RESEARCH REPORT
SWINE HEALTH

Title: Rapid detection and epidemiological surveillance of African swine fever using oral fluid – **NPB #15-125**

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Date Submitted: 2.09.2018

Industry Summary:

African swine fever (ASF) is a highly lethal and contagious disease of domestic and wild pigs caused by the ASF virus. ASF is commonly found in Africa, but in 2007 spread to Russia and Eastern Europe, and continues to spread within domestic pigs and wild boar populations. ASF is a serious risk to the global pig population and a challenge for the animal health sector in other countries including the U.S. There is no vaccine available for ASF; therefore, rapid and accurate diagnostic tools will be critical for control and surveillance in the event of an outbreak in the U.S. Oral fluid-based diagnostics provide a possible solution by allowing rapid pen-based sampling to monitor disease progression and intervention measures during disease outbreaks. Oral fluids are an aggregate sample collected from a group of pigs using rope chew sampling. Oral fluid sampling of groups is simple, inexpensive, safe, and less labor and animal intensive than individual sampling methods. This sampling method is user-friendly and the technology is easily transferrable to farmers and animal care specialists.

The purpose of this project was to compare and validate oral fluids (pooled sample) for the laboratory diagnosis of ASF infections in pigs. In doing so, we evaluated commercially-available diagnostic tests for detecting antibodies to the ASF virus and virus DNA. In the event of the detection of ASF in North America, the demand for testing will rapidly overwhelm national testing capacity. The only way to deal with this anticipated demand is through the use of commercial kits. The second aim of this project was to compare different specimen types, including oral fluids, to assess their suitability for diagnostic testing using our tests.

In order to produce diagnostic specimens to test in the laboratory, we infected pigs experimentally with the ASF virus within a high biocontainment animal facility. Blood samples, along with oral fluids were collected at various time points to test for virus and antibodies. We found that the appearance of antibodies and virus in oral fluids occurred at a similar time to blood, which is a traditional diagnostic specimen, but invasive to collect. Therefore, our results indicate that oral fluids have the potential to serve as a cheap and rapid sample type for diagnosing ASF infections. We also identified the best commercial kits available for sensitive antibody and DNA testing. Importantly, antibody tests were shown to be less sensitive than a non-commercial reference test, highlighting the need for more research and development in this area.

The results from this study have enabled us to identify sensitive diagnostic tools that are available commercially and can be applied to a variety of scenarios, such as diagnosis of infection during an outbreak, surveillance, and proof of freedom from ASF. The information we have generated will assist the swine industry and animal health decision makers concerning the tools and surveillance strategies available to deal with the potential emergence of ASF in North America. The results derived from this project have also underlined the need for future research on improved ASFV diagnostics.

Keywords: African swine fever, oral fluids, diagnostics, detection, surveillance

Scientific Abstract:

African swine fever (ASF) is a highly lethal and contagious disease of domestic and wild pigs. ASF is endemic to Africa, however in 2007 the ASF virus (ASFV) spread to Eastern Europe and subsequently spread to nine other countries, causing devastating impacts to their pork industries. If ASF were to emerge in the USA, the availability of accurate diagnostic tools and effective surveillance strategies would be critical for rapid detection and control. Oral fluid-based diagnostics provide a possible solution by allowing pen-based sampling. Using specimens derived from an experimental ASFV contact-infection model, we compared the diagnostic performance of a selection of commercially available diagnostic kits and OIE-recommended tests for the detection of specific antibodies and viral DNA. A comparison of diagnostic performance as a function of specimen (oral fluid vs. serum) was also performed. In three groups of pigs (n=6, n=8, n=16), two, two and four "donor" pigs, respectively, were inoculated intramuscularly with a moderately pathogenic strain of ASFV (Malta/78) and then returned to the remaining (contact) pigs in each group. The donor pigs developed severe disease between 6 and 11 days post infection (dpi) and were euthanized having reached predetermined humane endpoints. Contact pigs began showing clinical signs from day 5 post infection, which progressed to severe clinical signs between 14-35 dpi, at which point the majority of pigs were euthanized. Oral fluids collected with cotton rope, oral swabs, and faeces (pen) were collected daily and blood was taken every 2-3 days. Serological testing for ASFV antibodies in serum and oral specimens was completed using commercially available ELISAs from Ingenasa, IDvet and Svanova, and compared to the 'gold standard' immunofluorescence antibody (IFA) test. The IFA test proved to be more sensitive than the ELISA for detecting serum antibody, however, was comparable to a prototype IDvet Oral Fluids indirect ELISA. These results highlight the need for further development and commercialisation of sensitive ELISAs. Two commercial PCRs (Ingenasa qPPA and the IDEXX RealPCR ASFV DNA test) were also evaluated and compared with an OIE-recommended assay. All three assays showed similar levels of sensitivity for detecting ASFV DNA in blood. However, the IDEXX PCR was markedly more sensitive for detecting ASFV DNA in oral swabs or fluids. This study supports the potential use of oral fluids for surveillance testing, and has identified sensitive diagnostic tools to assist the swine industry and animal health decision makers concerning the tools and surveillance strategies available to deal with the potential emergence of ASFV in North America.

