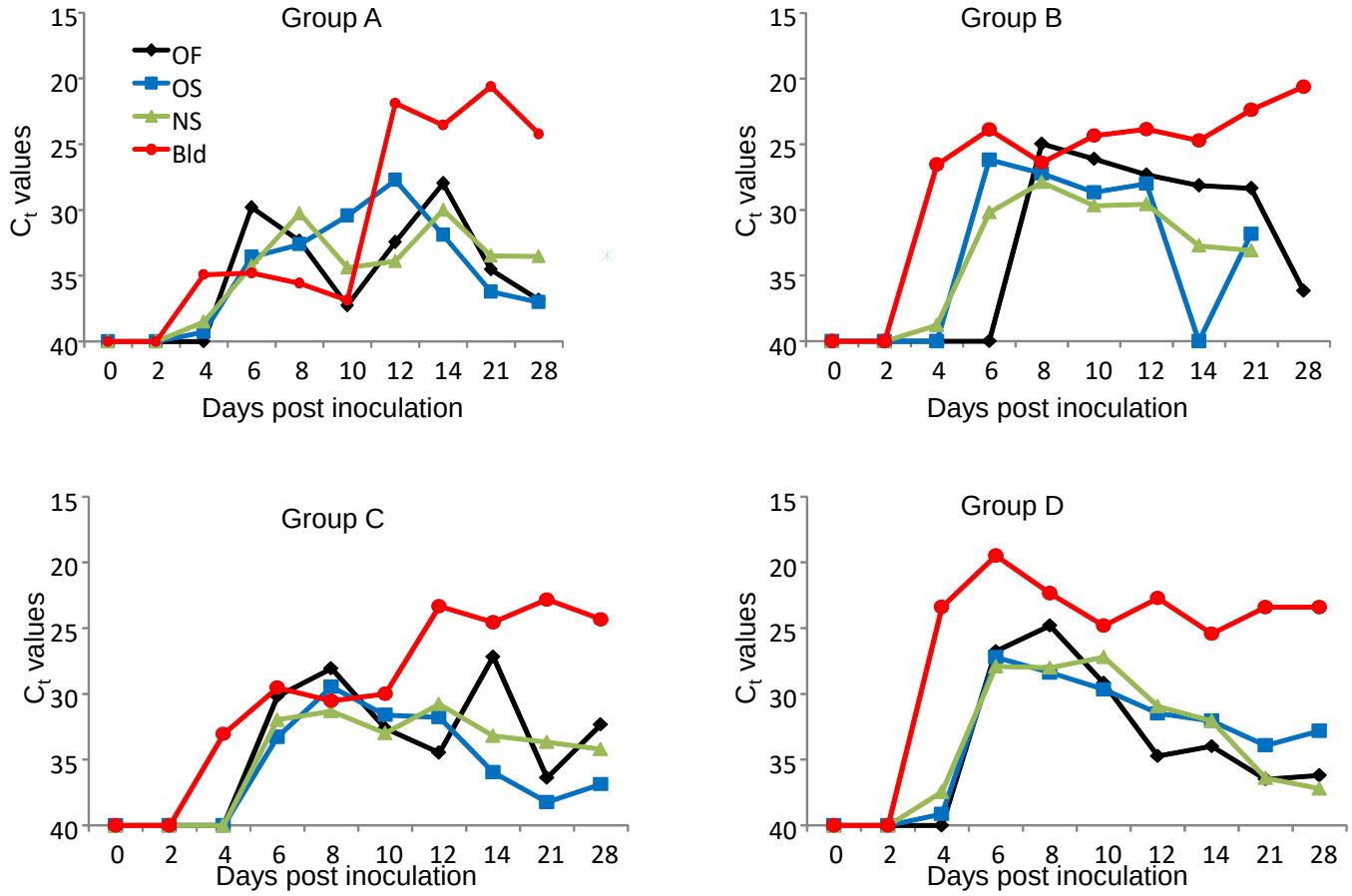

Figure 5: Detection of ASFV in various sample types by real-time polymerase chain reaction. This figure is a graphic representation of table 4. OF, oral fluid; OS, oral swab; NS, nasal swab, Bld, blood (whole blood).

c) Integration and validation of a fully automated multiplex assay for simultaneous detection of FMDV, SVDV, CSFV and ASFV.

