Dynamics of African swine fever virus (ASFV) infection in domestic pigs infected with virulent, moderate virulent and attenuated genotype II ASFV European isolates.

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**Summary (298 words)**

This study aimed to compare the infection dynamics of three genotype II African swine fever viruses (ASFV) circulating in Europe. Eighteen domestic pigs divided into three groups were infected intramuscularly or by direct contact with two haemadsorbent ASFVs (HAD) from Poland (Pol16/DP/ OUT21) and Estonia (Est16/WB/Viru8), and with the Latvian non-HAD ASFV (Lv17/WB/Rie1). Parameters such as symptoms, pathogenicity, and distribution of the virus in tissues, humoral immune response, and dissemination of the virus by blood, oropharyngeal and rectal routes were investigated. The Polish ASFV caused a case of rapidly developing fatal acute disease, while the Estonian ASFV caused acute to subacute infections in the presence of surviving animals. In contrast, animals infected with the ASFV from Latvia developed a more subtle, mild, or even subclinical disease. Oral excretion was sporadic or even absent in the attenuated group, whereas in animals that developed an acute or subacute form of ASF, oral excretion began at the same time as in the blood, or even 3 days earlier, and persisted up to 22 days. Regardless of virulence, blood was the main route of transmission of ASFV and infectious virus was isolated from persistently infected animals for at least 19 days in the attenuated group and up to 44 days in the group of moderate virulence. Rectal excretion was limited to the acute phase of infection. In terms of diagnostics, the ASFV genome was detected in contact pigs from oropharyngeal samples earlier than in blood, independently of virulence and, together with blood, both samples could cover a longer range to detect ASFV infection. The results presented here provide quantitative data on the spread and excretion of ASFV strains of different virulence among domestic pigs that can help to better focus surveillance activities and thus increase the ability to detect ASF introductions earlier.

**Key-words:** African swine fever; genotype II; virulence; virus shedding.

# Introduction

African Swine Fever (ASF) is one of the most complex infectious swine disease. It is currently considered as a great problem in animal health, representing the most serious challenge for pig sector worldwide. Due to the large economic implications on international trade of pigs and pork products it causes, ASF is on the list of notifiable diseases of the World Organisation for Animal Health (OIE). In recent years, it has affected up to four continents, where more than 75% of the world's pig population is present. ASF is caused by the ASF virus (ASFV), a large and complex enveloped double-stranded DNA virus that affects both domestic and wild suidae. To date, is the only member of the *Asfarviridae* family (Alonso *et* al., 2018; Galindo et al., 2017) with 24 genotypes identified to date (Malogolovkin *et* al., 2019; Quembo *et* al., 2018).

Since the last century, where the disease was present as endemic in more than 20 sub-Saharan African countries (Mulumba-Mfumu *et* al., 2019) and in Sardinia (Cappai *et* al., 2018; Laddomada *et* al., 2019), the situation has been getting worse. In 2007 the ASFV genotype II arrived at a Black Sea harbour in Georgia (Rowlands *et* al., 2007), from where the disease spread quickly west and northwards reaching the European Union (EU) in 2014 (Gallardo *et* al., 2014). Since then, 12 EU countries have reported the presence of ASF, including Lithuania, Poland, Latvia and Estonia (2014), Czech Republic and Romania (2017), Bulgaria and Hungary (2018), Belgium and Slovakia (2019) and Greece and Germany in 2020. Except in Czech Republic and Belgium that regained its ASF-free status, ASF continues to be reported in both wild boar and domestic pigs in the other countries, including in the east of Germany. Data published by the EU's Animal Disease Notification System (ADNS) since 2020 up to May 2021 show a total of 17,680 reported cases in wild boar (in eastern and central EU countries) and 1,720 outbreaks in domestic pigs (affecting eastern EU regions). In wild boar, the presence of the disease increased in 2020, exceeding the total for 2019 by 58%, while in domestic pigs, in most affected EU countries, a clear decrease in the number of outbreaks is observed due to the application of strict sanitary measures.

The worst case scenario was yet to come, when the ASFV showed its devastating power in China, the world's largest pig producer. In August 2018, China reported the presence of the disease (Ge *et* al., 2018), together with a rapid spread in large administrative areas (21 provinces, 5 municipalities and 5 autonomous regions, and Hong Kong (SAR)). As of June 2021, the virus has spread to 13 Asian countries affecting Mongolia, Vietnam (all provinces and municipalities), Cambodia, Democratic People's Republic of Korea, Laos (all provinces and municipalities), Myanmar, Philippines, Republic of Korea, Timor-Leste, Indonesia, India and Malaysia, as well as Papua New Guinea in Oceania (FAO 2021). The reasons for this uncontrolled spread of the virus are related not only to the absence of vaccines or treatments against ASF, but also to the illegal transport of pigs or pork products and the complex structure of the pig industry in China that positions the legal trade as a high risk factor for the spread of the ASFV (Gao *et* al., 2020; O´Hara *et* al., 2020). The lack of knowledge of different aspects of the disease, mainly related to its complex epidemiology, and the transmission capacity of the virus have contributed to its rapid spread.

Without an effective vaccine available, early detection of the disease remains crucial together with strict sanitary measures, including stamping out and movement restrictions. However, these measures have a considerable economic impact. To better combat the disease, more knowledge is needed about the circulating viruses in different scenarios and sub-scenarios. The clinical and pathological parameters as well as ASFV shedding and excretion are essentials to get a better understanding of the disease dynamics and transmission routes within infected and healthy animals/herds. Improving knowledge of the disease dynamics is essential for early recognition of ASF in the field and confirmation through laboratory diagnosis, which contributes to establish a quick response with a better control strategies to fight the disease.