Introduction:

With increasing globalization, the presence of transboundary animal diseases anywhere in the world is a serious risk to U.S. agriculture. ASFV was introduced into Georgia in 2007 and spread rapidly to neighboring countries, including the Russian Federation (RF), by movement of wild boar and infected pork meat (FAO, 2008; Sánchez-Vizcaíno et al., 2015). Initially limited to wild boar and the free-range pig production systems in the South of the RF, ASFV rapidly spread to the commercial pig production sectors in the North, then to

the West via wild boar and domestic pigs. ASFV reached Ukraine in 2012 and Belarus in 2013. Subsequently, cases have been reported in five EU countries (2014: Lithuania, Poland, Latvia and Estonia; 2017: Czech Republic) in both wild boar and domestic holdings. Under the present circumstances, the likelihood of eradicating ASFV from the RF and affected Eastern European countries is remote and ASFV will continue to threaten the food security of the whole of Eastern and Central Europe. Southeastward spread from the RF also threatens China – the world's largest producer of pigs. This worsening situation represents a serious risk to the global pig population and a challenge for the animal health sector in other countries including the USA. For this reason it is critical for US decision-makers, industry, and diagnostic laboratories to be prepared to deal with an incursion of ASFV. If such an event were to occur, accurate and cost-effective surveillance measures will be the key to the efficient eradication of ASFV and the rapid economic recovery of the swine industry.

ASF surveillance using oral fluids to enable early detection holds great potential to minimize the spread of disease. In the event of an ASFV outbreak in North America, the key to rapid detection and control is immediate access to accurate diagnostic tools and an efficient surveillance (sampling) strategy. The major barrier to efficient surveillance is the complexity and cost of collecting and testing statistically appropriate numbers of samples. Oral fluid-based diagnostics provide a possible solution to this problem by allowing for pen-based sampling.

Oral fluid is a mixture of saliva and oral mucosal transudate that contains pathogens and antibodies. Oral fluids are an aggregate sample collected from a group of pigs. As such, it represents the contribution of the pigs that chew on the rope (Olsen et al., 2013). Group sampling is cost-effective, diagnostically sensitive and specific, and practical, i.e., enables the surveillance of large populations of swine. Oral fluid sampling is simple, inexpensive, safe, and less labor and animal intensive. The method is user-friendly and the technology easily transferrable to farmers and animal care specialists. The ease of use and cost effectiveness of oral fluid enables multiple sampling to monitor disease progression and intervention measures and enables rapid sampling during disease outbreaks.

Oral fluids have been used to for the laboratory diagnosis of swine pathogens such as foot-and-mouth disease virus, porcine circovirus type 2 and porcine reproductive and respiratory syndrome virus (Kittawornrat et al., 2012; Prickett et al., 2008; Vosloo et al., 2013). Several PCR- and antibody-based diagnostic assays are currently available in commercial and public laboratories for the detection of ASFV using various diagnostic sample matrices, including oral fluid, serum, and feces (Aguero et al., 2003; King et al., 2003; Zsak et al., 2005). Stimulated by the global spread of ASFV, additional assays are under development. The current standard for detection of ASFV is real-time PCR (RT-PCR). Previous DHS-supported research (#HSHQDC-11-X-00528 and #2010-ST-061-AG0002) showed that oral fluids are a suitable sample for a highly sensitive RT-PCR. However, limiting the testing options to PCR is the diagnostic equivalent of "all eggs in one basket".

Serum antibody detection has historically been used in combination with virus detection in ASFV surveillance and control programs in the Iberian Peninsula and Sardinia, particularly as a tool for the detection of ASFV carrier animals. Research conducted by members of our research team confirmed that ASFV antibodies can also be detected in oral fluid (Mur et al., 2013). More recently, NPB-supported research (#13-048) resulted in the development of a "dual" ASFV antibody ELISA (manuscript in preparation). In brief, IgG antibodies were detected by 12 days post infection (DPI) in both serum and oral fluid specimens. Evaluation of 200 serum and oral fluid samples submitted to the Iowa State

University Veterinary Diagnostic Laboratory from U.S. swine herds (i.e., a known ASFV-negative population) showed specificities of 99.5% and 100% for serum and oral fluid samples, respectively. Thus, the test detected ASFV antibodies in both oral fluid and serum samples and is highly specific for both specimen types. Thus, screening (oral fluid) and confirmatory (serum) testing may be done simultaneously using the same ELISA kit.