The first step in assay development was the optimization of the CARD RT-PCR amplification component. Optimizations included reduction in reaction volume from 25 µL to 15 µL resulting in improved thermodynamics of the reaction, ensuring that the reagents reached the correct temperatures during the denaturation, annealing and elongation phases of the PCR cycle. In addition, 100 µg/mL of bovine serum albumin (BSA) was added to prevent inhibition, block adsorption to vessel walls and stabilize the polymerase. Furthermore, the volume of polymerase and extracted nucleic acid used in the reaction were optimized to reduce the effects of any inhibitors that may be present in the sample. Also reverse primers were modified by adding an internal spacer between the 5'-biotin group and the reverse primer sequence. The optimized CARD RT-PCR amplification had sensitivity comparable to bench-top thermocyclers.

Next, the array-based detection subcomponent was optimized. The optimal hybridization temperature was determined by assessing the hybridization of target pathogen amplicons on unmounted off-CARD filters on the bench-top at 40°C, 45°C, 48°C and 50°C according to the manufacturer's specifications. Some probes performed better at lower hybridization temperatures, prompting the selection of a hybridization temperature of 40°C to maximize the reactivity and specificity of the assay.

A panel of 21 laboratory amplified target viruses and 4 non-targets were correctly detected and differentiated (figure 13) by the multiplex CARD® assay. No cross reactivity was observed between probes and the non-targets.

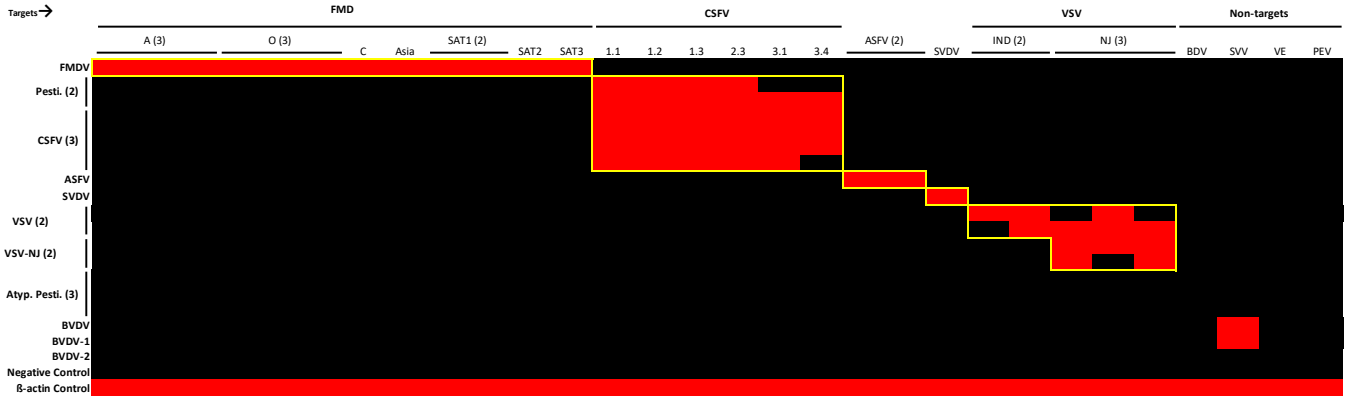

Figure 6: Summary of results from on-CARD testing with the Encompass MDx workstation. Areas in red indicate a positive probe with a fluorescent intensity above 30, while those in black are negative and below 30. Areas contained within a yellow box indicate expected positive sample to probe interactions.

