RESEARCH REPORT
SWINE HEALTH

REVISED

Title: Evaluation of meat exudate (juice) as a diagnostic sample for the detection of African swine fever (16-022)

Investigator: Juergen Richt

Institution: CEEZAD/Kansas State University

Date Submitted: 2019-08-30

Industry Summary: African swine fever (ASF) is one of the most important viral diseases of swine, and it can have severe socio-economic impact in countries with significant commercial pig industry. ASF has been traditionally restricted to Africa, and never occurred in North America. However, the recent emergence of ASF in Europe and China has increased the risk of introducing ASF into the North America. The causative agent, ASF virus (ASFV), is a highly stable virus and therefore can survive for long time in contaminated meat and meat products. ASFV-contaminated pork products are one of the main sources of introduction of this virus into ASF free countries.

Whole blood and tissues from animals are the routine samples tested for the presence of ASF. These samples may not be available all the time, and one may require testing alternative sample types. Meat juice is such an alternative non-traditional sample type which can be easily obtained from muscle tissues collected at slaughterhouses, road kills and supermarkets, and at the border from legally and illegally imported meat and meat products.

In this project we investigated meat juice as alternative sample for ASF detection. A total of 58 pigs were used in the studies and infected with ASFV with different lethality – highly lethal, moderately lethal and low-lethal. When the animals started to show fever or antibodies in their blood they were euthanized, different muscle samples were collected and tested for the presence of ASFV-specific markers. The results show that meat juice can be successfully used to detect ASFV genomic material and anti-ASFV antibodies. Current serological confirmatory diagnostic tests for ASF use viral proteins prepared from ASFV-infected cells and therefore can only be performed at high-containment BSL-3 laboratories. This study evaluated three commercially available serological assays which use recombinant ASFV antigens, and therefore can be used by veterinary diagnostic laboratories throughout the world.

The findings from this study provide additional tools to strengthen capabilities of US and Canadian diagnostic laboratories to detect ASF early and thereby prevent a potential ASF outbreak in North America. In the event of an outbreak, meat juice can be assayed with commercially available serological assays at any veterinary animal health laboratory. This will facilitate business continuity and eradication of the disease in case of the ASF outbreak.

Keywords: African swine fever, meat juice, exudate, ELISA.

Scientific Abstract: African swine fever (ASF) is one of the most important highly contagious viral diseases of pigs. ASF is an OIE notifiable disease that seriously affects local and international trade of live swine and pork products. ASF is endemic in the African continent, on the island of Sardinia, in Eastern Europe, Russia and in various South East Asian countries, e.g. China. ASF has never been reported in North America, however recent spread of ASF into Europe and Asia has increased the risk of introducing ASF into the US and Canada. Potential spread of ASF to North America is perceived as a serious risk for the pig industry.

Whole blood, serum and tissues from animals are the routine samples tested for the presence of ASFV nucleic acids and/or antibodies to ASFV. These samples however may not be available all the time, and therefore testing of alternative sample types might be required. Meat juice/exudate is such an alternative non-traditional sample that can be easily obtained from muscle tissues collected at slaughterhouses, road kills and supermarkets, and at the border from legally and illegally imported meat and meat products.

In this project we investigated meat juice as alternative sample type for detection of ASFV nucleic acids, ASFV live virus and antibodies to ASFV. A total of 58 pigs were used in the studies infected with highly virulent, moderately virulent or low-virulent ASFV strains. When the animals started to show fever, viremia or antibodies in their blood, they were euthanized and different muscle samples were collected and tested. Animals infected with highly virulent ASFV strain Malawi LIL 18/2 developed clinical signs and all died within 7 days post infection. The amount of ASFV nucleic acids and live virus in meat juice samples was comparable to that of the whole blood samples collected on the same day. No ASFV-specific antibodies were detected in serum or meat juice from these animals. Animals infected with the moderately virulent ASFV strain Malta'78 developed clinical signs but some animals survived up to 25 days post infection. Meat juice samples from these animals showed ASFV nucleic acids in meat juice samples comparable to that of the whole blood samples collected on the same day. Animals surviving beyond 8 days post-infection, developed antibodies to ASFV which could be detected in both serum and meat juice. Animals infected with the low virulent strain ASFV OURT88/3 developed no clinical signs but seroconverted with high titers of anti-ASFV antibodies that could be detected in serum and meat juice. These results showed that meat juice can be successfully used to detect ASFV genomic material and anti-ASFV antibodies.

Current serological confirmatory diagnostic tests for ASF use viral proteins prepared from ASFV-infected cells and therefore can only be performed at high-containment BSL-3 laboratories. In this study we evaluated three commercially available serological kits -INgezim PPA Compac ELISA, ID Screen® assay and INgezim Indirect ELISA. Based on the results it was concluded that the INgezim PPA Compac kit can only be used to detect anti-ASFV antibodies in serum samples not in meat juice samples. The remaining two kits can be used to detect anti-ASFV antibodies in serum and meat juice samples.

The findings from this study provide additional tools to strengthen capabilities of US and Canadian diagnostic laboratories to detect ASF early and thereby prevent a potential ASF outbreak in North America. In the event of an outbreak, meat juice can be assayed with commercially available serological assays at any veterinary animal health laboratory. This will facilitate business continuity and eradication of the disease in case of the ASF outbreak.

Introduction: African swine fever (ASF) is a highly contagious, devastating viral disease of pigs. Pigs infected with highly virulent strains of ASFV develop high fever, leukopenia, dark red to purple discoloration of the skin, and high (~ 100%) mortality within 7-10 days post infection. Moderately virulent strains cause severe clinical signs but some animals recover (70-90% mortality). Low–virulent strains cause chronic mild disease (slight fever for 2-3 weeks) or no clinical signs with seroconversion. These chronically infected animals could develop several recurrent mild episodes and eventually die during an acute episode (Sánchez-Vizcaíno et al., 2015).

ASF is an OIE notifiable disease that seriously affects local and international trade of live swine and pork products. ASF is endemic in the African continent, where infected warthogs and bush pigs present asymptomatic infections, whereas domestic European pigs suffer severe clinical signs and high mortality. ASF has spread to other countries many times. ASF spread to Portugal in 1957 and again in 1960. From Portugal it quickly spread to Spain, France, Italy, Belgium and the Netherlands. Later, the virus spread to Malta, Cuba, Brazil, Haiti and the Dominican Republic. ASF was eradicated through culling from most European regions by 1999, except from the island of Sardinia where it is still endemic. In 2007, an ASF outbreak appeared in the Republic of Georgia as a result of swill feeding and rapidly spread throughout the Caucasus region, affecting Armenia, Azerbaijan and the Russian Federation. From this region, ASFV gradually spread to Ukraine (2012), Belarus (2013), Estonia, Latvia, Lithuania, and Poland (2014), Moldova (2016), Czech republic and Romania (2017) and Hungary, Bulgaria and Belgium (2018). In August 2018, ASF was first reported in China and currently is widely spread across China and recently was detected in Mongolia, Laos, Cambodia, and North Korea (2019).
ASF has never been reported in North America, however recent spread of ASF into Europe and Asia has increased the risk of introducing ASF into the US and Canada. Potential spread of ASF to North America is perceived as a serious risk for the pig industry. The benefit of preventing ASF introduction into the US alone was estimated to be worth almost US $2.5 billion (Rendleman & Spinelli 1994).