Importance to Stakeholders

Traditionally, ASFV surveillance has been based on collecting and testing individual pig nasal swab and/or serum samples, but the labor and cost of this approach makes it unacceptable for routine use. For the reasons outlined above, a more efficient and effective surveillance system could be based on collecting and testing oral fluid specimens. In addition, the limited availability of validated commercial kits for use in oral fluids will limit its routine use in surveillance. The purpose of this project was to compare and validate oral fluid (pooled sample) for the molecular and serological detection of ASF. The results have enabled us to identify the most sensitive PCR- and ELISA-based diagnostic tools available commercially that can be applied to a variety of scenarios (e.g. negative cohort testing, diagnosis of infection during an outbreak, surveillance, vaccine monitoring, proof of freedom from ASF). The information we have generated on specimen and assay performance will inform the swine industry and animal health decision makers concerning the tools and surveillance strategies available to deal with the potential emergence of ASFV in North America. More specifically, this work will enable decision makers to make an informed selection of the best combination of specimen and test for different ASFV disease control scenarios. The results derived through this project have underlined the need for future research on improved ASFV diagnostics, particularly in the development of more sensitive ELISA tests.

Objectives:

Aim 1. Compare the diagnostic performance of available ASFV RT-PCR and ELISAs, including commercially available kits.

Aim 2. Compare diagnostic performance as a function of the diagnostic specimen tested [oral fluid vs. oral swabs (PCR and ELISA), vs. blood (PCR), vs. serum (ELISA) vs. fecal specimens (PCR and ELISA)].

Materials & Methods:

Ethics Statement. Animal work was approved by the CSIRO Australian Animal Health Laboratory (AAHL) Animal Ethics Committee (permit number 1795). All procedures were conducted per the guidelines of the National Health and Medical Research Council as described in the Australian code for the care and use of animals for scientific purposes, 8th edition (2013).

Virus. The Malta/78 strain of ASFV was used in this study, which was originally isolated from Malta in 1978 from the spleen of an infected pig (Wilkinson et al., 1981). ASFV Malta/78 has been characterized as moderately virulent in phenotype and belonging to genotype 1. All *in vitro* and *in vivo* work involving live ASFV was conducted within biosafety level 3 facilities at the AAHL. Prior to inoculation in pigs, virus was cultured in primary porcine bone marrow cells, as previously described (Jaing, et al., 2017).

Experimental infection of pigs. Groups of 5–6 weeks old Landrace cross domestic pigs were used in two separate infection trials. Pigs in both experiments were acclimatized in

the animal rooms for a period of approximately one week prior to inoculation. During this time, baseline samples were collected, including oral and pen fecal swabs, serum, blood. Fecal samples were collected from the floor of the pen and represent a group or aggregate sample. Temperatures were also recorded during the pre-challenge period and pigs undertook 'rope training', in which they were encouraged to chew on a cotton rope used for oral fluid collection. Ropes for oral fluid collection consisted of unraveled three-strand cotton rope (~12 mm wide and 20 cm long). Oral fluids from ropes were collected as previously described (Kittawornrat et al., 2013). Pigs were lightly anaesthetized for all blood and serum collections. The first experiment involved eight pigs: two donor pigs were physically separated from the remaining animals and inoculated intramuscularly (i.m.) with $10^{3.8}$ $TCID_{50}$ of virus. After 24 hours, the inoculated pigs were returned to the contact animals. Oral swabs and rectal temperatures were taken from each pig daily, as well as pen fecal samples. Blood samples were collected from day 3 (day 2 contact) and then on alternate days. The second experiment was performed in the same way as Experiment 1 except that four pigs were directly inoculated i.m. and returned to twelve contact pigs after 1 hour. Following inoculation, pigs were monitored daily then twice daily or more following the appearance of clinical signs. The clinical scoring system was employed in combination with qualitative endpoint criteria as previously described (Jaing, et al. 2017).