ASF is a disease transmitted by numerous ways, either by direct contact, between infectious and susceptible domestic pigs and wild suids by soft ticks of *Ornithodoros* spp., or by indirect transmission via contaminated wild boar habitat (Chenais *et al.*, 2018). The virus is very resistant to environmental conditions enabling the transmission by indirect contact, through ASFV contaminated pork, people, vehicles and fomites (Blome *et* al., 2020; Costard *et* al., 2009; Gallardo *et* al., 2015a; Gaudreault *et* al., 2020: Olensen *et* al., 2020; Penrith *et* al., 2009). ASFV transmission involves virus shedding by infected pigs in all excretions and secretions, with particularly high levels in oral-nasal ﬂuid (de Carvalho Ferreira *et* al., 2012, 2013b; Ekue *et* al., 1989; Greig and Plowright, 1970; Guinat *et* al., 2014) and occasionally in faeces (de Carvalho Ferreira *et* al., 2012; McVicar, 1984). These viral shedding parameters have been mainly quantified in experimental studies using virulent genotype II strains such as Georgia 2007/1, Estonia 2014 or China 2018 (Pig/HLJ/18) ASFVs (Davies *et* al., 2015; Guinat *et* al. 2014, 2016; Nurmoja *et* al., 2017; Pietschmann *et* al 2015; Zhao et al., 2019). Moreover, there is a clear evidence of the presence of genotype II ASFV strains of different virulence ranging from virulent to attenuated strains co-circulating within Eastern-Central Europe and more recently in Asia (Sun *et* al., 2021). The current ASFV infections range from acute to chronic or even subclinical forms of the disease with recovered and a potential number of long-term animals reported (Gallardo *et* al., 2018, 2019a; Nurmoja *et* al., 2017; Pershin *et* al., 2019; Sargsyan *et* al. 2018; Zani *et* al., 2018). Although shedding of the attenuated Latvia ASFV in wild boar has been recently published (Kosowska *et* al 2020), to our knowledge, no detailed information is available about the dynamics of virus shedding and excretion of different genotype II ASFV strains from domestic pigs that are currently circulating in Europe. Therefore we present a comparative study in domestic pigs experimentally infected with three ASFV genotype II viruses originating from three ASF endemic countries in Europe (Latvia, Estonia, Poland), including the attenuated and non-haemadsorbing (HAD) Latvian strain (Gallardo *et* al., 2019a: Barasona *et* al., 2019). The parameters analysed comprised virus excretion patterns, along with clinical and pathological findings, viraemia, and antibody response. The domestic pigs included in the experiment were infected by intramuscular inoculation or by direct contact, resembling the natural route of infection. The implications of these disease parameters for disease early detection and transmission are discussed.

1. **Materials and methods.**
   1. **Virus isolates and cell culture.**

For the experimental *in vivo* studies three genotype II ASFV isolates were used. These viruses were obtained from field clinical samples collected between 2016 and 2017 in Estonia, Poland and Latvia (Table1). The Estonian Est16/WB/Viru8 is a haemadsorbing (HAD) virus strain isolated after 3 passages in porcine blood monocytes (PBMs) (Carrascosa et al., 2011, OIE 2019) from the serum collected from a wild boar hunted in Lääne-Viru county, in Estonia in November 2016. The Polish Pol16/DP/OUT21 is also a HAD virus strain obtained from a serum sample of a domestic pig slaughtered during the outbreak occurred in Leśna, in September 2016. Virus was passaged three times into PBMs. The Latvian Lv17/WB/Rie1 ASFV was included in this study for comparison as an attenuated and non-HAD genotype II strain. This isolate, obtained from the serum of a hunted wild boar in Latvia in 2017, was previously described by Gallardo *et* al., (2019a) and tested in domestic pigs in an experimental trial.

Titrations of ASFV stocks and of PCR-positive samples were performed on PBM (Carrascosa *et* al., 2011) to monitor the end-point dilution and viral titers (Reed and Munchen, 1938) were determined as the amount of virus causing haemadsorption (for HAD isolates) or cytopathic effect through the use of immunoperoxidase staining (for the non-HAD isolate) in 50% of infected cultures (HAD50/ml or TCID50/ml, respectively).

The MS-stable monkey kidney cell line (ECACC, 91070510) was used for the preparation of the ASFV MS-adapted E70 isolate (E70 MS 48); coated 96-well plates. These plates were then used as the antigen in the indirect immunoperoxidase test (IPT) (OIE 2019).

* 1. **Experimental design.**

Eighteen 3- months’ old European hybrid pigs, 20-25 kg weight, were used in the three independent parallel experiments. The *in vivo* experiments were conducted under biosafety level 3 (BSL3) conditions at the animal facilities of INIA-CISA, in accordance with the EC Directive 2010/63/UE and approved by the Spanish Ethical and Animal Welfare Committee (Ref nº PROEX/125/16). The design of the animal experiments is described in **table 1**.

Upon arrival to the BSL3 facilities, the animals were individually ear-tagged and divided into three experimental groups, with six pigs per group. Each group was placed in a separate pen. Two pigs per group were intramuscularly (i.m.) inoculated with 10 HAD50 of the Est16/WB/Viru8 ASFV (Estonia group), the Pol16/DP/OUT21 ASFV (Polish group) and the attenuated Lv17/WB/Riel ASFV isolate (Latvian group). The four remaining pigs per group were housed together with the inoculated pigs as in contact (exposed) pigs. By 58 days after virus inoculation (dpi), two pigs of the Latvian group (PW13 and PW14) were separated in another pen to be used in a follow-up protection trial and were challenged with a virulent and HAD-genotype II ASFV strain (Lv17/WB/Zieme3) as previously described (Gallardo *et* al., 2019a). Since these two animals (PW13 and PW14) were fully protected against the virulent strain and no significant differences in terms of clinical signs and viraemia were found amongst the challenged and non-challenged pigs (Gallardo *et* al., 2019a), we considered all of them within a single group, denominated as “the attenuated Latvian group”.

* 1. **Clinical evaluation and sampling.**

Clinical signs were recorded daily and expressed with a quantitative clinical score obtained by adding values for eight clinical signs recorded on a daily basis, as previously described by Gallardo *et* al. (2015b, 2017); fever parameters, anorexia, recumbence, skin haemorrhage or cyanosis, joint swelling, respiratory distress, ocular discharge and digestive findings were assigned points on a severity scale of 0 to 3 (most severe). The sum of the points was recorded as the clinical score (CS), which was also used to define humane endpoints. Blood-EDTA, serum, oropharyngeal (OPS) and rectal (FCS) cotton sterile dry swabs (DELTALAB) were collected twice a week starting at 3 days post infection (dpi) or 3 dpe (days post exposure) and on the last day on their study period. Negative control samples were collected on day 0, the day of inoculation. Twenty one different types of tissues and organs were obtained from each necropsied animal, namely, liver, spleen, tonsil, heart, lung, kidney, submandibular, retropharyngeal, inguinal, popliteal, mesenteric, mediastinal, gastro-hepatic, splenic and renal lymph nodes, bone marrow, diaphragmatic muscle and intra-articular tissues of joints. Pericardial and peritoneal fluids were collected for antibody testing.