There is no vaccine available for ASF, and therefore rapid diagnosis of ASF is exceptionally important to control the spread of infection. Clinical signs of ASF are not pathognomonic, and therefore laboratory confirmation is essential. Laboratory confirmation of ASF is done on whole blood and/or tissue samples (spleen, lymph nodes, tonsil and kidney) collected from suspected animals. ASFV is highly stable virus and can survive in the environment and in pig carcasses and meat products for a long time. Therefore, the main method of ASFV spread from country to country is via uncooked fresh and processed pork products. Meat juice (muscle exudate) recovered from skeletal muscle samples from pigs has been shown to be an alternative sample type for detection of a number of viral, bacterial and protozoan pathogens. Meat juice has also been used for detection of biomarkers of health status of pig herds. The potential of using meat juice to detect classical swine fever virus (CSFV) in pigs has been evaluated. In pigs experimentally infected with high- and low-virulence strains of CSFV, viral RNA was detected in meat juice, however its quantity was lower than in corresponding serum samples. Based on these results, it was concluded that meat juice constitutes a useful alternative sample type for detection of CSFV, when blood and/or target organ material are not available (Lohse et al., 2011, Kaden et al., 2009). Meat juice has been also used to detect the presence of hepatitis E viral RNA in meat samples collected from wild boar (Ivanova et al., 2015). In porcine reproductive and respiratory syndrome (PRRSV)-infected pig herds, haptoglobin and C-reactive protein in meat juice have been implicated as valuable biomarkers of herd health (Gómez-Laguna et al., 2010, Gutiérrez et al., 2015). Meat juice has been a useful sero-surveillance tool for a number of viral (CSF, influenza A, porcine circovirus 2, Aujeszky's disease and PRRS viruses), bacterial (Salmonella spp., Mycoplasma hyopneumoniae, Yersinia enterocolitica) and protozoal (Trichinella spp. and Toxoplasma gondii) diseases of swine (De Lange et al., 2003, Le Potier et al., 1998, Fabisiak et al 2013) Wacheck et al., 2012, Meemken et al., 2014, 09).

In 2010, McKillen et al., reported testing of a small number (n=6) of meat juice samples collected from quadriceps muscles of 6 pigs infected with low-virulent ASFV strains Malta/78 and MwLil 20/1. All 6 samples were identified as positive for ASFV by real-time PCR assay (McKillen et al., 2010).

The objective of this project was to conduct a comprehensive evaluation of the suitability of meat juice as a potential diagnostic sample for virological and serological detection of ASF in pork. Pigs were infected with highly, moderately and low virulent ASFV strains and meat samples were collected and tested for presence of live virus, viral nucleic

acids and antibodies to ASFV. Virus isolation was carried out by inoculating porcine alveolar macrophage cultures and the presence of viral nucleic acids was confirmed by real-time PCR assay. For serological detection, three commercially available serological kits were used

Objectives:

1. Generate meat-juice samples from high-virulent, moderately-virulent and low-virulent ASFV-infected pigs
2. Evaluate the virological, molecular and serological diagnostic potential of meat juice for detection of ASF
3. Evaluate sensitivity and specificity of two commercially available ASF serological kits for detection of anti-ASFV antibodies in serum and meat juice samples.

Materials & Methods:

Viruses: The haemadsorbing Malawi LIL 18/2 isolate (Haresnape et al., 1984), the haemadsorbing ASFV Malta'78 isolate (Wilkinson et al., 1978) and the non-haemadsorbing ASFV OURT88/3 isolate were used as the highly-virulent, moderately-virulent and low-virulent strains, respectively. The ASFV Malta'78 and OURT88/3 isolates were kindly provided by Dr. Linda Dixon at the Pirbright Institute, Pirbright, UK. All the viruses were propagated in swine peripheral blood mononuclear cells (PBMC) and titrated on porcine alveolar macrophages followed by immunostaining.

Animal experiments: A total of four independent animal experiments were conducted under the animal-use document (AUD) C-1016-009 approved by the Animal Care Committee at the Canadian Science Centre for Animal and Human Health. In all experiments, 4-5 weeks old Landrace-Large white cross-bred grower pigs were used. The pigs were purchased from a local farm in Manitoba, Canada and transported in a heated truck to the National Centre for Foreign Animal Disease (NCFAD) in Winnipeg, Canada. Upon arrival they were randomly assigned into groups and housed in biocontainment-level 3 animal cubicles with ad libitum food and water supply. The animals were monitored daily and given 7 days of acclimatization period before they were used in the experiments.

Experiment #1: Prior to beginning of this study, the NCFAD Laboratory did not have well characterized low or moderately virulent ASFV strains. Therefore, prior to the use of ASFV OURT/88/3 and ASF Malta'78 strains in a large animal experiment, a small experiment with eight 4-5 week old pigs was carried out. The pigs were randomly assigned to two pens (4 pigs per pen), and pigs in one pen (Pig# 1-4) were inoculated with OURT88/3 and those in the second pen (Pigs# 5-8) were inoculated with ASFV Malta'78. For both viruses, a dose of $1X10^5$ $TCID_{50}$ in 4 ml of culture media was used with 2 ml administered orally and 1 ml per each nostril. The animals inoculated with OURT88/3 were re-infected 4 weeks after the first infection. After inoculation, animals were monitored twice daily and rectal temperatures recorded. Whole blood samples and oral and nasal swabs were collected from each pig on each sampling day and after they died/were euthanized, whole blood (in EDTA) and serum samples, tissues samples (spleen, lymph nodes and tonsils) and muscle samples were collected. The muscle samples were cut into pieces of approximately 2 cm × 2 cm × 2 cm, put into plastic bags and frozen at –20°C.

Experiment #2: Highly virulent Malawi LIL 18/2 infection study. For this experiment, 14 pigs (Pig # 33-46) were used. Upon arrival they were randomly assigned to three pens (6, 4, and 4), and after one week of acclimatization each pig was inoculated with $1X10^5$ $TCID_{50}$ of ASFV Malawi LIL18/2 in 4 ml of culture media via the oronasal route (2 ml orally and 1 ml per nostril). After inoculation animals were monitored twice daily, rectal temperatures recorded and whole blood samples and oral and nasal swabs were collected daily from each pig. When the pigs started to show fever they were euthanized (2 animals per day), and whole blood (in EDTA), serum, spleen, lymph nodes and muscle tissues were collected, processed and stored as described above.

Experiment#3: Moderately virulent Malta'78 infection study. A total of 24 pigs (Pigs# 9-31) were used in this experiment. According to the literature, ASFV Malta'78 infection is not 100% lethal in pigs. However in the preliminary Malta'78 experiment, all 4 pigs inoculated with Malta'78 succumbed to infection by 12 days post infection. Therefore in this experiment it was decided to inoculate the pigs with 10 times less virus. Pigs were randomly assigned to 4 pens (6 pigs per group), and each pig received ASFV Malta'78 at $1X10^4$ $TCID_{50}$ in 4 ml of culture media via the oronasal route (2 ml orally and 1 ml per nostril). After inoculation, animals were monitored twice daily, rectal temperatures recorded and whole blood samples and oral and nasal swabs were collected daily from each pig. When the pigs started to show fever, they were euthanized (2 animals per day), and whole blood (in EDTA), serum, spleen, lymph nodes and muscle tissues were collected from each pig. The muscle samples were cut into pieces of approximately 2 cm × 2 cm × 2 cm, put into plastic bags and frozen at –20°C until use. Animals that didn't develop clinical signs by 13 days post infection were reinfected with $1X10^5$ $TCID_{50}$ of ASFV Malta'78 in 4 ml of culture media via oronasal route (2 ml orally and 1 ml per nostril) on day 13 post infection. Following the second inoculation the remaining animals developed clinical signs and died within 12 days.

Experiment#4: Low virulent OURT88/3 infection. For this experiment, twelve pigs (Pigs # 63-74) were used. Pigs were divided into 3 pens (4 pigs in each) and after one week of acclimatization each pig was inoculated with $5X10^6$ $TCID_{50}$ (10 times more virus than in the previous experiment) in 4 ml of culture media via oronasal route (2 ml orally and 1 ml per nostril). The animals were monitored daily for body temperature and development of clinical signs. Whole blood, and serum samples, and oral and nasal swabs were collected from each pig on days 0, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 17, 18, 20, 21, 23, 24, 25 and 26 post infection. When pigs started to develop anti-ASFV antibodies in the serum, they were euthanized (2 animals per day), and samples were collected as described above for previous animal experiments. Animals that did not seroconvert two weeks after the first inoculation were boosted with the same dose of OURT88/3 on day 13 post infection. All the animals seroconverted after the boost.

Meat juice exudate from the muscle samples was collected after thawing the frozen muscle samples at 4 degrees overnight and used for subsequent analyses.

All the animal experiments were conducted under the guidelines of the Canadian Council for Animal Care.