PCR testing. Real-time PCR was performed on extracts of whole blood, oral fluids, and oral and pen fecal swabs. Viral DNA was extracted using the Ambion MagMAXTM−96 Viral Isolation Kit and the MagMAXTM Express-96 Magnetic Particle Processor. As a reference 'gold standard' assay, the OIE-recommended real-time PCR assay (King assay) described by King et al. (2003) was employed using the following reaction mix: 5 µL of DNA template, sense and antisense primers (900 nM) and TaqMan® probe (250 nM), and AgPath-ID one-step RT-PCR reagents (ThermoFisher) in a 25 µL reaction volume. Thermocycling conditions were 45 °C 10 min, 95 °C 10 min, and 45 cycles of 95 °C 15 sec, 60 °C 45 sec. Tests with a cycle threshold (Ct) ≥45 were considered negative.

The commercially-available kits evaluated were the Ingenasa qPPA and the IDEXX RealPCR ASFV DNA test. The latter test was provided by IDEXX as a prototype, which was made commercially available in 2017. A second prototype version of the Ingenasa qPPA kit was also supplied that was modified for improved sensitivity for detecting ASFV DNA in oral fluids. PCR test kits were kindly supplied by Dr. Patricia Sastre (Ingenasa) and Dr. Valerie Leathers (IDEXX).

Serological testing. Serum samples for antibody testing were heat-inactivated at 60 °C for 1 hr prior to serological testing. Commercially available ELISA kits were compared with the AAHL in-house immunofluorescence antibody (IFA) test, based on the OIE-recommended test (Jaing et al., 2017). For ELISAs, both competitive (C-ELISA) and indirect (i-ELISA) formats were used (Table 1), following manufacturer's instructions. In some instances, prototype kits were used that were made available specifically for this project by the manufacturers. Serum and OF specimens collected before and after virus challenge were used for ELISA testing. We thank Dr. Patricia Sastre (Ingenasa), Dr. Loic Comtet (IDvet) and Prof. Malik Merza (Boehringer Ingelheim Svanova) for kindly providing ELISA kits for evaluation.

Quality Control. Laboratory diagnostics at AAHL was performed in the Diagnostic Virology laboratory, which is a National Association of Testing Authorities (NATA, Australia)-accredited laboratory, operating under a quality assurance system, following ISO9001:17025 standards.

Table 1. Commercial ELISAs used in this study.

Kit/test	Format	Recommended sample type
ID.vet ID Screen	Competitive	Serum, plasma
ID.vet ID Screen	Indirect	Serum, plasma, meat juice, blood filter paper
ID.vet Oral Fluids[a]	Indirect	OF
Svanova ASFV-Ab	Indirect	Serum, plasma, oral fluids[b]
Ingenasa INgezim PPA Compac 1.1PPA.K.3	Competitive	Serum
Ingenasa INgezim ASF 11.ASF.K.1	Indirect	Serum, oral fluid, meat juice

[a] Prototype

[b] Optimization needed for oral fluids

Results:

Clinical outcome of experimental infections.

Clinical scores for pigs following inoculation with ASFV are summarized in Fig. 1. Direct-inoculated and contact pigs showed signs and lesions characteristic of pathogenic ASF. Clinical scores were observed from day 3 PI in the direct inoculated pigs, progressing rapidly until pigs reached humane endpoints between days 6 and 11 post-inoculation. Onset of significant clinical signs in contact pigs occurred between days 10 and 12, gradually increasing thereafter. Humane endpoints were reached in all but one animal, from day 13 to 22 post-inoculation. Two pigs survived until 35 days until one reached human endpoint and the remaining animal was euthanized for welfare reasons. Clinical signs included fever (>41 °C; Fig. 1), skin lesions, diarrhea (sometimes bloody), conjunctivitis, decreased activity, reduced body condition, swollen joints and lameness, dyspnea. Gross pathology was characteristic of ASF, and included enlarged, congested lymph nodes, accumulation of fluid in the abdominal, thoracic and pericardial cavities, multifocal sub-capsular petechiae on kidneys, distention of the wall of the gall bladder with blood, and congested lungs.