Samples from experimentally infected animals were then tested on the prototype integrated assay to assess detection of viruses in clinical samples (Table 5). The fully integrated multiplex assay successfully detected FMDV, SVDV, ASFV and CSFV in OF. Virus genome detection in OF was comparable to singleplex qRT-PCR assays except for ASFV which detected fewer samples than singleplex real-time PCR.

Virus	Samples	Animal	Sample Type	dpi	CARD Result	qRT-PCR results	qRT-PCR Ct
FMDV	O UKG 11/2001	Group A	Oral Fluids	2	+	+	23.6
				3	+	+	23.1
		Group B		3	+	+	19.4
		Group C		3	+	+	23.1
		Group D		-1	-	-	40.0
				1	+	+	24.4
				2	+	+	24.1
				3	+	+	22.8
				4	-	+	29.9
				5	+	+	24.6
				6	-	-	40.0
				7	-	-	36.1
				21	-	-	40.0
		Group A		2	+	+	23.6
				3	+	+	23.1
		Group B		3	+	+	19.4
		Group C		3	+	+	23.1
SVDV	UK 27/72	Group A	Oral Fluids	0	-	-	40.0
				1	+	+	29.3
				2	+	+	24.4
				3	+	+	25.8
				4	+	+	22.7
				5	+	+	26.0
				6	+	+	25.6
				7	+	+	28.2
				14	-	+	31.4
				21	-	-	37.8
				28	-	-	40.0
		Group D	Oral Fluids	0	-	-	40.0
				1	+	+	29.7
				2	+	+	23.6
				3	+	+	24.5
				4	+	+	25.2
				5	+	+	21.2
				6	+	+	25.1
				7	+	+	28.0
				14	-	+	28.9
				21	-	+	34.8
				28	-	-	40.0
CSFV	Diepholz	Group A	Oral Fluids	0	-	-	40.0
				6	-	-	40.0
				7	-	-	40.0
				14	-	+	35.2
				20	+	-	37.8
ASFV	Malawi	Group A	Oral Fluids	31	-	-	40.0
				33	-	-	40.0
				38	-	-	40.0
				42	-	+	33.2
		Group D	Oral Fluids	31	-	-	40.0
				33	-	-	40.0
				38	-	-	40.0
				42	-	+	29.9
			Oral Swab	40	+	+	27.2
				42	+	+	25.0
				44	+	+	21.2
			Nasal Swab	40	+	+	31.7
				42	+	+	24.7
				44	+	+	18.4
			Whole Blood	35	-	-	40.0
				40	+	+	20.6
			Oral Swab	35	-	-	40.0
				37	-	-	40.0
				40	+	+	30.3
				42	+	+	27.3
				44	+	+	22.3
			Nasal Swab	40	+	+	28.6
				42	+	+	21.2
				44	+	+	19.5
			Whole Blood (1/10)	40	+	+	23.0
			Whole Blood (1/4)	40	+	+	23.0
			Whole Blood (1/2)	40	+	+	23.0

Table 5: Summary of clinical sample results from this study

The limit of detection of the assay was different for each target (Table 6).

Virus	Limit of Detection (TCID50/mL)
FMDV	25.7
CSFV	6309.6
SVDV	1241.1
ASFV	498.8

Table 6: Limit of detection of CARD assay

d) Validation/methods comparison studies for detection of antibodies to FMDV, CSFV, ASFV and SVDV in OF and serum specimens collected during the convalescent phase of infection.