Quantitative real-time polymerase chain reaction (qPCR): The amount of ASFV genomic material in whole blood and meat juice samples was quantified using a published TaqMan qPCR assay that specifically amplifies a conserved region of the ASFV p72 gene (Tignon et al., 2011). Total nucleic acid was extracted from the whole blood and meat juice samples using the MagMAX™ Pathogen RNA/DNA Kit (Life Technologies, Burlington, ON) and the MagMAX Express-96 Magnetic Particle Processor (Life Technologies), following the manufacturers' protocol. The qPCR was carried out using the TaqMan™ Fast Virus 1-Step master mix (Life Technologies) on the Applied Biosystems 7500 Real-Time PCR Instrument (Life Technologies) using the following cycling conditions: 50°C for 10 min, 95°C for 3 min followed by 40 cycles of 96°C for 3 sec and 60°C for 30 sec. TaqMan qPCR assay for beta-actin as an internal gene was developed in-house and used to ensure nucleic acid extraction and absence of PCR inhibitors in the samples.

ELISA: Three commercially available ELISA kits were used in this study. Antibodies to ASFV in serum samples were tested using INGEZM PPA COMPAC blocking ELISA Kit (R.11.PPA.K.3, Ingenasa, Madrid, Spain) which uses a monoclonal antibody (MAb) specific to African Swine Fever Virus (ASFV) VP72 protein. This kit is not recommended for testing meat juice samples due to strong background observed with meat juice samples. Two indirect ELISA kits, INGEZM ASFV Indirect (11.ASF.K1, Ingenasa) and ID Screen® ASF Indirect (ID.Vet, Grabels, France) were used to test the anti-ASFV antibodies in meat juice samples. The ID Screen® ASF Indirect

is a multi-antigen indirect ELISA kit for the detection of antibodies against the ASFV P32, P62 and P72 proteins. INGEZM ASFV Indirect is a prototype ASFV indirect ELISA kit developed by Ingenasa, Madrid, Spain. All three ELISA assays were performed according to the manufacturer's protocols.

Virus Isolation: Virus was isolated from a limited number of whole blood and meat juice samples on monolayers of porcine alveolar macrophage cells.

Results:

1. Generate meat-juice samples from high-virulent, moderately-virulent and low-virulent ASFV infected pigs

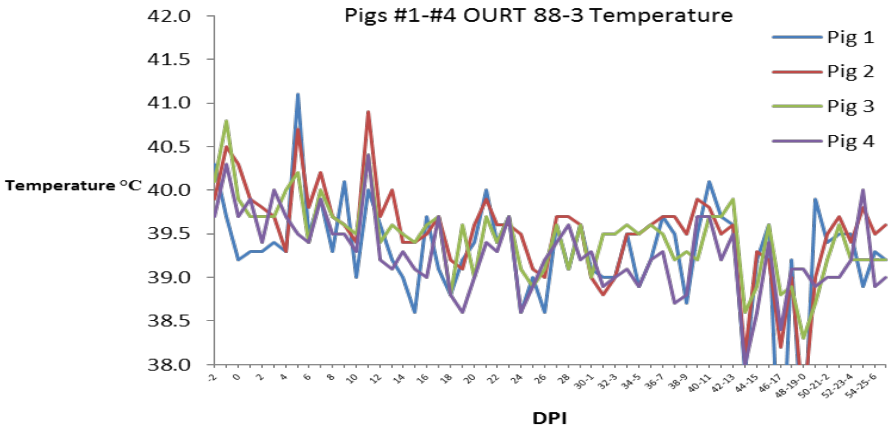
Four animal experiments were conducted at the NCFAD in Winnipeg, Canada.

Experiment #1: Preliminary characterization of OURT/88/3 low-virulent and Malta'78 moderately-virulent ASFV strains.

The four animals (#1-4) inoculated with OURT/88/3 did not develop any clinical signs (Figure 1A). They were re-infected with OURT/88/3 at 29 dpi with the same dose and no clinical signs developed even after the second inoculation. On 48 day post-primary inoculation, they were challenged with ASF Malta'78 ($1X10^5$ $TCID_{50}$ in 4 ml) oronasally. No animal developed fever or other clinical signs and the animals were euthanized on 56 dpi. Whole blood, serum, nasal, oral and rectal swabs were collected from each animal every fourth day and meat samples from a large number of muscle tissues were collected from each animal at necropsy

All four animals (#5-8) inoculated with Malta'78 developed fever (rectal temperature above 40.5°C for at least two consecutive days, Figure 1B) and clinical signs such as diarrhea, vomiting, shivering, depression, ataxic, paleness and drooling white foam. ASFV nucleic acids in whole blood were detected by 4 days post infection. One of the animals died by 6 dpi, and the remaining animals died by 13 dpi.

(A)



(B)

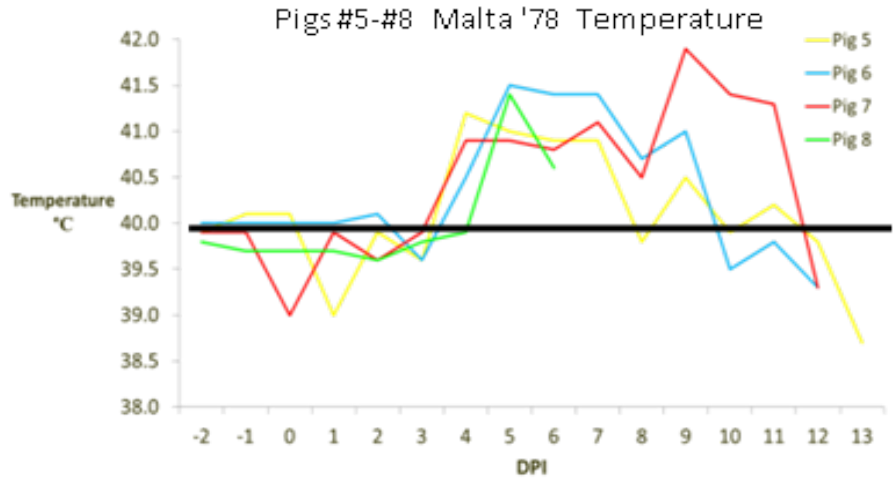


Figure 1. Rectal temperatures of pigs inoculated with ASF OURT/88/3 (A) and Malta'78 (B)

Experiment#2: Generation of meat juice samples from pigs infected with highly-virulent ASFV Malawi LIL 18/2 strain.

Fourteen 4-5 week old pigs (#33-46) were inoculated with the ASF Malawi LIL 18/2 strain and observed twice daily for clinical signs of ASF. All animals, except one, developed fever by 3 dpi (Figure 2). In addition to fever, infected pigs also displayed depression, diarrhea, nasal discharges and vomiting. Whole blood, serum, nasal and oral swabs were collected from each animal daily after the challenge. Whole blood samples were analyzed by ASFV real-time PCR on the same day to identify if animals are showing viremia. All animals started to show viremia starting 3 dpi. After pigs started to show fever (3 dpi), two animals were euthanized daily and meat samples from muscle tissues (biceps femoris, brachialis, masseter and diaphragm) were collected.

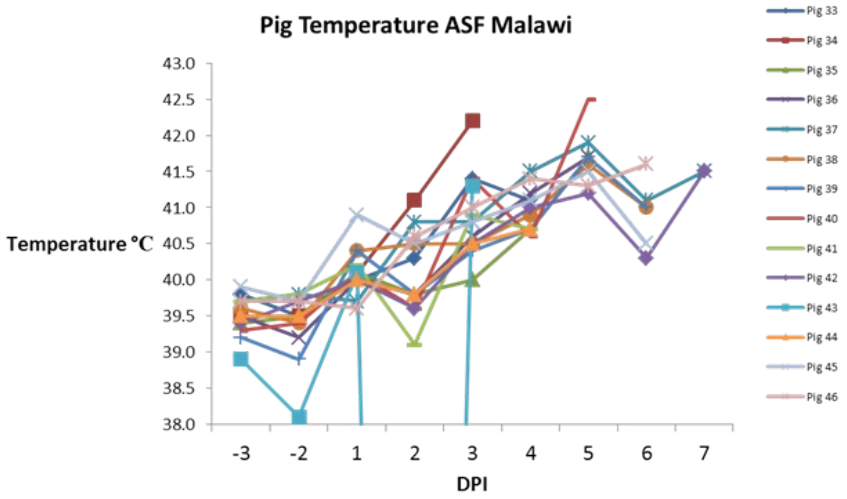


Figure 2. Rectal temperatures of 14 pigs inoculated with ASFV Malawi LIL 18/2 strain.