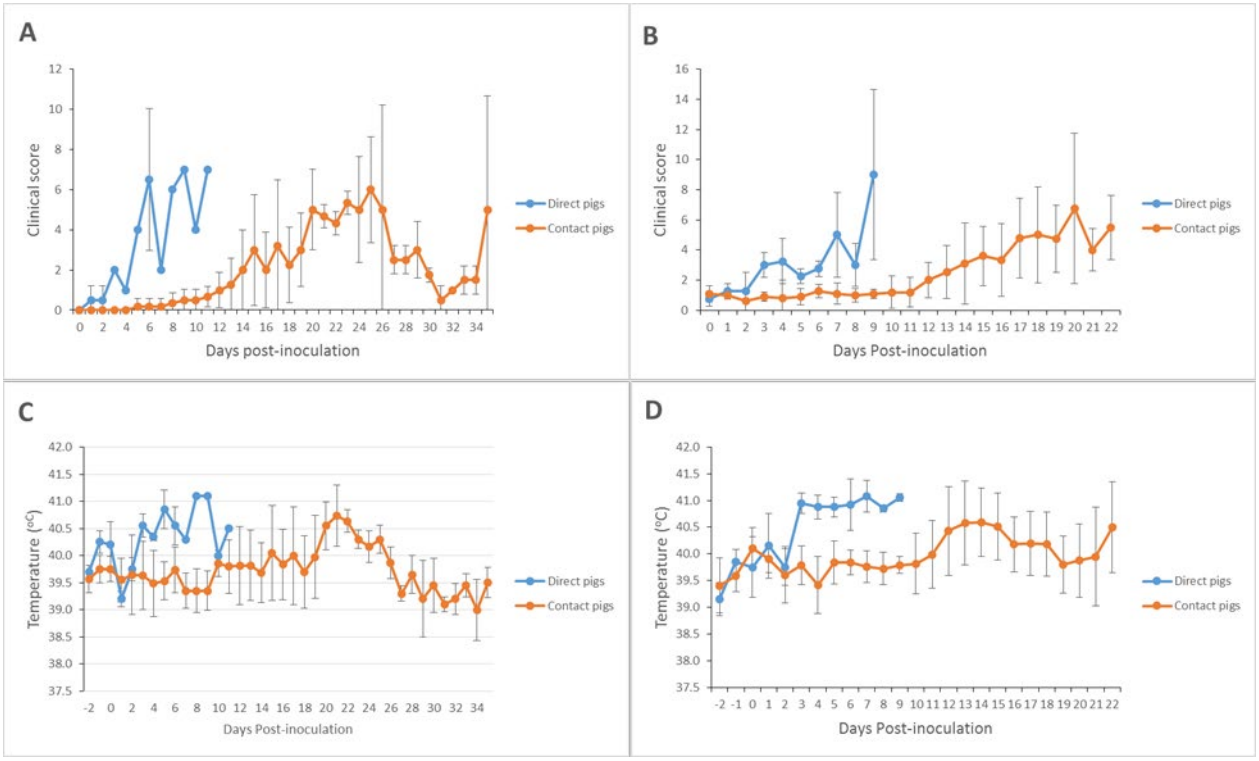

Fig. 1. Clinical scores (A, B) and rectal temperatures (C, D) of pigs inoculated with ASFV. Experiment 1 (n = 8 pigs; A, C) and Experiment 2 (n = 16 pigs; B, D). Error bars shown are standard deviation of the mean.

Comparison of the diagnostic performance of commercially available ASFV PCR and ELISAs.

i. PCR. To compare commercial kits with the reference King assay, a testing strategy was employed whereby whole blood or oral swab specimens that tested positive or indeterminate in the King assay were subsequently tested using commercial kits. This was done to minimize costs and for practical reasons (viz., limitations on kit numbers). All oral fluid sampled post challenge were tested using all three kits. In preliminary studies using the King PCR assay, pen fecal samples were found to be unsuitable for detecting ASFV DNA. To optimize the available commercial PCR kits, it was decided to focus on blood and oral samples.

Summary of PCR testing is provided in Table 2. The results for PCR testing of whole blood samples from ASFV-infected pigs showed that the IDEXX assay was able to detect a slightly higher percentage of samples that were positive in the King reference assay compared to the Ingenasa test (95% vs 90.6%). Using oral swabs from individual pigs, the IDEXX test out-performed the reference test by detecting 100% of samples compared to 88.2% for the King PCR. The Ingenasa PCR was unable to detect any of the positive oral swab specimens. Oral fluids collected from day 1 PI to the end of each experiment were tested, including negative specimens. A similar pattern observed for oral swabs was found, with the highest percentage of positive specimens detected by the IDEXX test (80%), followed by the King PCR (46.7%), while only 4.3% of samples were positive using the Ingenasa test.

Subsequently, a modified Ingenasa assay was provided by the manufacturer and tested using selected blood and oral specimens. This test performed significantly better than the previous version for all specimens, detecting ASFV DNA in 8 of the blood samples that previously tested negative (remaining sample not tested). Similarly, for oral fluids, 37 samples that were previously negative or indeterminate, were re-tested and 30 (81.1%) of these were positive in the modified assay. Using oral fluids, this test was comparable to the King reference assay, with 48.6% of the 34 samples testing positive.

ii. Serology. Serum and oral fluids specimens collected pre- and post-challenge were tested using commercial ELISAs manufactured by IDvet, Svanova and Ingenasa (Table 1). The performance of these assays was compared with the reference IFA test. The table below (Table 3) summarise the results for serological testing using kits from each of the manufacturers.