* 1. **Sample analysis**

*ASFV detection*. DNA was extracted from organ homogenates, blood, OPS and FCS using the High Pure PCR Template Preparation kit (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany). Briefly, 10% (w/v) clarified homogenized tissue suspensions were prepared in phosphate-buffered saline (PBS). Cotton swabs were soaked in 2 ml of phosphate buffer saline (PBS), vortexed for approximately 15 seconds, incubated for one hour at room temperature, and decanted in a microcentrifuge for 10 minutes. Supernatants were filter with MINISART filters 0.45m and then treated with 0.1 % of gentamicin sulphate 50mg/ml (BioWhittaker) during 1h at 4±3ºC prior to use for virus detection. For amplification of the ASFV genomic DNA, the Universal Probe Library (UPL) real-time PCR (Fernandez-Pinero *et* al., 2013; OIE 2019) was carried out using undiluted extracted DNA for each sample. Samples with recorded Ct < 40.0 were considered positive, while samples with no recorded Ct value were considered negative. Virus isolation (VI) and titration were performed using PBM cells and titers were estimated by end-point dilution, as described in point 1.1.

*ASF antibody detection:* Detection of ASFV-specific antibodies was performed in serum using the IPT and the commercial ELISA (®INGENASA-INGEZIM PPA COMPAC K3 INGENASA, Madrid, Spain). The tissue exudates of liver, lung, kidney, heart, spleen, bone marrow and diaphragmatic muscle, and ascetic and pericardial fluids (if present) were tested for antibody detection using the IPT. The ASFV antibody titers were determined in all positive samples by end-point dilution using the IPT (OIE 2019).

* 1. **Statistical Analysis**

For the comparative analysis of the samples positives by PCR with respect to the virulence of ASFV and the type of sample analysed, only samples taken from 3 to 76 dpi / dpe were considered, which were divided into two periods, i) samples taken from 3 at 20 days where all the groups were compared and, ii) samples taken from day 21 in which only the moderate and attenuated virulence groups were compared. In summary, a total of 181 paired blood, OPS, and FCS samples were included in the comparative study (22 from the virulent group, 56 from the moderate virulent group, and 103 from the attenuated group). Statistical analyses were performed using XLSTAT software (Data Analysis and Statistical Solution for Microsoft Excel, Addinsoft, Paris, France 2017). To establish the correlation between ASFV-virulence groups *vs* type of sample and percentage of positives, two-way ANOVA was used while the Tukey test served to determine the statistical significance of the differences between individual means values. Mann–Whitney U test was used to compare different ASFVs and the positive results of the PCR in blood, OPS and FCS samples in the two groups compared after 21 days-period. A *p-*value < 0.05 was set as the statistically significant level.

1. **RESULTS.**
   1. **Comparative clinical outcomes of animals infected with the HAD and non-HAD genotype II ASFV isolates.**

All inoculated and in-contact animals were successfully infected independently of the strain used although differences were found in the clinical outcome of the disease (**Table2**).

After incubation period of 4.5 ± 0.7 days, inoculated pigs (IP) with HAD Polish ASFV showed increased temperature (>41°C), lethargy and reduced appetite, and died or were euthanized at 8 dpi. The IP with the HAD Estonia ASFV started with mild fever (40-40.5°C) at 5.5 ± 0.7 dpi and died or were slaughtered at 10 dpi reaching also temperatures (>41°C) . This group had higher clinical scores (up to score 8.5) than Polish group (up to score 5.5) with the cyanosis in the ears and tail and the skin hemorrhages prevailing. (**Figure 1A**).

Thein-contact pigs (CP) within the Polish group started to show mild fever (40-40.5°C) at 9±1 dpe, around 4-5 days later than the IPs. Except in one animal, a worsening of clinical signs was recorded at day 13 with increased body temperature (from 41 to 41.5°C), recumbence, anorexia, generalized body congestion and cyanosis, and were euthanized or died at 14-15 dpe. In one CP the onset of the disease was slightly delayed to finally succumb at 20 dpe. The domestic pigs exposed to the HAD-Estonia ASFV showed similar clinical signs from 13 dpe, except pig #E6 that had a peak of fever (40.7°C) at day 9. Clinical scores subsequently increased in 2 out of the 4 CP (50%), which were euthanized between 15 and 20 days after the initial exposure with acute to subacute clinical signs. Opposite to that observed in the inoculated group, the domestic pigs naturally infected with the Polish ASFV had higher clinical scores (up to 13) than those obtained within the Estonia CP group (up to 9.5) (**Figure 1B**).

Two remaining in-contact animals infected with the HAD-Estonia ASFV (#E2 and #E6) developed similar clinical signs from 13 dpe (albeit less intense in pig #E6) but they survived the disease. High fevers of 41 °C were detected as early as 13 dpe and lasted up to 22-25 days before temperatures decreased to within normal limits (<40 °C). Mild cyanosis in ears, swollen of joints of the rear legs and conjunctivitis was then subsequently observed between 16 to 25 dpe. After an apparent recovery by day 29, the clinical signs reappeared around days 37-41 in both animals. Generalized lymphadenopathy and swelling of joints were the most noticeable clinical signs at that time, but the body temperatures were within the normal range (<40°C). A worsening of the clinical signs was observed at 65 dpe in one pig (#E6) with severe necrotic areas in the joints accompanied by the appearance of cyanosis in the ears and severe ocular discharge, reaching a high clinical score of 15 at the time of death at 76dpe. The other domestic pig (#E2) was also slaughtered at 76 dpe to compare the virus distribution in tissues (**Figure 1B**).