Experiment #3: Generation of additional meat juice samples from pigs infected with moderately-virulent ASF strain Malta'78.

Out of 24 pigs inoculated with $1X10^4$ $TCID_{50}$ of ASF Malta'78 strain, 5 animals (#15, 20, 22, 25 and 32) developed clinical signs and died by 11 days post infection. One animal (#18) died during the acclimatization period due to non-relevant illness. Following the second inoculation the remaining animals developed clinical signs and died within 12 days. In addition to fever, the affected animals also displayed depression, diarrhea, nasal discharges and vomiting. Whole blood, serum, nasal and oral swabs were collected from each animal on days 3, 5, 6, 7, 10, 11, 12, 14, 17,19, 20, 21, 22, 23, 24, and 25 post primary infection. Whole blood samples were analyzed by ASFV-specific real-time qPCR on the day of collection to identify animals showing viremia. Pigs which developed fever and/or viremia were euthanized (two pigs per day) and meat samples (biceps femoris, brachialis, masseter and diaphragm), as well as other samples, were collected.

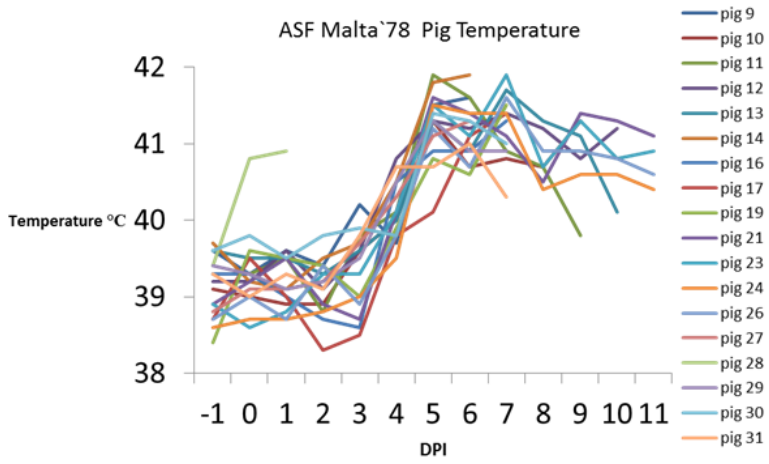


Figure 3. Rectal temperatures of pigs inoculated with ASFV Malta'78. The rectal temperatures of five animals (#15, 20, 22, 25 and 32) which developed clinical signs and died by 11 days post infection are not represented here.

Experiment #4: Generation of additional meat juice samples from pigs infected with low-virulent ASF strain OURT/88/3.

Twelve pigs (#63 -74) were divided into two pens (6 pigs per pen) and each pig was infected with $1X10^6$ $TCID_{50}$ of OURT/88/3 per pig oronasally (2 ml orally, 1 ml into each nostril). Animals were observed twice daily for clinical signs of ASF. None of the animals developed fever or other clinical signs of ASF. On day 13, Pig #68 and #71 were euthanized and samples were collected. On 14 dpi, 10 animals (63, 64, 65, 66, 67, 69, 70, 72, 73 and 74) that did not seroconvert (based on the INGEZM ASF PPA Compac assay) were boosted with $1X10^6$ $TCID_{50}$ of OURT/88/3 per pig oronasally.

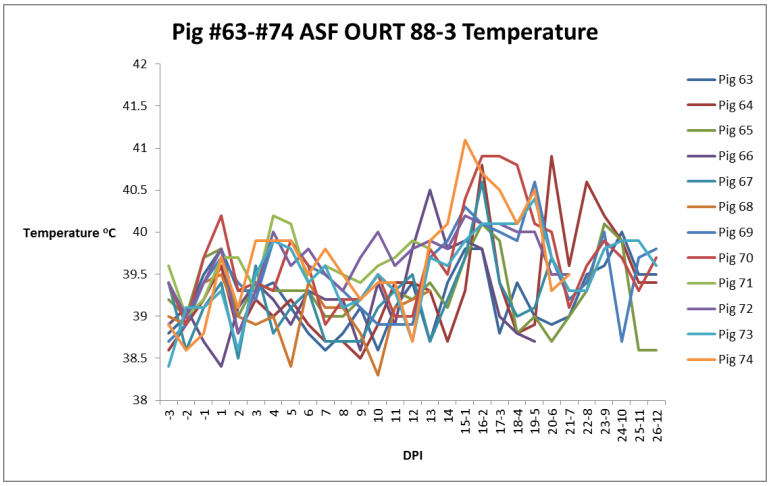


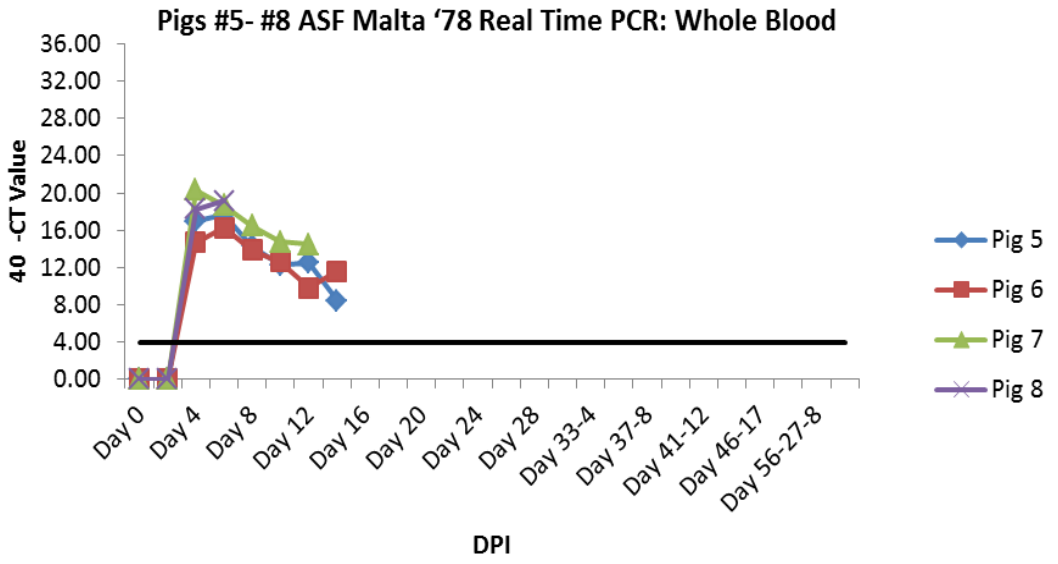
Figure 4. Rectal temperatures of 12 pigs inoculated with ASFV OURT/88/3.

2. Evaluate the virological and serological diagnostic potential of meat juice for detection of ASF

Experiment #1: Preliminary experiment with ASFV OURT/88/3 and ASFV Malta'78 strains.

In pigs inoculated with Malta'78, ASFV genomic DNA was detected in whole blood by 4 dpi and animals remained viremic until they died (Figure 5A). ASFV DNA was readily detected in meat juice samples and the CT values were comparable to that to the whole blood samples collected at the final bleed (Figure 5B). There was no obvious difference in the amount of ASFV DNA between the muscle types. However tongue, diaphragm and masseter muscles provided the best volume of meat juice compared to the other muscle types. These muscles were also the easiest to collect at necropsy and caused the least damage to the carcass value.