Foot-and-mouth disease virus

The competitive ELISA for antibodies to FMDV 3B nonstructural protein was unsuccessful at detecting anti-3B antibodies in OF. Further work on this assay was therefore abolished and focus was shifted to anti-FMDV IgA ELISA. The results of this experiment have been published (Senthilkumaran C, Yang M, Bittner H, Ambagala A, Lung O, Zimmerman J, Giménez-Lirola LG, Nfon C. Detection of genome, antigen, and antibodies in oral fluids from pigs infected with foot-and-mouth disease virus. *Can J Vet Res. 2017 81(2):82-90.*)

Swine vesicular disease virus

Experiment 1:

The results of this experiment have been published (Senthilkumaran C, Bittner H, Ambagala A, Lung O, Babiuk S, Yang M, Zimmerman J, Giménez-Lirola LG, Nfon C. Use of Oral Fluids for Detection of Virus and Antibodies in Pigs Infected with Swine Vesicular Disease Virus. *Transbound Emerg Dis.* 2016 Sep 15. doi: 10.1111/tbed.12563).

Experiment 2:

The SVDV cELISA, IgM and IgA response patterns in experiments 2 were similar to experiment 1 (Table 7 and Figure 7 A and B).

		DPI 0	DPI 1	DPI 2	DPI 3	DPI 4	DPI 5	DPI 6	DPI 7	DPI 14	DPI 21
Group A	Pig 109	0	0	0	0	27	84	94	96	98	99
	Pig 110	0	0	7	0	25	83				
	Pig 111	0	0	0	11	82	92	91	96	99	
	Pig 112	0	0	1	0	51	85	93	94	98	
Group B	Pig 113	0	0	0	0	17	60	95	93	97	98
	Pig 114	0	3	7	37	91	99	85	99	99	99
	Pig 115	0	0	0	8	66	86	99	91	95	
	Pig 116	0	0	0	3	71	91	89	96	95	95
Group C	Pig 117	0	0	0	7	43	89				
	Pig 118	0	0	0	4	56	92	94	95	99	97
	Pig 119	4	0	0	28	85	95	95	96	95	
	Pig 120	0	0	0	27	65	91	96	97		
Group D	Pig 121	0	0	0	0	35	78	95	93	97	
	Pig 122	0	0	0	6	58	89	90	97	98	98
	Pig 123	0	2	4	42	54	96	98	97	97	97
	Pig 124	0	16	6	11	91	90	95	96	96	
Oral fluids	Group A	16	14	0	17	14	24	29	39	51	36
	Group B	4	16	17	10	1	17	36	44	49	30
	Group C	12	17	19	14	20	21	52	30	44	6
	Group D	21	21	22	8	12	18	34	48	33	26

Table 7: Antibody detection in serum and oral fluids from SVDV-inoculated pigs. DPI, days post infection; antibody response measured as percent inhibition (PI), sera with PI ≥ 55 are considered positive for antibodies to SVDV (green cells) and oral fluids with PI ≥ 24 are considered positive for antibodies to SVDV (orange cells). Grey cells indicate the pig either died or was euthanized.

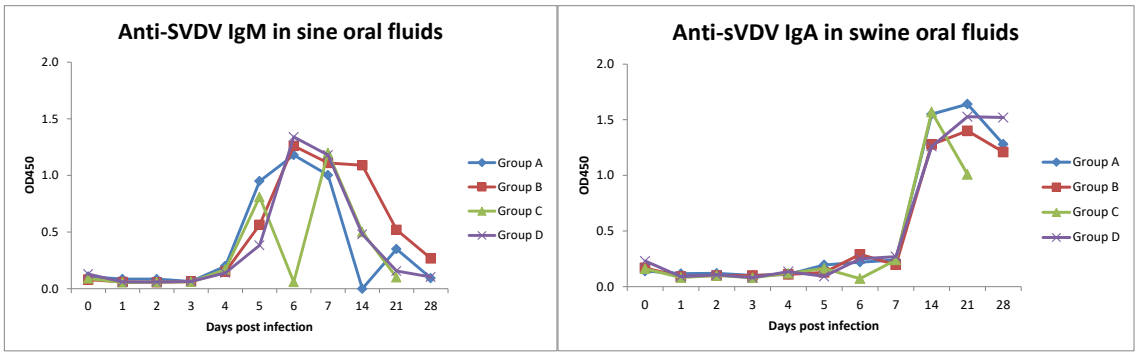

Figure 7: Anti-SVDV IgM and IgA detection in oral fluids by indirect ELISAs

Classical swine fever virus

Experiment 1:

A cutoff of 15% blocking (mean + 3 standard deviation) was established for anti-CSFV antibodies in swine OF after testing 303 negative OF. Positive antibody response in OF in experiment 1 (n = 1 group) was at 9 and18 DPI (Figure 8). However, antibody levels started rise at 5 DPI. The fluctuation in antibody levels between 9 and 13 DPI could be due to the OF collection and the level of interest each pig had in the ropes considering that some pigs may have been contributing more to this group sample than others.

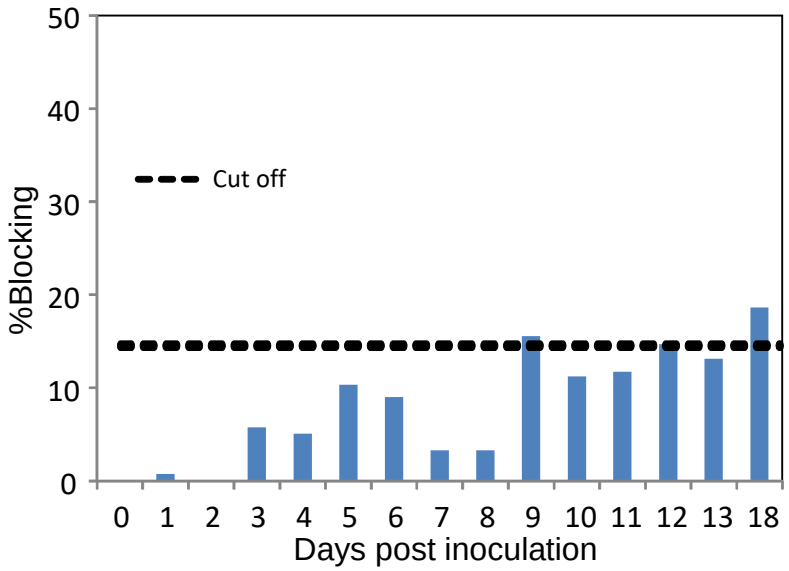

Figure 8: Anti-CSFV antibodies detection in oral fluids by the IDDEX blocking ELISA

Experiment 2:
Using the previously established cutoff of 15% blocking positive antibody response in OF in experiment 2 (n = 4 groups) started at 14 DPI in group B and 21 DPI for group C. Antibody response in groups A and D never rose above the cutoff (Table 8). Serum antibody response was also delayed, with positive detection starting at 14 DPI. The cutoff for serum antibodies considered positive is ≥ 30% blocking.

		DPI 0	DPI 1	DPI 2	DPI 3	DPI 4	DPI 5	DPI 6	DPI 7	DPI 14	DPI 21	DPI 28
Group A	Pig 61	0	4	5	0	12	9	1	3	19	30	45
	Pig 62	6	14	12	17	19	21	5	0	0	42	22
	Pig 63	3	3	6	7	12	10	0	0	0	24	
	Pig 64	2	2	3	7	7	2	6	9	25		
Group B	Pig 65	1	3	0	0	0	0	7	4	1	38	27
	Pig 66	2	0	0	4	9	4	3	3	22		
	Pig 67	0	0	0	6	5	0	5	0	15	31	
	Pig 68	1	6	2	12	15	5	5	6	32	44	42
Group C	Pig 69	5	2	1	5	13	2	1	0	14	62	66
	Pig 70	10	8	2	12	15	7	0	0	5	29	
	Pig 71	2	3	0	13	12	5	0	0	36	65	77
	Pig 72	2	1	0	0	0	0	1	4	31	57	68
Group D	Pig 73	0	1	0	0	0	1	9	3	48		
	Pig 74	0	3	0	4	7	0	0	2	22		
	Pig 75	0	5	6	6	8	2	11	4	27	42	
	Pig 76	5	4	1	6	6	2	4	0	12	19	
Oral fluids	Group A	0	2	16	1	12	7		0	1	10	12
	Group B	4	6	10	1	0	5	14	5	21	26	6
	Group C	0	0	2	0	3	0	11	2	7	35	36
	Group D	8	13	13	1	0	0	0	2	13		