Testing pre-challenge serum showed that all tests were highly specific, with no false-positives detected. Testing of post-challenge sera collected from day 3 post-inoculation revealed that the IFA test was the most sensitive, detecting 25.9% antibody-positive samples from a total of 174 sera tested. The two Ingenasa ELISAs and the IDvet C-ELISA were the next most sensitive assays, with around 16-17% samples testing positive. The IDvet i-ELISA and the Svanova i-ELISA were the least sensitive in our hands, detecting 9.2% and 6.4% positive samples, respectively.

Oral fluids collected by rope sampling in the pens were also tested using the commercially-available Ingenasa i-ELISA and a prototype IDvet ELISA designed for oral fluid testing. The Svanova ASFV-Ab kit was initially used for testing of both serum and oral fluid. This test is currently only recommended for serum testing and preliminary testing of oral fluids revealed technical issues that are being resolved with the manufacturer. Therefore, results are not shown for oral fluid testing using the Svanova

Table 2. Comparison of real-time PCR results between assays and sample types. All oral fluid (OF) samples collected post-challenge were tested in all three kits. Only whole blood (WB) and oral swabs (OS) specimens that tested positive or indeterminate in the King assay were subsequently tested using the commercial kits.

PCR Kit	Sample type	Pos	Neg
King	WB	96 (100%)	0
	OS	45 (88.2%)	6[a]
	OF	21 (46.7%)	25
Ingenasa qPPA	WB	87 (90.6%)	9[b]
	OS	0	51[c]
	OF	2 (4.3%)	44[c]
Modified Ingenasa qPPA	WB	NA	NA
	OS	NA	NA
	OF	17 (48.6%)	18[d]
IDEXX	WB	95 (100%)	0[e]
	OS	51 (100%)	0
	OF	36 (80%)	9[e]

[a] Includes six samples that tested as indeterminate (Ct >40-45) and were re-tested as negative;
[b] Includes two samples that tested as indeterminate (Ct >40-45);
[c] Includes one sample that tested as indeterminate (Ct >40-45);
[d] Includes six samples that tested as indeterminate (Ct >40-45);
[e] One sample not tested.

Table 3. Serological testing of serum and oral fluids (OF) from ASFV-infected pigs using commercial ASF ELISA kits and the AAHL IFA.

Test	Pre-challenge		Post challenge	
	Pos	**Neg**	**Pos**	**Neg**
Serum				
IFA	0	24	45 (25.9%)[a]	129
INgezim PPA Compac 1.1PPA.K.3 C-ELISA	0	24	28 (16.1%)[b]	142
INgezim ASF 11.ASF.K.1 i-ELISA	0	24	29 (16.7%)	145
ID.vet ID Screen C-ELISA	0	24	29 (16.8%)[c]	140
ID.vet ID Screen i-ELISA	0	24	16 (9.2%)[d]	157
Svanova ASFV-Ab i-ELISA	0	24	11 (6.4%)[e]	162
Oral fluids				
IFA	0	27	8 (30.8%)	18
INgezim ASF 11.ASF.K.1 i-ELISA	0	27	0[f]	26
ID.vet Oral Fluids i-ELISA	0	26	8 (30.8%)	18

[a] IFA samples positive at titre of ≥10; when cut-off of pos ≥20 applied, 42 (24.1%) samples were positive;
[b] Four additional serum samples tested as doubtful; 3 samples re-tested and confirm doubtful;
[c] Four additional serum samples tested as doubtful; 4 samples re-tested and only one was confirmed as doubtful, the remaining 3 were negative; one sample not tested (n = 173);
[d] Two additional serum samples tested as doubtful; one of two doubtful results tested as positive on repeat (counted in table), the other confirmed doubtful.
[e] One additional specimen not tested due to exhausted/insufficient volume.
[f] Two additional OF samples tested as doubtful; one of the two tested as negative on repeat; all false negatives were confirmed by re-testing.

ELISA. As for serum, all assays were specific when pre-challenge samples were tested, with no positives found. For oral fluids collected post-challenge (from day 3), results using the IDvet i-ELISA were comparable with the IFA reference test, with both tests detecting 8 positive samples. The Ingenasa i-ELISA was unable to detect antibodies in the oral fluids specimens tested.We also analysed the data to determine if there was a correlation between false negatives in the ELISA and antibody titre determined by the IFA test (Table 4). As expected, there was a general trend for false negative results to occur for serum with IFA titres <1280. However, even at higher titres, false negatives were observed in all ELISAs that were evaluated. The assays with the least number of

Table 4. Comparison of ELISA results with IFA serum antibody titres. Results for serum samples with an IFA titre ≥40 are shown.