(A)



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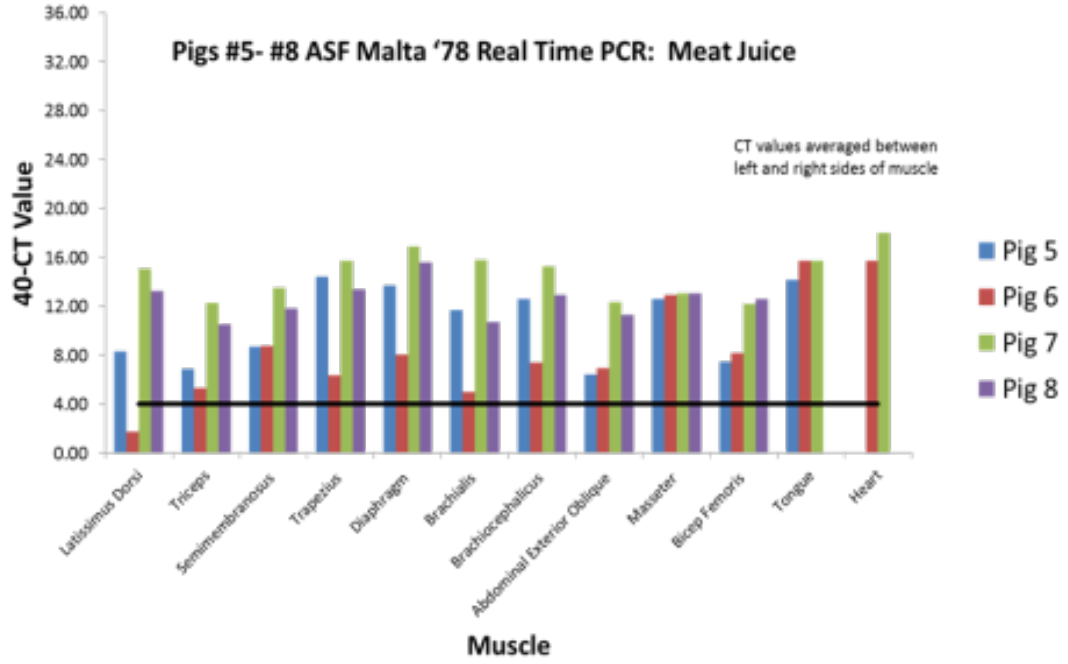


Figure 5: Real-time qPCR results for pigs inoculated with moderately virulent ASF strain Malta'78. (A) Whole blood (B) Meat juice. There were no tongue samples collected from Pig#8 and no heart muscle samples collected from pigs #5 and #8. Cut-off = 4.0

In pigs inoculated with the low-virulent ASFV strain OURT88/3, no ASFV genomic DNA was detected in whole blood samples. When they were challenged with the moderately virulent ASFV strain Malta'78, only Pig #4 on 56 dpi (last day of the study i.e. 7 days after Malta'78 challenge) showed weak positive qPCR results, indicating possible low level virus present in the blood. When meat juice samples from these animals were evaluated, only samples from tongue and heart of pig #4, and diaphragm of Pig #3 were weakly positive for ASFV genomic DNA (data not shown).

Experiment #2: Meat juice samples from pigs infected with highly-virulent ASFV Malawi LIL 18/2 strain.

Two pigs (#34 and 43) developed fever on day 3 and were euthanized. Both animals were positive for ASFV genomic DNA in whole blood and in meat juice samples. On 4 dpi, the other animals became viremic and ASFV DNA was detected in all meat juice samples tested (Figure 6). The amount of ASFV DNA in meat juice samples was slightly lower than that detected in whole blood samples at the time of necropsy. As previously observed, there was no difference in ASFV DNA levels in different meat juice samples tested (Fig. 6).

(A)

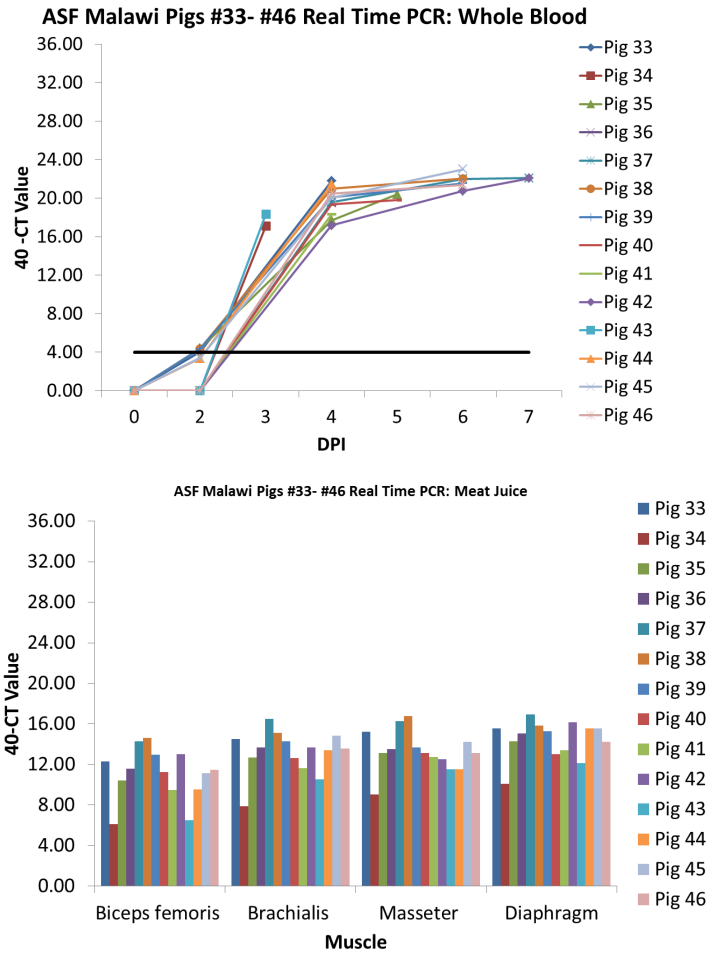
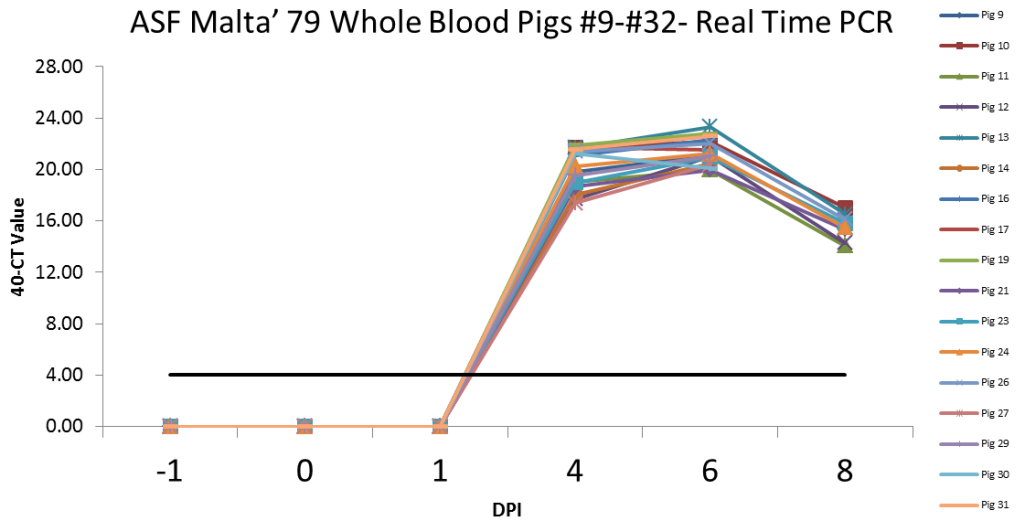


Figure 6: Real-time qPCR results for pigs inoculated with high virulent ASF Malawi LIL 18/2 strain. Cut-off = 4.

Experiment #3: Meat juice samples from pigs infected with moderately-virulent ASFV Malta'78 strain.

All Malta'78 inoculated pigs developed fever and viremia, including those that died after low dose inoculation (#15, 20, 22, 25 and 32) and all meat juice samples were positive for the presence of ASFV genomic material. The amount of ASF genomic material in whole blood on the day of necropsy was comparable to that detected in the meat juice (Figure 7).