Domestic pigs infected with attenuated and non-HAD Lv17/WB/Rie1ASFV developed non-specific clinical signs or, in some cases, remained apparently healthy across the entire observation period, even in the challenged group, as previously described by Gallardo *et* al., (2019a). In summary, in four out of the six infected animals, the most noticeable clinical reaction was a slight increase in body temperature (from 40 to 40.7°C as maximum) starting around 8dpi/11dpe, accompanied by the appearance of swelling of the joints that was more intense in the IP PW17, followed by the CP PW14. Two CPs (PW15 and PW18) did not develop any detectable clinical signs other than a weak peak of fever at 10 dpe in one animal (PW18). The animals were slaughtered at 25 dpe (PW16), 45 dpi (PW17), 101 dpe (PW15 and PW18) and 126 dpi/dpe (PW13 and PW14) to assess the virus presence in tissues (Gallardo *et* al., 2019a).

* 1. **Pathological findings and virus presence in tissues of animals infected with the HAD and non-HAD genotype II ASFV isolates.**

Pathomorphological changes compatible with an acute (Poland and Estonia) or subacute (Estonia) form of ASF were shown in all animals infected with the HAD Polish or Estonia ASFVs that died or were slaughtered between 8 to 20dpi/dpe. The vascular changes observed in the animals infected with the Estonia ASFV, mainly hemorrhages and edema, were more intense than those reported in the Polish group whereas, in the latter, the spleen was more affected. The ASFV genome was detected in all organs tested at this point in the pigs of both groups, and the virus was easily isolated in PBMs after one passage. In the pigs infected with the Estonia ASFV, titers were 1 log lower (average titer 105±0.2HAD50/ml, 5log10 [95% of confidence interval -CI- of 4.5–5.5]) than in the group of pigs infected with the Polish ASFV (average titer 106±0.3HAD50/ml, 6log10 [95% CI of 5.2–6.7]). The viral load in the tissues obtained from the CPs showed similar results with an average titer of 105±1HAD50/ml (5log10, [95% CI of 2.5–7.4]).

Pathological findings found in the surviving pigs within the in-contact Estonian group were in agreement with the clinical signs. Both of them were euthanized at 76 dpe, but whereas no significant lesions were found in the pig #E2, examination of the pig #E6 revealed the presence of ascites, petechial hemorrhages in the epicardium, endocardium and pleura, fibrinous pleuritic and pericarditis, pleural adhesions, necrotic pneumonia and edematous and hemorrhagic renal, gastro-hepatic and mediastina lymph nodes. In this pig (#E6), the ASFV genome was detected by PCR in 71.4% (15/21) tested organs and infectious ASFV could be recovered in 40% (6/15) of them, including the tonsil, retropharyngeal, gastro-hepatic, inguinal and submandibular lymph nodes and the intra-articular tissues of the left anterior limb, the latter yielding the highest viral load of 105 HAD50/ml (data not shown). Interestingly, despite no significant lesions were found in the pig #E2 at the time of slaughter, the virus genome was detected in 4 out of the 21 (19%) collected tissues (retropharyngeal and submandibular lymph nodes, bone marrow and tonsil), and the tonsil was found positive for infectious virus with a low titer of 102 HAD50/ml (2log10).

In the group of animals infected with the attenuated Latvian strain, fibrinous pericarditis and focal pneumonia associated with a secondary bacterial infection were found in the animals slaughtered at 25 dpe (PW16) and 45 dpi (PW17). No significant lesions were identified in the animals slaughtered at 101 dpe (PW15 and PW18) or 126 dpi/dpe (PW13 and PW14). The ASFV genome was detected by PCR in all tissue samples at 25 days, in 90.4% (19/21) at 45 days, and in 42% (9/21) at 101 days but only in pig PW15, while PW18 was negative. As previously described by Gallardo *et* al. (2019a), infectious ASFV was isolated from 16 (76.1%) and 6 (31.6%) different tissue samples at 25 and 45 dpi / dpe, respectively. At 101 days, only 2 tissues from pig PW15 were positives in virus isolation (retropharyngeal and submandibular lymph node). In the challenged animals slaughtered at 126dpi (PW13 and PW14), the presence of ASFV was demonstrated by PCR in 4/21 (33.3%) but the non-HAD ASFV could not be isolated, although the challenge virulent HAD ASFV strain was isolated from the tonsil as previously described (Gallardo *et* al., 2019a).

The comparative PCR and VI results obtained among three groups in tissues are showed in the **Supplementary file S1**

* 1. **Viraemia and virus shedding of animals infected with the HAD and non-HAD genotype II ASFV isolates.**

A total of 735 individual paired samples of blood, oropharyngeal (OPS) and fecal swabs (FCS) were analyzed, 84 from the Polish group (from here referred as “virulent group”), 186 from Estonia group (from here referred as “moderate virulent group”) and 465 from the Latvian group (referred as “attenuated group”). Samples were tested by real-time PCR and VI as described in point 1.4. Viraemia and excretion patterns were followed for a period of up to 20, 76 and 126 days in the virulent, moderate virulent or attenuated group, respectively. The comparative results are shown in **table 3.**

* + 1. *Viraemia and virus shedding in domestic pigs infected with the HAD and virulent Polish Pol16/DP/OUT21 ASFV***.**

In the IP, the HAD and virulent Polish ASFV strain was detected for the ﬁrst time by PCR at 3 dpi in blood, reaching the maximum virus titer of 6.81x107 HAD50/ml (7.8log10) at 7 dpi, which was the highest amongst all samples. Virus shedding started at 5 ± 1.6 dpi in OPS and FCS in all pigs. The OPS and FCS samples in all animals tested positives for VI at 7 dpi with titers of 1 and 2 logs lower than those obtained from blood (**Figure 2A**). Amongst the CPs, the viral DNA was first detected at 7 dpe in OPS in all animals but no virus could be isolated at this point (Ct values > 35). The mean for the initial ASFV genome detection in FCS and blood was of 8.5 ±1.3 dpe and 10 ±2.1 dpe, respectively. While the OPS and blood samples remained PCR-positive during the whole observation period, the detection of the ASFV genome in FCS was intermittent and, only one animal excreted virus in faeces (titer of 3.16x 104 HAD50/ml. 4.5log10) at 13 dpe. These data were consistent with clinical signs since this domestic pig had bloody diarrhea from day 11 until his slaughter, three days later. In contrast, infectious virus was detected in both blood and OPS since around 10 and 13 days, respectively, until the death in all domestic pigs. The mean highest titer in whole blood was of 8.11x107 HAD50/ml (7.9log10 [95%CI of 5.2–10.6]) obtained at 13 dpe, which was the highest amongst all samples. The mean maximum titers of OPS was of 1.72 x 105 HAD50/ml (4.3log10 [95% CI of 2.5–6]) at 10 dpe (**Figure 2B**).