Table 8: Antibody detection in serum and oral fluids from CSFV-inoculated pigs. DPI, days post infection; antibody response measured as percent blocking, sera with %blocking ≥ 30 are considered positive for antibodies to CSFV (green cells) and oral fluids with %blocking ≥ 15 are considered positive for antibodies to CSFV (orange cells). Grey cells indicate the pig either died or was euthanized.

African swine fever virus

The cutoff for serum considered positive for antibodies to ASFV is OD ≥ 0.32. Positive detection of serum antibody response to ASFV started at 10 DPI. Positive antibody response in OF (n = 4 groups) was only detected at 10 DPI in group B and D (Table9).

	Animal #	DPI 0	DPI 2	DPI 4	DPI 6	DPI 8	DPI 10	DPI 12	DPI 14	DPI 21	DPI 28
Group A	125	0.115	0.136	0.129	0.156	0.207	0.108	0.285	0.129	0.670	
	126	0.076	0.113	0.111	0.118	0.288					
	127	0.070	0.088	0.087	0.074	0.073	0.066	0.267	0.066	0.487	0.594
	128	0.107	0.145	0.149	0.135	0.105	0.133	0.249	0.113	0.487	0.548
Group B	129	0.069	0.105	0.112	0.105	0.116	0.085	0.092	0.327	0.545	
	130	0.081	0.097	0.108	0.079	0.285	0.446				
	131	0.121	0.134	0.155	0.113	0.294	0.397	0.367	0.407		
	132	0.056	0.157	0.132	0.118	0.265	0.385	0.372			
Group C	133	0.093	0.120	0.131	0.253	0.173	0.118	0.312	0.106		
	134	0.079	0.117	0.111	0.109	0.240	0.410	0.474	0.475	0.536	0.529
	135	0.070	0.126	0.140	0.135	0.268	0.365	0.332			
	136	0.107	0.121	0.142	0.194	0.129	0.110	0.166	0.097	0.650	0.645
Group D	137	0.083	0.106	0.095	0.085	0.289	0.413				
	138	0.056	0.081	0.075	0.096	0.231	0.305	0.271	0.313	0.434	0.532
	139	0.150	0.132	0.125	0.261	0.492	0.825				
	140	0.121	0.201	0.167	0.149	0.378	0.397	0.324			
Oral fluids	Group A	0.05	0.051	0.07	0.041	0.066	0.124	0.153	0.084	0.11	0.111
	Group B	0.076	0.027	0.063	0.193	0.064	0.403	0.125	0.088	0.01	
	Group C	0.036	0.035	0.053	0.063	0.068	0.096	0.124	0.093	0.062	0.18
	Group D	0.026	0.033	0.109	0.116	0.101	0.407	0.037	0.047	0.001	0.109

Table 9: Antibody detection in serum and oral fluids from ASFV-inoculated pigs. DPI, days post infection; antibody response measured OD values, sera with ODs ≥ 0.32 are considered positive for antibodies to ASFV (green cells) and oral fluids with ODs ≥ 0.2 are considered positive for antibodies to ASFV (orange cells). Grey cells indicate the pig either died or was euthanized.

e) Once methods have been established, perform a feasibility evaluation of whether PrimeStore® Molecular Transport Medium (MTM) will support antibody recovery for downstream serological testing on archived samples.

Oral fluids collected from SVDV infected pigs in SVDV experiment 1 at 2 and 21 DPI preserved in MTM were tested in parallel with corresponding OF stored at -70°C for anti-SVDV IgA by ELISA. MTM apparently interfered with antibody detection in OF, as well as increased the background for negative samples (figure 18). With these results, testing of MTM-preserved samples for antibodies was discontinued.