IFA Titre	No. of serum samples IFA pos.	Number positive (%)				
		INgezim PPA Compac 1.1PPA.K.3	INgezim ASF 11.ASF.K.1	IDvet ID Screen C-ELISA	IDvet ID Screen i-ELISA	Svanova ASFV-Ab i-ELISA
40	*4*	0	0	0[c]	0	0 (0%)
160	*10*	3 (30)[a]	4 (40)	5 (56)[d]	1 (10)	1 (11.1%)[h]
320	*2*	1 (50)	1 (50)	1 (50)	0 (0)	0
640	*3*	2 (67)	3 (100)	3 (100)	2 (67)	1 (33.3%)
1280	*1*	1 (100)	1 (100)	1 (100)	1 (100)	1 (100%)
2560	*13*	12 (92)[b]	11 (85)	11 (85)[e]	6 (46)[g]	5 (38.5%)
5120	*1*	1 (100)	1 (100)	1 (100)	1 (100)	1 (100%)
10240	*6*	6 (100)	6 (100)	5 (83)[f]	6 (100)	2 (33.3%)
40960	*2*	2 (100)	2 (100)	2 (100)	2 (100)	2 (100%)
False negatives:		**14**	**13**	**13**	**23**	**23**

[a] Three additional doubtful results for C-ELISA for samples with an IFA titre of 160; two samples were re-tested and result was confirmed;
[b] One additional doubtful result for the C-ELISA for samples with an IFA titre at 2560; result was confirmed by re-testing;
[c] One additional doubtful result for C-ELISA for samples with IFA titre of 40; re-test for this sample was negative;
[d] Only 9 samples tested. One additional doubtful result for C-ELISA for sample with an IFA titre of 160; re-test for this sample was negative;
[e] One additional doubtful result for the C-ELISA for sample with an IFA titre at 2560; re-test for this sample was negative;
[f] Two samples tested as doubtful in the i-ELISA; re-test for these samples confirmed one as doubtful and the other as positive;
[g] One additional sample tested as doubtful and confirmed by re-test;
[h] One specimen not tested due to exhausted/insufficient volume.

false negative results were the two Ingenasa ELISAs and the ID.vet C-ELISA, while the most false negatives were found using the IDvet i-ELISA and the Svanova i-ELISA.

Comparison of diagnostic performance as a function of the diagnostic specimen tested.

The collection of multiple specimens from infected pigs allowed for direct comparison of the detection of ASFV in oral fluid, oral swabs and blood/serum by PCR and the detection of ASFV antibody. The levels of antibodies in serum versus oral fluids showed a similar pattern (Fig. 2), albeit with much lower titres found in oral fluids, ranging from 10 to 40, compared to titres of up to 40,960 in serum.

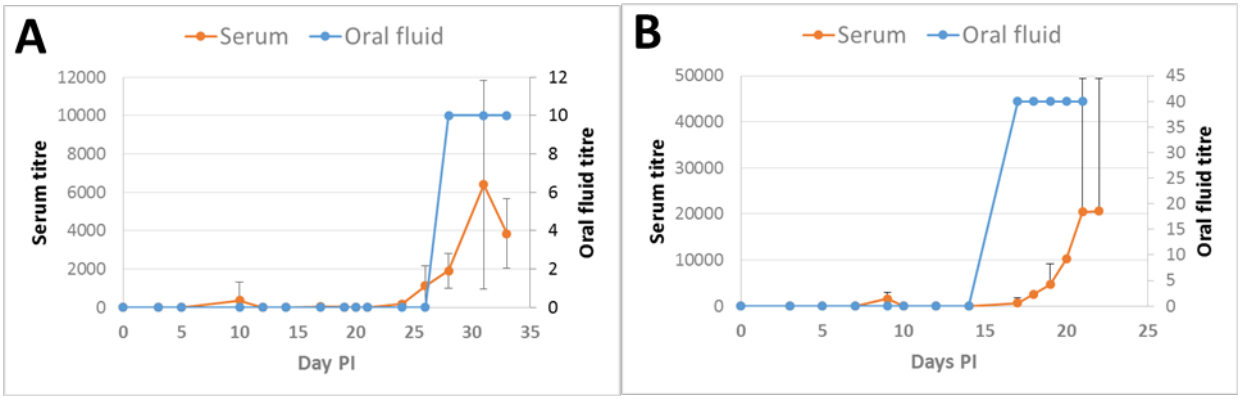

Fig. 2. Comparison of the detection of antibodies in serum and oral fluids using the IFA test. Geometric mean titres are shown on the y axes. Results from Experiment 1 (n = 8 pigs) and Experiment 2 (n = 16 pigs) are shown in A and B, respectively. Errors bars, SD of the mean.