(A)



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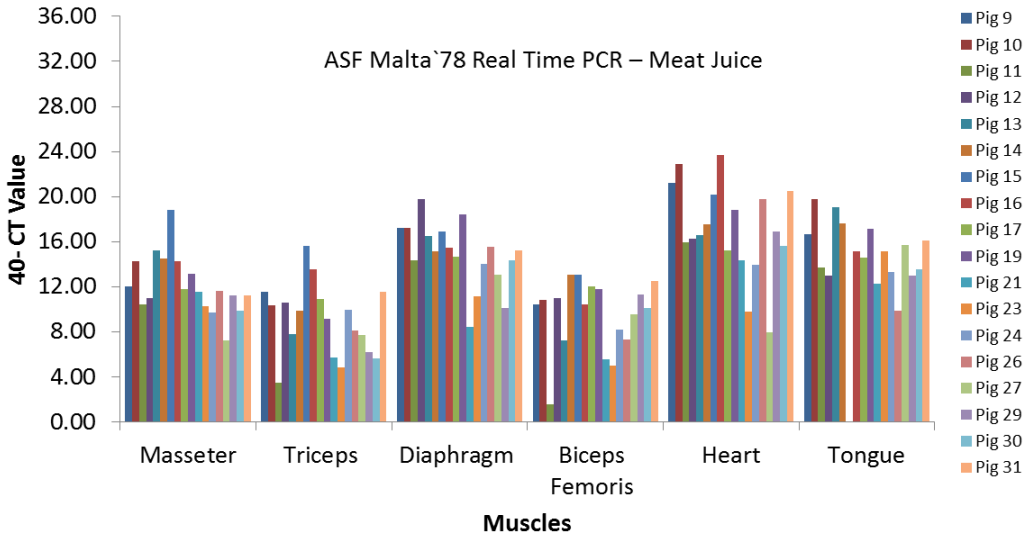


Figure 7: Real-time qPCR results for pigs inoculated with moderately virulent ASF Malta'78. Whole blood (A) and Meat Juice (B). Cut-off = 4.

Experiment #4: Meat juice samples from pigs infected with low-virulent ASFV OURT/88/3 strain.

Similar to the previous OURT/88/3 experiment #1, none of the animals developed fever post inoculation. One pig (#64) showed weak positive results in real-time qPCR after the second inoculation. No ASFV genomes were detected in meat juice samples (data not shown). In order to identify potential sites of OURT/88/3 replication, spleen, tonsil, and bone marrow samples from these animals were tested. Pigs 64 and 65 showed weak positive qPCR results in spleen samples but not in other tissue samples.

3. Evaluate sensitivity and specificity of three commercially available ASF serological kits for detection of anti-ASFV antibodies in serum and meat juice samples.

The potential of meat juice as a sample for ASF serology was evaluated using three commercial ASF serological kits: INgezim ASF Indirect ELISA, INgezim ASF PPA Compac and ID Screen® ASF Indirect kits. The INgezim PPA Compac kit is recommended for ASF serological diagnostics by the ASF European Reference Lab (EURL), Madrid, Spain and widely used. It has

been validated for specificity and sensitivity using serum samples collected from Africa, Europe, and the USA (only for specificity). This kit is only recommended for serum samples and not for meat juice samples. The INgezim ASF Indirect ELISA kit is a prototype.

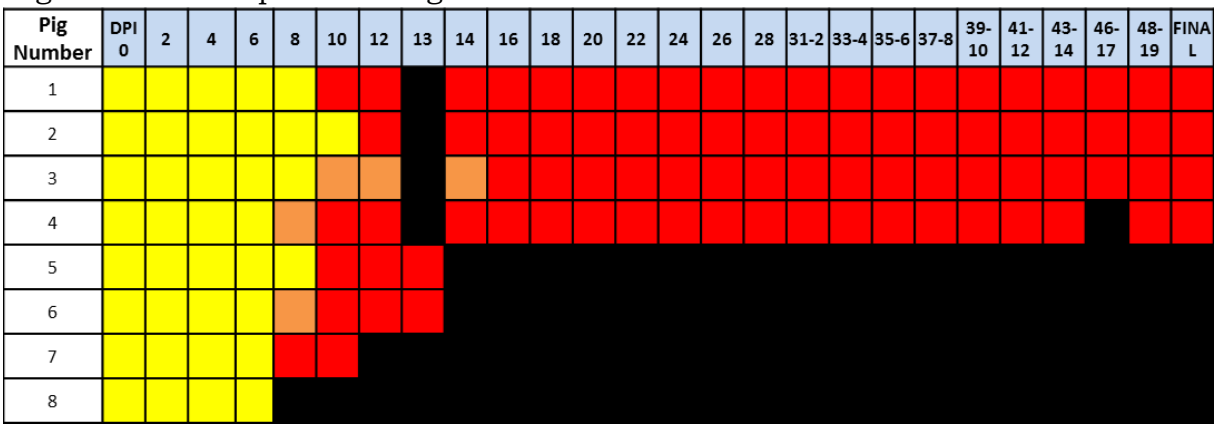
3.1. Samples from the pilot experiment with pigs infected with moderately virulent or low-virulent ASFV strains.

Serum collected from pigs infected with ASF OURT88/3 (Pigs # 1-4) and ASF Malta'78 (Pigs # 5-8) were tested using three different ELISA kits (Figure 8A). Based on the EURL-recommended blocking ELISA INgezim PPA Compac, all pigs that survived beyond 10 days post-infection, irrespective of the virulence of the virus, developed serum antibodies to ASFV. The indirect INgezim ELIZA detected pig #3 serum on 6 dpi and pig #1 serum on 8 dpi as positive. Both serum samples tested negative by the immunoblotting test and were therefore considered false positive. The ID Screen® Indirect kit was not able to detect positive animals until 12 dpi and all animals were positive by 16 dpi.

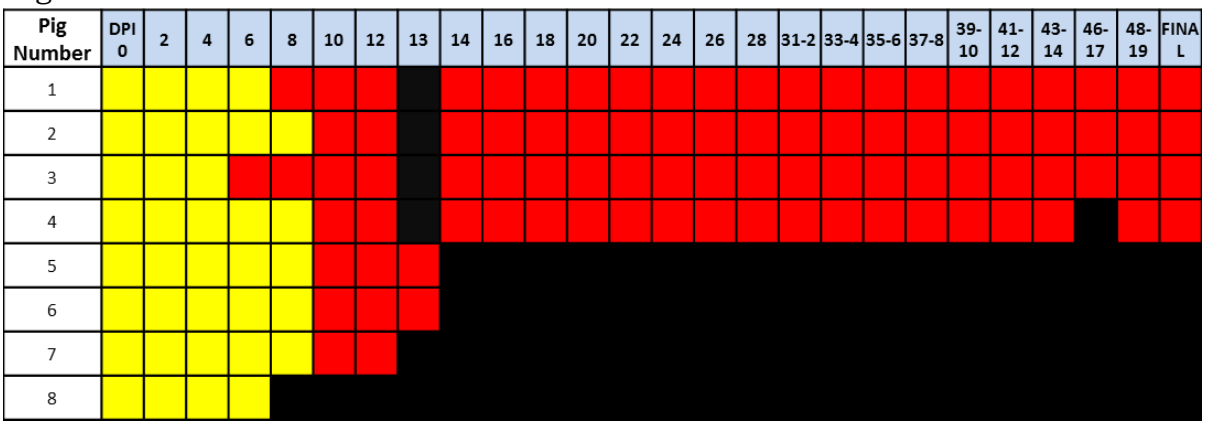
When the meat juice samples were tested, the animals that showed positive antibodies in serum samples were positive with the meat juice samples irrespective of the type of muscle type tested (Figure 8B). Pig #8, which died on 6 dpi, had no anti-ASFV antibodies in the meat juice and in the serum.