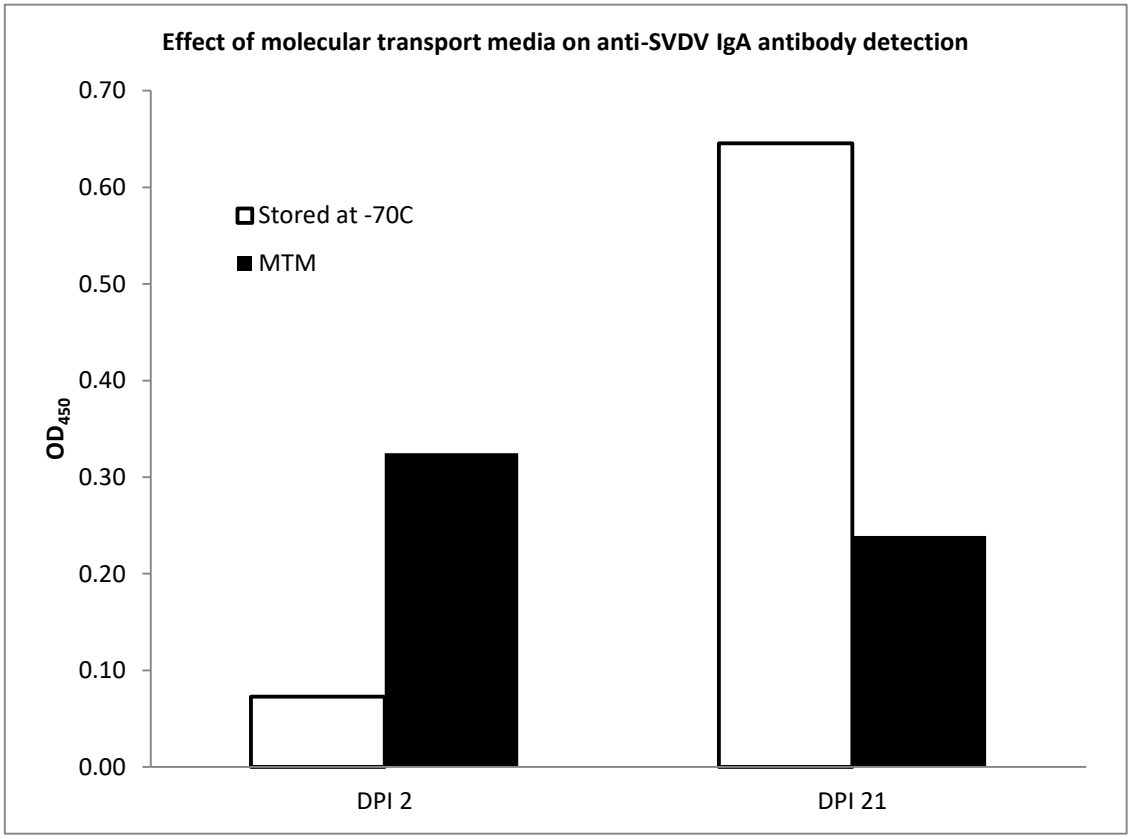

Figure 9: Effect of molecular transport media (MTM) on anti-SVDV IgA antibody detection. DPI= days post infection

IX. Discussion:

Some of the data in this report has previously been discussed in two publications (Senthilkumaran et al. 2016 and 2017).