* + 1. *Viraemia and virus shedding in domestic pigs infected with the HAD and moderately virulent Estonia Est16/WB/Viru8 ASFV.*

Within the group of pigs inoculated with the moderate virulent HAD Estonian ASFV, all animals were positive for ASFV genome and infectious virus independent of the samples tested. Viral DNA in blood was positive at 3 dpi followed by OPS and FCS at 7 dpi. Virus shedding started at 7 days in blood, OPS and FCS, yielded similar titers in OPS and in blood (3.1x107 HAD50/ml, 7.5log10), while in FCS the virus titer was four logs lower (1.58x104HAD50/ml, 4.2log10).The detection of genome and infectious ASFV was constant in the samples tested throughout the sampling period (from the ﬁrst appearance until the day of death or euthanasia) **(Figure 3A)**.

In the in-contactpigs that succumbed to the infection within the first three weeks **(Figure 3B)**, the ASFV genome was initially detected in OPS at 10 days, followed by blood at 11.5±1.3 days and FCS at 13 days. Infectious virus was isolated in OPS from day 10 -three days earlier than in blood- until the death of the pigs, showing a mean titer of 1.61x104 HAD50/ml (3.7log10 [95% CI of 2.2-5.1]). As expected, the highest titer of ASFV of about 6.5x108 HAD50/ml (8.8log10) was from blood samples (13dpe). No virus could be isolated from faeces **(Figure 3B).** In animals that survived to infection **(Figure 3C)**, a peak of viraemia was detected by PCR in the pig #E2 at 7 dpe (Ct = 37.62), prior to the weak peak of fever found at 9 dpe, but turned negative until day 13. At that point both animals became PCR positive until 72 (#E2) and 76 (#E6) dpe in the days analysed. The infectious ASFV was recovered from day 13 in all blood samples and lasted for a month (until day 44) with titers of up to 106HAD50/ml (6log10) The OPS samples were weak PCR positives (Ct average 37) from 7 to 52 dpe. Virus was isolated from a 10 days-window (7-20 dpe) but only in the pig #E2 (average titer of 3.3x104 HAD50/ml, 4.2log10 [95%CI of 2.9-5.4]). Fecal samples from these animals were PCR and VI-negative with the exception of one sporadic detection at 16 dpe (#E2) found positive for ASFV genome and VI with low virus level (3.16x103 HAD50/ml, 3.5log10) **(Figure 3C).**

* + 1. *Viraemia and virus shedding in domestic pigs infected with the non-HAD and attenuated Latvian Lv17/WB/Rie1 ASFV.*

The domestic pigs infected with the non-HAD attenuated Latvian ASFV showed variable results depending on the animal and the samples tested. Initial genome detection was identified by PCR from both blood and OPS samples taken at 3 dpi from the IP PW17 and lasted until the death of the animal (45 dpi). Infectious virus was recovered from blood samples over a period of 19 days (3–22 dpi) with a titer of 4.76 × 109 TCID50/mL (9.7log10) (Gallardo *et* al., 2019a). No virus could be isolated from PCR-positive OPS samples. The other IP (PW13) was used for the protection trial done at 58 dpi as previously described (Gallardo *et* al., 2019a). Prior to the challenge, and in agreement with the clinical signs, the IP PW13 had a shorter viraemia (7 to 22 dpi) and virus shedding (7-17dpi). The maximum peak of titer was 3.4 × 106 TCID50/m (6.5log10)at day 14. After challenge, and despite the absence of clinical signs, the blood started to be PCR-positive at 91 dpi (33 days post-challenge) and lasted until the slaughter at 126 days. Sporadic shedding of the virulent ASFV was detected at 94 dpi -36 days after challenge- (titer 6.81x105 HAD50/mL, 5.9log10). The OPS samples taken from this animal throughout the experiment were occasionally weak positive by PCR (Ct> 35) on days 10, 14, 17, 31, 66, 70, 80, 98 and 101 but negatives in VI **(Figure 4A).**

Large differences were observed between pigs infected by direct contact with the attenuated strain. The pig that had more pronounced clinical signs of a chronic form (PW14) started to be PCR positive in OPS samples at 10 dpe followed by blood at 14dpe. Viraemia lasted for 35 days (14 - 49 dpe) with virus shedding over a 7-days period (14-22 dpe) and a maximum titer of 3.4 × 107 TCID50/mL (7.5log10)at day 14. OPS samples were positive by PCR intermittently until day 35 but negative in VI. This pig was used for the challenge experiment, but no virus was detected in any samples after challenge, except a weak positive in OPS by PCR (Ct =37.69) on day 80. In the pigs that did not develop clinical signs, the ASFV genome could be intermittently detected at a low level (Ct>35) in OPS samples in all pigs, but in blood in only two pigs. Infectious virus was not detected in these sample types except in pig PW18. Interestingly, despite the negative viraemia result in the latter, OPS samples were positive by PCR from 7 up to 29 dpe with sporadic virus shedding on day 14 (virus titer of 104 TCDI50/mL, 4log10). Occasionally, weak PCR positive values were obtained in faecal samples from both inoculated and in contact animals, but virus could not be isolated in any case (**Figure 4B**).

* + 1. *Comparative analysis of viraemia and virus shedding amongst groups infected with the HAD and non-HAD genotype II ASFV isolates.*

Given the *p*-value of the F statistic computed in the two-way ANOVA procedure, and given the significance level of 5% (one-way ANOVA; F = 24.026, *p*= 0.005), the information brought by the ASFV and the type of sample tested provided significant differences in the percentage of positives by PCR. The overall analysis of the results showed the highest percentages of positive samples in the virulent group in OPS (77.3%), while was in blood (80.4%) in the moderate virulence group. However, similar percentages of positivity (around 30 to 35%) were obtained in both type of samples in the attenuated group but significantly lower than those observed in the other groups. Significant differences (*p*<0.05) were observed when FCS were analyzed and only in the virulent group the positivity reached more than 50% (table 4).