ASFV DNA was detected in blood from day 3 in directly inoculated pigs and from day 7 in contact pigs. Shedding in oral fluid was detected by PCR from day 5, corresponding to virus detected in oral swabs from direct inoculates. PCR-positive oral swabs from contact pigs were first detected on day 7 post-inoculation. Therefore, although there was a delay of 2 days between detection of viral DNA in blood versus oral samples for directly inoculated pigs, this was synchronous for contact pigs.

Discussion:

We have compared gold standard laboratory diagnostics with commercially available diagnostic kits for the detection of antibodies to ASFV and viral DNA using samples collected from experimentally-infected pigs. This study has shown that for serum, the OIE-recommended IFA test was more sensitive than the ELISAs tested. This is consistent with previous comparisons of ASF serological tests (Gallardo et al., 2015), and underscores the necessity for further development of sensitive commercial ELISAs that are able to match the performance of the IFA test. The latter requires handling of 'live' virus antigen by skilled technicians within biocontainment facilities that are currently limited in the U.S. In the event of an outbreak, the availability of sensitive commercial tests will enable rapid roll-out of diagnostic capability to front line veterinary diagnostic laboratories for ASF diagnosis and surveillance. It was notable that for oral fluids testing, the ID.vet Oral Fluids i-ELISA performed at comparable levels of sensitivity to the IFA test, albeit using

relatively low sample numbers. However, this assay was provided as a prototype and at the time of writing, it is not known when this will be available commercially.

Using limited numbers of oral fluid samples, we found that the antibodies to ASFV could be detected in these samples with similar kinetics as serum antibodies, albeit at much lower titres. These results support the use of the oral fluids as a group specimen for diagnostic testing. However, further testing with additional antibody-positive oral fluids with matched serum samples will be required to fully validate this approach.

Our evaluation of two commercially available PCR kits demonstrated that both the Ingenasa and the IDEXX kits have comparable levels of performance compared to the OIE-recommended King assay for testing whole blood for ASFV DNA. In contrast, for oral fluid testing the IDEXX RealPCR ASFV DNA test was superior to the King assay and the Ingenasa qPPA kit, which failed to detect any oral swabs and only 2 of 46 oral fluid specimens. A modified Ingenasa kit subsequently provided by the manufacturer for oral fluid testing showed marked levels of improvement, but still lagged behind the IDEXX kit in terms of sensitivity. When this study was conceived, only the IDEXX and Ingenasa assays were available commercially. However, other commercial kits are now available, such as the QIAGEN virotype® ASFV PCR. It will be important to evaluate these assays for their utility to test oral fluids.

For this study, we chose to use a moderately virulent strain of ASFV for the experimental infections, which has a relatively slow clinical progression, compared to highly virulent, enabling the collection of both virus- and antibody-positive specimens. The use of a highly virulent strain would have enabled the detection of viral DNA, but pigs rarely (or weakly) seroconvert before death/humane endpoint. Conversely, the use of a low pathogenic strain would have allowed the collection of seropositive sera and oral fluids, the levels of virus in the blood and saliva would have been expected to be low or absent. Our approach therefore enabled us to maximize sample collection to address the objectives. It should be noted, however, that the Malta/78 strain of ASFV is a genotype I virus. ASF viruses belonging to this genotype circulate in Africa where the virus is endemic, but this does not represent the current outbreak virus in Europe (genotype 2), which may differ antigenically from genotype 1 viruses. Future work should evaluate commercial ELISAs using serum and oral fluids derived from pigs that have seroconverted from genotype 2 ASFV infections, to confirm the performance of these tests.

This study supports previous studies that indicate that oral fluids have strong potential for surveillance testing. The pooling of samples accelerates the rate of testing during an outbreak, while conserving reagents that might be in scarce supply. The incorporation of oral fluid, as a pooled sample from a pen of pigs, satisfies the user need for sample collection methods that are non-invasive and can be collected on an as needed basis. We have evaluated and identified the sensitive PCR and ELISA kits that are available commercially. This information will assist the swine industry and animal health decision makers concerning the tools and surveillance strategies available to deal with the potential emergence of ASFV in North America.

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