The cost of sampling individual animals, the requirement for high numbers in case of diseases with low prevalence, and the risks involved in restraining animals for sample collection from individual animals for disease surveillance has driven research on the use of OF for swine disease surveillance. Part of the objectives for this study were to evaluate the utility of OF for FMDV, SVDV, CSFV and ASFV detection. We have successfully detected the genome of these viruses in OF from groups of infected pigs by qRT-PCR. Interestingly, for FMDV and SVDV, genome detection in OF was stronger and more prolonged compared to oral and nasal swabs. Additionally, FMDV and SVDV RNA detection in serum was of very short duration compared to OF. Importantly, SVDV detection in OF was possible even in groups of pigs with subclinical disease. Recent strains of SVDV have evolved to cause subclinical disease (Bellini et al. 2007) and therefore can be missed if detection is based on clinical signs. This report adds to the growing body of literature on the use of OF for detection of swine infectious diseases (Prickett et al. 2008; Romagosa et al. 2012; Mur et al. 2013; Vosloo et al. 2013; Grau et al. 2015). Our data indicates a likely superiority of OF over swabs and serum for FMDV and SVDV detection. Viremia in pigs infected with FMDV is usually transient while virus shedding in oral and nasal secretions may be prolonged (Nfon et al. 2010; Alexandersen et al. 2003). Therefore extended detection of FMDV and SVDV in OF relative to serum is not surprising. However, the strong detection of these viruses in OF compared to oral swabs is novel and likely due to the fact that chewing on ropes allows for enhanced transudation

from capillaries, sampling of wider surfaces, and collection of more concentrated fluids than is achieved with swabs. Additionally, swabs are usually dipped into collection media which may dilute any viral particles present, thereby reducing the level of detection. Virus isolation is the confirmatory test for most virus detection assays. FMDV and SVDV were isolated from OF at high titres compared to individual and average isolation from OS. However, virus was also not isolated from OF at some time points despite the presence of virus genome which could be attributed to reduced levels of virus at later time points. Additionally, antibodies are likely present in OF starting at DPI 6 leading to be virus neutralization and thus the inability to infect cell cultures for virus isolation.

Contrary to the vesicular disease agents (FMDV and SVDV), the haemorrhagic disease agents (CSFV and ASFV) detection in OF was delayed, weaker in some cases compared to serum/blood. Given the data, blood and serum are the sample of choice in case any of these haemorrhagic diseases is suspected. However, there is still potential for using OF if the goal is herd level surveillance.

Mucosal immunity to FMDV, SVDV, CSFV and ASFV in swine is not fully understood and OF provide an opportunity for evaluating mucosal antibody response to these viruses. Antibodies in OF can be secreted locally or derived from systemic antibodies through leakage. Indeed, plasma cells present in salivary glands and duct-associated lymphoid tissue (DALT) secrete IgA into OF. Although the bulk of IgM and IgG in OF originates from blood, IgM and IgG are also produced in plasma cells located in salivary glands, tonsils, and other DALT (Nair et al. 1986; Decorte et al. 2014). Antibodies in OF were detectable by C-ELISA (SVDV), blocking ELISA (CSFV) and indirect ELISA (ASFV). Additionally, our isotype ELISAs detected specific IgM (SVDV) and IgA in OF (FMDV and SVDV). Given that IgA is the predominant mucosal antibody isotype (Decorte et al. 2014; Pacheco et al. 2010), the serological assays reported here can be used to detect antibody response to FMDV, SVDV, CSFV and ASFV in OF. Our data is consistent with previous reports for specific IgM and IgA detection in OF for viruses endemic in North American swine, including influenza A virus (Panyasing et al. 2013), PRRSV (Kittawornrat et al. 2013; Kittawornrat et al. 2012), and porcine circovirus type 2 (Prickett et al. 2011).

In summary, FMDFV, SVDV, CSFV and ASFV genome and infectious virus, as well as antibodies to these viruses are detectable in OF from infected pigs. However, there is the possibility that virus detection in OF may vary for different virus species, between serotypes of the same virus, under different experimental settings, and/or sample handling and testing conditions. Collectively, these data reveal the potential of swine OF in replacing serum and other sample types for routine virological and serological surveillance of swine herds for FADs. With further validation, the assays developed and/or evaluated in this study could be used for testing for FADs in swine OF.

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