Among the samples collected within the first period (3-20 days), the highest proportion of PCR-positive samples was obtained in OPS with percentages of 77.3% in the virulent group, 69.2% in the moderate virulence group and 50% in the attenuated group. In the case of bloods, no significant differences (*p*>0.05) were detected between virulence and moderate virulence groups with similar percentages (61.5 to 68.2%), while in the attenuated group this percentage decreased to 37.7%. After day 20, there was a significant difference in the percentage of PCR positives blood samples (*p* <0.05) between the moderate and attenuated virulence groups. Only 29% of the blood samples tested in the attenuated group had positive a PCR results, while 97% were positive in the moderate virulence group. In contrast, we did not observe significant differences amongst OPS positive samples (*p*>0.05) between both groups with similar percentages (30% *vs* 32%). The percentage of positives in feces was less than 50% in all cases, except in the virulent group, even during the first twenty days regardless of the period tested.

VI was done only on PCR positive samples. The virus was easily isolated from the blood during the first three weeks in the non-HAD attenuated (100%) and in the HAD-virulent (86.7%) and moderate virulent (81%) groups. After this period, the infectious virus was only detected in 31% and 10% of the PCR positive blood samples. In contrast, in OPS and FCS, the virus was isolated within the first 20 days only in the HAD ASFVs, but not in the attenuated group, where it was sporadic.

The comparative positivity results obtained by PCR and VI with regard to the type of samples tested, the ASFV strain used and the day periods are summarized in **table 4.**

* 1. **Seroconversion of animals infected with the HAD and non-HAD genotype II ASFV isolates.**

For serological examination, serum samples were taken from all animals at different time points and analysed with two different methods: IPT and commercial blocking ELISA. In addition, a total of 7 fluids including tissue´s exudate (*n=5*) obtained from heart, lung, liver, kidney and spleen and fluids collected from the abdominal cavity (*n*=1) and pericardium (*n*=1), were tested by IPT to determine the presence of antibodies. The comparative results are shown in **table 5.**

In the "virulent group", only one pig in contact (25%) was ELISA positive from 16 dpe. By IPT, 83.3% of the animals (5/6) were positive from 7 dpi or 11.5 ± 1.2 dpe in serum. Furthermore, 100% of the infected pigs with the virulent Polish virus had antibodies in the lung, 83% (6/7) in liver, kidney, heart and spleen, 67% (4/6) in ascitic fluid and 50% (3/6) in pericardial fluid.

For domestic pigs infected with the Estonian strain ("moderately virulent group") that died within the first 20 days, 50% (2/4) were IPT positive in serum samples from day 7 (IP) or 16 (CP). All were ELISA negative. In contrast, liver, lungs, spleen, ascites, and pericardial fluids were positive in all four domestic pigs. No significant differences were found when compared with the virulent virus strain, although the mean antibody titer was 1 log higher than that obtained in the virulent group, either in serum or in tissues and fluids (data not shown). In the two pigs that survived the infection, a strong antibody response was detected by ELISA and IPT from day 16 to slaughter on day 76 (mean antibody titers 1: 163.840 5.21 Log10). Within the attenuated group, antibodies were detected in all infected domestic pigs at 7 dpi (IP) and 19.5 ± 3.6 dpe (CP) that produced antibody titers >1: 163,840 5.21 Log10. All tissues and fluids were also positive for antibody detection.

1. **DISCUSSION**

ASF has become without any doubt, the number one threat for the swine industry, significantly affecting the global commerce equilibrium. Although ASF is registered as one of the most important transboundary animal diseases (TADs), notifiable to OIE, the wide re-occurrence of outbreaks in various regions of the world indicates that many questions are still poorly solved concerning the biology of this devastating disease. The ASFV strains circulating in Europe and Asia of p72 genotype II (Gallardo *et* al., 2014; Ge *et* al., 2018; Kim *et* al., 2020; Mai *et* al., 2021; Malogolovkin *et* al., 2020; Rajukumar et al., 2021; Vilem *et* al., 2020), which are generally highly virulent both in domestic pigs and wild boar causing acute disease with almost 100% lethality in animals of all ages and sexes (Blome et al., 2020; Pikalo *et* al., 2019; 2020). However, one major concern is its increasing and continuous trend to appear and reappear in a clinically very mild or in a completely unapparent form in areas where the disease is endemic (Lamberga *et* al., 2018, 2020; Nurmoja *et* al., 2020; Sánchez-Vizcaíno *et* al., 2012, 2015). By being unnoticed for a time, such mild infections may spread to large populations of pigs, causing serious social, sanitary and economic consequences (Gallardo *et* al., 2018, 2019b; Nurmoja *et* al., 2017, 2020; Sargsyan *et* al., 2018; Sehl *et* al., 2020; Sun *et* al., 2021). Considering the varying ASF clinical manifestations observed in Europe in areas where genotype II is circulating for years, this study aimed to expand and improve the current knowledge in the ASFV infections by experimental inoculation or direct contact infection (resembling a natural route), using three genotype II ASFV isolates exhibiting different virulence~~,~~ including the previously analysed non-HAD and attenuated Latvia strain (Gallardo *et* al., 2019a; Barasona *et* al., 2019).