(A) Serum samples

INgezim PPA Compac Blocking ELISA



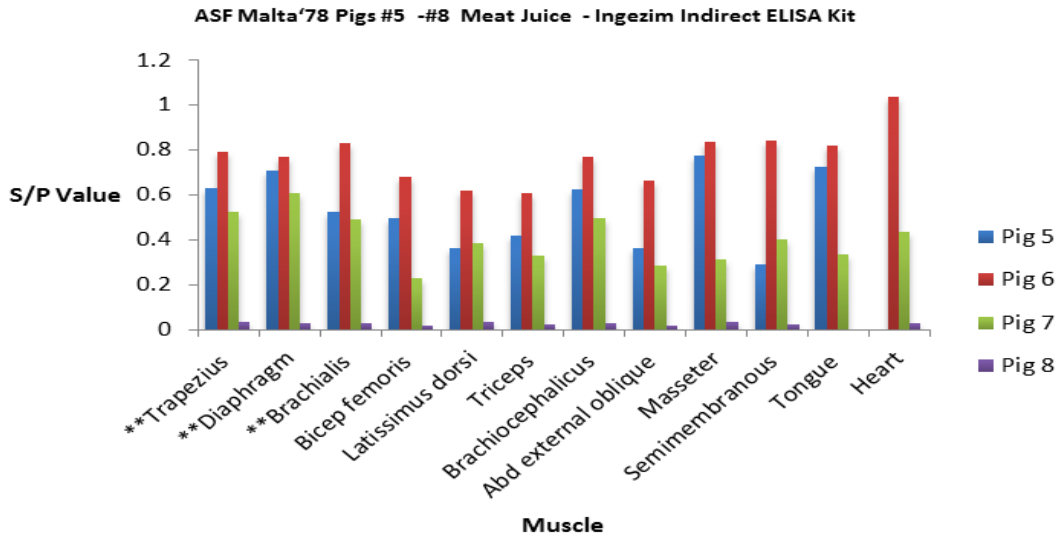
INgezim Indirect ELISA



ID Screen® Indirect ELISA

Pig Number	DPI 0	2	4	6	8	10	12	13	14	16	18	20	22	24	26	28	31-2	33-4	35-6	37-8	39-10	41-12	43-14	46-17	48-19	FINAL
1																										
2																										
3																										
4																										
5																										
6																										
7																										
8																										

(B) Meat juice samples



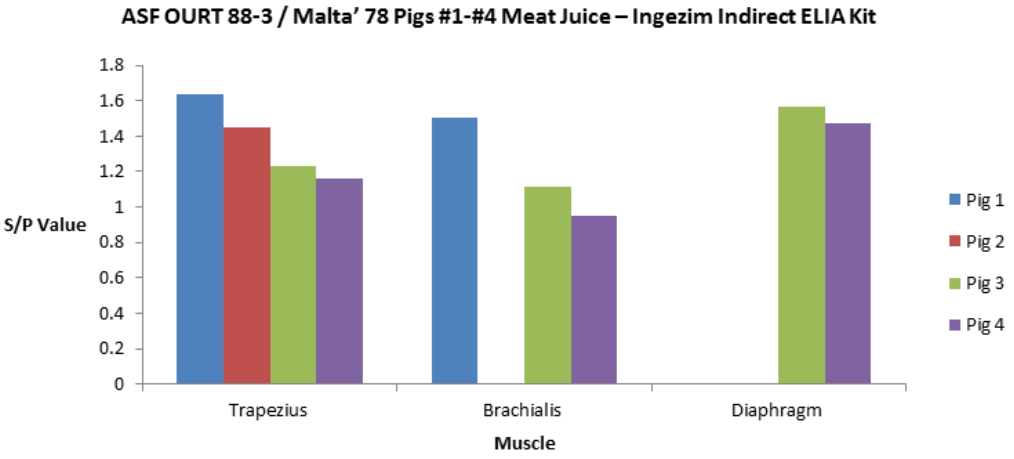


Figure 8. (A) ASFV antibodies in serum samples. Serum samples were tested using all three ELISA kits, and meat juice samples were tested using INgezim Indirect ELISA kit. Yellow= Negative samples; Red = Positive; Black = Animal died/euthanized or not done. (B) ASFV antibodies in meat juice samples. Cutoff for the INgezim Indirect ELISA= 0.2 S/P value. There were no brachialis muscle samples from pig #2 and no diaphragm muscle samples from pig #1 and #2 available for testing.

3.2 Pigs infected with highly-virulent ASFV strain.

The serum samples collected from pigs inoculated with Malawi LIL 18/2 were tested with all three ELISAs. None of the animals had detectable levels of anti-ASFV antibodies in serum and therefore meat juice samples from these animals were not tested by the ELISA (Data not shown).

3.3 Pigs infected with moderately-virulent ASFV strain.

One hundred and eighty one serum samples collected from pigs infected with the Malta'78 strain were tested using the INgezim PPA Compac and INgezim Indirect ELISA kits (Figure 9). At present, these serum samples are still being tested using the ID Screen® Indirect kit. None of the pigs (#15, 20, 22, 25 and 32) that died before the second ASFV inoculation developed anti-ASFV antibodies according to both ELISA kits, including pig #15 which survived up to 11 days post infection. Following the second inoculation, pigs that died within 1-7 days post second inoculation also did not develop any ASFV antibody response. The results from both INgezim kits were comparable. The meat juice samples are still being tested by the INgezim ASF Indirect ELISA kit.

INgezim PPA Compac INgezim Indirect ELISA

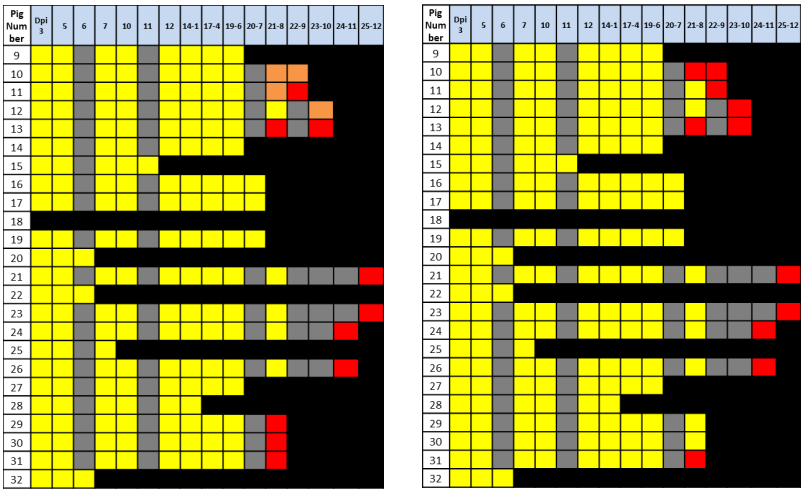


Figure 9. ASFV antibodies in serum from pigs infected with ASFV Malta'78. For labelling see Figure 8.

3.4 Pigs infected with low virulent ASFV strain OURT/88/3.

One hundred and eighty seven serum samples were collected from pigs inoculated with the ASFV OURT/88/3 strain and tested using the INgezim PPA Compac and ID Screen® kits (Figure 10). According to these tests, two pigs (#68, 71) seroconverted by days 11-13 post-primary infection (Fig. 10A). Meat juice collected from these two pigs was readily detected by the ID Screen® kit (Fig. 10B). Meat juice samples from the remaining pigs were all tested positive by the ID Screen® kit except the samples from Pigs #72 and 73. The reason for this is not known, but could be due to a generally observed lower sensitivity of the ID Screen® kit compared to the other kits. The meat juice samples are presently being tested by the INgezim Indirect ELISA kit.

(A)

INgezim PPA Compac

Pig #	DPI 0	6	7	8	9	10	11	12	13	14	16	17	18	20	21	23	24	25	26
63	4.96	9.93	8.03	25.9	37.8	38.2	33.4	41.6	39.5	33.9	35.2	39.9	42.1	58.7	61.7	73.9	70.5	71.2	
64	10.5	10.4	13.5	24.8	26.3	32.9	19.6	26.1	19.9	15.9	13.6	15.1	17.3	73.6	74.7	75.4	69.1	70.1	
65	14.5	17.2	1.91	16.8	23.6	21.4	10.7	16.6	11.5	8.45	8.42	8.2	14	40.5	48.8	69.9	62.3	58	
66	5.58	9.09	3.81	24.7	37.6	37.9	34.4	42.4	47.3	43	50.3	58.1	64.5						
67	9.76	15.2	3.05	14.7	13.1	17.8	6.54	10.4	10.2	5.67	10.4	7.46	12.6	54.8	66.5				
68	9.53	13.8	17.2	33.8	48.8	51.2	52	54.6	63.5										
69	13.8	18.6	5.31	24.8	22.5	30.1	12	24.6	29.7	15.4	13.4	11.9	12.4	56.7	57.5	81.9	70.2	75.9	70.6
70	8.64	14.7	4.14	19.2	17.4	21.4	14.4	22.2	28.8	12.9	12.1	10.7	15.1	40.1	56.1	44	43.7	60.7	60.2
71	10.3	11.1	17.6	43.6	49.5	48.7	50.6	51.8	63.4										
72	11.3	15.6	7.99	22.6	29.7	32.3	25	28.8	34.7	20.9	21.2	22	22.2	46.4	63.8				
73	11.9	11.5	7.45	25	27.2	37.3	46.8	44.4	49.5	35.9	25.1	35.3	44.8	59.6	63.3	65	62.2	64.9	62.8
74	13	11.4	3.22	19.5	19.7	18.6	19.2	11.6	26.3	16.1	16.2	28.3	39.6	83.4	85.1				