Domestic pigs in all three groups, both inoculated and in direct contact, were successfully infected with the corresponding ASFVs, but clinical signs and pathological findings varied markedly amongst the three groups. As previously described, the Latvian non-HAD ASFV strain (Lv17/WB/Rie1) is a low virulence ASFV that causes very weak or unapparent clinical symptoms in domestic pigs and all animals survived the infection (Gallardo *et* al. 2019a). In contrast, the HAD ASFV collected from a domestic pig in Poland (Pol16/DP/OUT21), induced acute lethal disease in both inoculated and in direct contact pigs, with clinical signs and pathological lesions comparable to previous studies with highly virulent genotype II strains. (Blome *et* al., 2013; Gallardo *et* al., 2017; 2018; Nga *et* al., 2020; Pikalo *et* al., 2019, 2020; Rodriguez-Bertos *et* al., 2020; Sánchez-Cordón *et* al., 2020; Sánchez-Vizcaíno *et* al., 2012, 2015; Zhao *et* al., 2019). Domestic pigs infected with the Estonia HAD ASFV (Est16/WB/Viru8) had similar clinical signs as those observed in the Polish group although with longer incubation period (5-13 days). Two contact pigs within this group survived, but one of them developed a persistent infection with reappearance of clinical signs from day 41, that led to death on day 78. Similarly to that observed with other moderately virulent ASFVs, the death of domestic pigs infected with the Estonia ASFV occurred at two different stages: (a) during the initial phase with the pigs dying at 7–20 after infection with similar clinical signs to those observed in the acute clinical form, and (b) during the “recovery” phase, with chronic clinical signs (de Carvalho Ferreira *et* al., 2012; Gallardo *et* al., 2018; Petrov *et* al., 2018; Salguero *et* al., 2020).

By comparing the distribution of the virus in the tissues of pigs that survived after primary infection, either with the moderate virulence Estonia virus or with the attenuated Latvian strain, the infectious virus was cleared more or less rapidly from the target organs, such as bone marrow, spleen or kidney. However, it persisted for more than two months in primary replication sites, such as tonsils and lymph nodes, or in secondary replication sites, such as intra-articular tissues. This long-term detection of ASFV in animals infected with both attenuated and moderate virulent virus strains was comparable to previous studies. In pigs infected with the moderately virulent strains from the Dominican Republic (DR'79) or Malta, or with the attenuated and non-HAD NH/P68 Portuguese ASFV, the virus was detected in lymph nodes and/or tonsils for long periods of time, even up to 13 weeks after infection (Gallardo *et* al., 2015b Leitao *et* al., 2001; McVicar, 1984; Oura *et* al., 1998). Similar findings have been recently described in a pig (chronic form) that survived after the infection with the moderate virulent genotype II Polish ASFV. Viral DNA was detected in tonsil and lymph nodes after one month of being infected but not in target organs such as the spleen, liver or bone marrow (Walczak *et* al., 2020). Localized virus presence in lymphoid tissues that occur to some extent in any of the categories of survivors, could suggest either the likelihood of persistent infection, or that pigs undergo several reinfections with the same strain. Since not fully neutralizing antibodies are produced the virus is usually present where primary virus replication occurs (Colgrove *et* al. 1967; Wilkinson *et* al., 1981). The reason for this could be that the animals are kept in a contaminated environment during the experimental trials and have, therefore, the highest probabilities to be re-infected. However, all together these data suggest that other tissues should be also considered as target samples in the surveillance programs (Beltran-Alcrudo *et* al., 2017).

Given that one of the main concerns of the ASFV is transmission to susceptible hosts, we were particularly interested in evaluating the routes of shedding of ASFVs of different virulence to pigs in direct contact within the same pen. Within this study, blood was clearly the main route of ASFV transmission, although with clear differences amongst groups. Consistent with previous studies, in animals that developed an acute or subacute form of ASF, the virus was isolated from the blood at the same time as OPS, or in some cases about 3 days later, but with titers 1 to 2 log higher and for a longer period (44 days *vs* 22 in OPS) (de Carvalho Ferreira *et* al., 2012; Petrov *et* al., 2018; Walczak *et* al., 2020; Zani *et* al., 2018; Zhao *et* al., 2019). The pigs infected with the attenuated Latvian strain were able to eliminate the infectious virus from the blood in a shorter interval between 7 and 19 days, depending on the animal, but with similar titers (104TCID50 / ml) to those of the moderately virulent group. Studies on the domestic pig behavior reported that common social interactions, such as feeding or mating, generally cause skin injury and if this induces any bleeding, then this appears to give rise to more bites and licking (Kittawornrat *et* al., 2011). Therefore, blood would easily contaminate the environment, especially in a within-pen transmission scenario (Cádenas-Fernández *et* al., 2019). In contrast, within this group, oral excretion was sporadic or even absent, and only one pig was able to excrete the virus orally. Therefore, although sporadic shedding of attenuated viruses throughout the respiratory tract cannot be excluded (Gallardo *et* al. 2015b), it is questionable to what extent this is really relevant with attenuated strains given the high virus dose (104 TCDI50/ml) generally required for oral infection (McVicar, 1984; Ståhl, *et* al., 2019). These findings suggest that the risk of oral transmission, which is the natural route of infection, is much lower with attenuated viruses than with highly virulent or moderately virulent strains. However, transmission through contaminated blood cannot be ruled out. Finally, the elimination of the ASFV through the feces seems to be limited, being more important in the acute phase of the infection caused by virulent strains, although it generally occurs two or even four days later than in blood (Guinat *et* al., 2014). After the acute phase, the presence of the virus in the stool decreases rapidly, making it an unreliable diagnostic sample. In addition, it must be taken into account that the half-life of the virus in the field is strongly affected by the enzymes (proteases and lipases) produced by bacteria that colonize the feces, so the survival of the virus in the field is not comparable with the estimates obtained under laboratory conditions (EFSA 2018).