ID Screen® Indirect ELISA

Pig#	DPI 0	6	7	8	9	10	11	12	13	14	16	17	18	20	21	23	24	25	26
63	1.01	0.27	1.04	10.36	21.13	27.42	34.07	34.52	39.94	41.45	41.96	44.48	50.08	40.71	50.57	43.68	48.75	42.77	
64	0.74	0.38	0.54	0.16	1.00	1.18	1.57	1.51	1.53	2.38	9.76	16.05	27.82	69.65	81.38	78.29	84.70	65.20	
65	0.10	0.40	-0.42	0.94	0.57	0.34	0.90	0.41	0.37	0.50	0.34	0.69	1.11	27.06	38.33	46.94	56.57	40.24	
66	0.65	0.20	0.82	3.64	9.72	15.01	16.71	28.34	35.71	50.88	56.85	56.51	59.41						
67	0.72	0.57	0.42	0.64	0.82	0.54	1.20	1.23	2.17	2.65	2.44	2.90	5.64	51.92	59.59				
68	0.91	0.64	1.23	8.70	21.97	31.06	30.84	18.33	34.08										
69	0.49	0.81	1.03	0.96	1.46	5.51	8.87	14.51	18.66	19.35	20.85	22.91	21.59	60.84	77.18	78.92	76.34	80.95	72.01
70	0.25	0.65	0.26	0.03	0.63	0.94	3.96	6.89	9.44	12.65	12.26	14.26	17.56	70.07	91.81	92.85	83.11	84.69	87.98
71	0.46	0.79	2.66	5.88	15.77	22.78	32.23	43.30	46.33										
72	0.21	0.26	0.44	1.47	3.81	6.78	12.79	20.06	30.46	32.15	30.33	29.87	31.98	38.50	48.65				
73	0.36	0.51	0.87	2.81	9.68	20.54	27.48	34.74	34.10	35.09	32.66	31.54	35.33	36.93	36.27	40.85	40.57	42.14	38.98
74	0.27	1.00	1.20	3.42	8.58	15.37	20.56	29.31	30.59	30.58	34.30	48.40	60.51	71.05	68.39				

(B)

ID Screen® Indirect ELISA

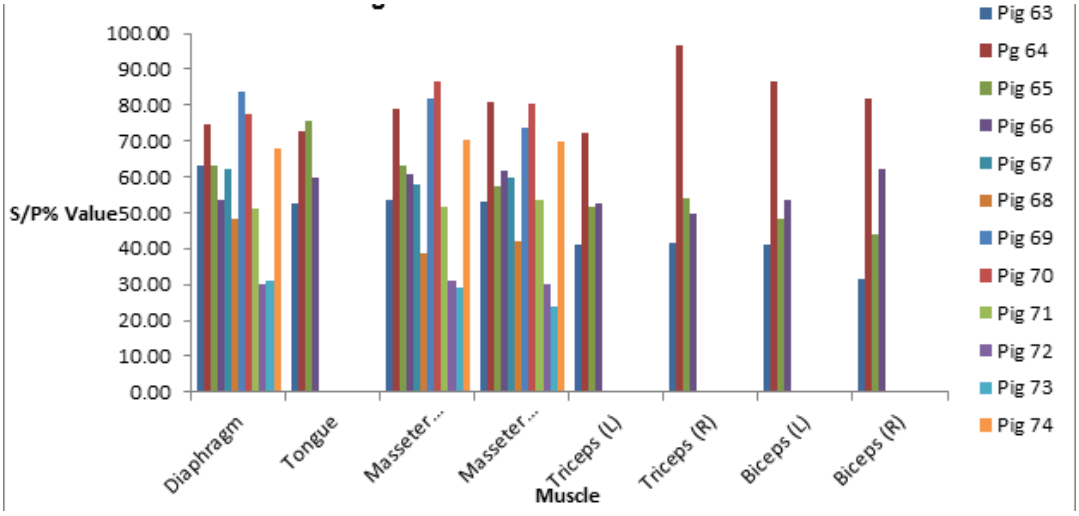


Figure 10. ASFV antibodies in serum (A) and meat juice (B) from pigs infected with ASFV OURT88/3. Cut off values: ID Screen® assay: S/P ≤30% - Negative (yellow); 30-40% - Doubtful (white) and ≥40% Positive (red). INgezim PPA Compac: S/P%≤40% - Negative (yellow); 40-50% – Doubtful (white) and ≥50% Positive (red). The numbers in the table are the % of inhibitions for INgezim PPA Compac and S/P ratios for the ID Screen® Indirect ELISA.

Discussion:

ASFV is an environmentally highly resistant virus and can survive in the environment and in pig carcasses and meat products for a long time. Therefore, one of the main methods of ASFV spread is via uncooked fresh and processed ASFV-contaminated pork products. Meat juice is an easily obtainable sample from muscle tissues collected at slaughterhouses, road kills, supermarkets, and at the border from legally and illegally imported meat and meat products. Using three ASFV strains with high, moderate and low virulence, we demonstrated that meat juice can be used as an alternative sample to detect ASFV genomic material and/or antibodies to ASFV. **Importantly, infectious ASFV was successfully isolated on primary alveolar macrophage cells from all PCR positive meat juice samples.**

In this study we used three commercial ELISA kits: The INgezim PPA Compac ELISA, ID Screen® assay and the INgezim Indirect ELISA kits. The INgezim PPA Compac kit can only be used for serum samples and not for meat juice samples. The remaining two kits can be used to detect anti-ASFV antibodies in meat juice samples.

Highly virulent ASFV leads to death before the animals seroconvert, and therefore, meat juice samples or serum may not be of any use for serodiagnosis, but can be used for viral nucleic acid detection. When pigs are infected with moderately virulent ASFV strains, such as Malta'78 or some of the ASFV strains now circulating in parts of Europe, meat juice can be a useful sample type to detect both ASFV nucleic acids and ASFV antibodies. Low virulent strains result in low levels of intermittent viremia, and therefore detection of viral nucleic acid is not possible, but antibodies can be detected.

Testing of the remaining meat juice samples with the INgezim ASF Indirect ELISA kit (INGENASA, Spain) could not be completed due to the high background signals observed with the new kits received. The high background resulted in false positive results with some of the meat juice samples collected from control animals (Fig. 11A). This issue was communicated to INGENASA and the company is working with us to resolve the background issue of the kit.

The above described results will be summarized and submitted to a peer-reviewed journal for publication.

(A) New INgezim ASF Indirect ELISA kit

Sample	**net OD**	**S/P**	**Interpretation**
Negative control	0.16415	0.00	
Positive control	3.1774	1.00	
neg meat juice P89	0.71905	0.1842	Negative
neg meat juice P42	1.5003	0.4434	False Positive
P53 masseter	2.5486	0.7913	Positive
P53 heart	3.6856	1.1687	Positive
P51 tongue	4	1.2730	Positive
P58 serum	3.976	1.2650	Positive

(B) Old INgezim ASF Indirect ELISA kit

Samples	**net OD**	**S/P**	**Interpretation**
Negative control	0.0737		
Positive control	1.6617		
neg meat juice P89	0.08465	0.0075	Negative
neg meat juice P42	0.0872	0.0096	Negative
P53 masseter	0.48865	0.2613	Positive
P53 heart	0.9169	0.5310	Positive
P51 tongue	1.5345	0.9199	Positive
P58 serum	1.8204	1.0999	Positive

Figure 11. ELISA results on meat juice samples using the new (A) and old (B) INgezim ASF Indirect ELISA kits

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