From the diagnostic point of view, the ASFV genome was generally detected by PCR, in OPS samples from the contact pigs before they developed viraemia, while it was in the blood of the inoculated pigs. This is consistent with previous studies in which ASFV genome could be detected within oral and pharyngeal swabs 1–2 days prior to systemic dissemination due to primary replication in the tonsil and retropharyngeal lymph nodes in the natural infection route (Howey *et* al., 2013; de Carvalho Ferreira *et* al., 2012, 2013, 2014; Greig and Plowright, 1970; Guinat *et* al., 2014; Kosowska *et* al., 2020; Petrov *e*t al, 2018; Pietschmann *et* al., 2015). These observations, along with other results obtained in several studies, support the importance of OPS as a relevant type of sample to complement the surveillance programs for the early diagnosis of infected pigs (Elnagar *et* al., 2021; Flannery *et* al., 2020; Guinat *e*t al., 2014; Olensen *et* al., 2020). However, the overall analysis of the results, showed that the highest percentage of positivity was obtained in blood samples independent of the strain assayed, confirming their valuable use in the diagnosis of the disease. Our results contrast somewhat with recent studies that reported a low number (11%) of PCR-positive blood samples in wild boars oronasally infected with the attenuated Latvian strain over a 30-day observation period whereas in our study this percentage increased to 43% (Kosowska *et* al., 2020). The reason could be that in our study, the animals that presented the highest percentage of PCR positive blood samples (77%) were those infected by the intramuscular route. In pigs in contact, the natural route of infection, similar percentages to those described above (24%) were obtained, although still 10 points higher (Kosowska *et* al., 2020). This could be because we used a different PCR method, the UPL real-time PCR (Fernandez *et* al., 2013; OIE 2019), which has been shown to be more sensitive in detecting a low viral DNA load than the real-time PCR method of King (King *et* al., 2003) used in the study by Kosowska (Gallardo *et* al., 2015c; 2019b).

The serology results also provided differences between the three groups. According to previous studies, all animals infected with the attenuated strain developed a high antibody response from the week of infection, which was maintained until the end, that is, more than 4 months (Boinas *et* al., 2004; Gallardo *et* al, 2015b; Leitao *et* al, 2001). No differences were found between pigs infected with the HAD-ASFVs that died in the first 20 days, although the mean antibody titer was 1 log higher in the moderately virulent group compared to the virulent group, both in serum and in tissue exudate. The surviving animals infected with the Estonia ASFV developed a strong antibody response from 14-21 days to the end of the experiment, similar to that observed in animals infected with strains of moderate virulence (Gallardo et al., 2018). Furthermore, it is important to note that specific antibodies against ASFV were detected in tissues using IPT in all infected pigs, even those acutely infected with a negative serum result. These findings confirm the usefulness of IPT as a diagnostic tool to analyze any type of porcine material such as blood, tissue exudates or body fluids, in the surveillance of wild boar when in most cases serum are not available (Gallardo et al., 2015c, 2019).

In conclusion, this study confirms previous expectations of the presence of genotype II ASF of different virulence co-circulating in EU countries. The Polish ASFV caused a case of rapidly developing fatal acute disease, while the Estonian ASFV caused acute to subacute infections in the presence of surviving animals. In contrast, animals infected with the ASFV from Latvia developed a more subtle, mild, or even subclinical disease. Comparative data on clinical manifestations, pathological signs, ASFV tissue distribution, and humoral immune response and transmission parameters reveal many aspects in practice that could be useful for the early diagnosis of ASF, with special attention to the detection of very mild or unapparent clinical manifestations. The present study shows that in the case of ASFVs of high and moderate virulence, virulence influences the appearance, intensity, duration and evolution of the disease and, therefore, the degree of spread, depending not only on the virus, but of the animal. With regard to attenuated ASFV, virus shedding clearly occurs in a shorter time interval and only contaminated blood appears to be relevant. In terms of diagnosis, oral swabs provide a useful means of early detection regardless of virulence and, together with blood, could cover a longer range for detecting ASFV infection. IPT remains the best option for serological determination; however, in most cases, it is not suitable for routine work, since it is time-consuming and requires specialized trained staff (Gallardo et al., 2019b).

Other research groups reported on comparative *in vivo* analysis of ASFV strains of genotype II in wild boar (Kosowska *et* al., 2021) but none of the previous investigations provided such a comprehensive analysis of various virulence genotype II ASFVs in domestic pigs as the present study.

**ACKNOWLEDGEMENTS**

This study was supported by the INIA (projects RTA2015-00033-C02-01, AT2015-002) and the European Union Reference laboratory for ASF (grant nº UE- LR PPA/03). We would like to thank all the staff at the INIA-CISA who work in the animal facilities department.

**Conﬂict of interests**

The authors declare no conﬂict of interest.

**Data availability statement.**

The data that support the findings of this study are available from the corresponding author [CG] upon reasonable request.

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**FIGURE LEGENDS**

**Figure 1:** Clinical score (right X-axis) overlapped with percentage of survival (left-X-axis) in the domestic pigs infected with the haemadsorbing (HAD) **Polish (red color)** or **Estonia (blue color)** genotype II ASFVs in the group of  **(A)** inoculated domestic pigs and **(B)** in contact domestic pigs. Means per day post inoculation (dpi) or exposure (dpe) are shown for all infected animals.

**Figure 2:** Relationship between ASFV genome detection by PCR and virus shedding assessed by VI, in **(A)** pigs inoculated with ASFV Pol16/DP/OUT21 and **(B)** pigs exposed by direct contact. Lines indicating ASFV genome detection in **whole blood (▬)**, **oropharyngeal swabs (OPS) (▬)** and **fecal swabs (FCS)** **(▬)** are expressed on the left X-axis. The VI (bars) are expressed on the right X-axis. Means per day post inoculation (dpi) or exposure (dpe) are shown for all infected animals.

**Figure 3:** Relationship between ASFV genome detection by PCR and virus shedding assessed by VI, in **(A**) pigs inoculated with ASFV Est16/WB/Viru8, **(B)** pigs exposed by direct contact that died within 20 days after exposure (#E3 and #E4) and **(C)** survivors in contact pigs slaughtered at 76dpe (#E2 and #E6). Lines indicating ASFV genome detection in **whole blood (▬)**, **oropharyngeal swabs (OPS) (▬)** and **fecal swabs (FCS)** **(▬)** are expressed on the left X-axis. The VI (bars) are expressed on the right X-axis. Means per day post inoculation (dpi) or exposure (dpe) are shown for all infected animals.

**Figure 4:** Relationship between ASFV genome detection by PCR and virus shedding assessed by VI, in **(A**) pigs inoculated with ASFV Lv17/WB/Rie1, **(B)** pigs exposed by direct contact. Lines indicating ASFV genome detection in **whole blood (▬)**, **oropharyngeal swabs (OPS) (▬)** and **fecal swabs (FCS)** **(▬)** are expressed on the left X-axis. The VI (bars) are expressed on the right X-axis. Means per day post inoculation (dpi) or exposure (dpe) are shown for all infected animals.