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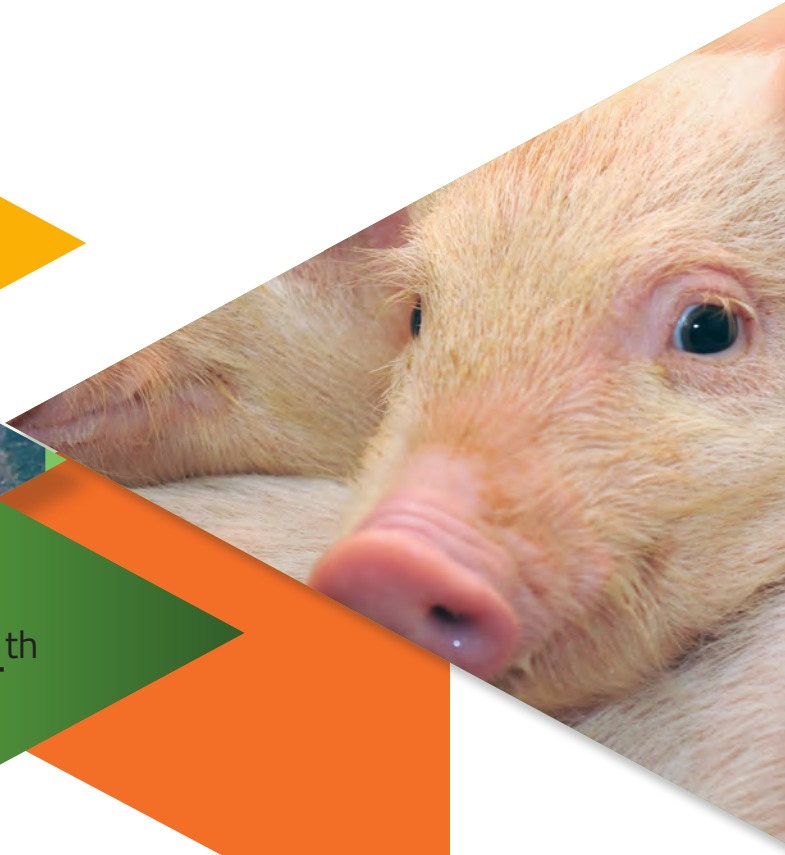

# Proceedings IPVS2022

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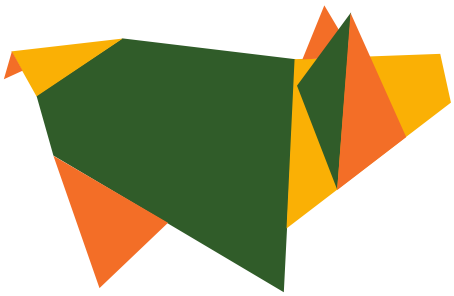

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# Update on ASF diagnosis and current circulating strains

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**Summary**

Knowledge of and attempts to control African swine fever (ASF) date back at least a century and it is now the first disease for which a viral etiology, ASF virus (ASFV), threatens the global swine industry in all over the world and affects the five continents. ASFV infects domestic and wild members of the Suidae family, causing a wide variety of symptoms, from chronic or persistent infection to acute haemorrhagic fever, causing up to 100% mortality in their sharper forms. There is no commercialized vaccine available against the ASFV, and current control measures consist of strict animal quarantine and culling procedures. The virus is very stable and spreads easily through infected pigs, contaminated pork products, and fomites, or by transmission by the *Ornithodoros* vector. The establishment of endemic ASFV infections in wild boar populations further complicates the control of the disease. Since its first description in Kenya in 1921, ASFV remained exclusively on the African continent until the end of the 1950s. It was in 1957 that ASFV emerged for the first time in Europe, spreading to South America, but was eradicated in mostly in the mid-1990s. In 2007, a highly virulent genotype II ASFV strain emerged in the Caucasus region and subsequently spread to the Russian Federation and Europe, where it has continued to circulate and spread. In 2018, ASFV jumped to China and spread to several neighbouring countries in Southeast Asia. More recently, and after 40 years of silence, the ASFV emerged in America, affecting the Dominican Republic and Haiti in 2021. The high morbidity and mortality associated with ASFV, the lack of an effective vaccine, and the complexity of the virus, as well as its epidemiology, make this pathogen a serious threat to the global swine industry and national economies. Topics covered by this review include the genetic characteristics of ASFV, its biological properties, with particular attention to the evolution of virulent to moderate and attenuated, strains, current and future diagnostic strategies, diagnostic gaps and their relevance.

## ASFV genetics, epidemiology and genotype II epidemic history

The ASFV genome is approximately 170 to 190 kilobase (kb) pair and is divided into the left variable region (38 to 48 kb), the conserved central region (approximately 125 kb), and the right variable region (13 to 22 kb). The differences in size between the strains are due to insertions or deletions of the genes of the 5 multigene families (MGF), although variations in the conserved central region related to single nucleotide polymorphism (SNP) or to the presence of tandem repeat sequences (TRS) have been described. These variable regions are important for phylogenetic studies of ASFV. Comparative analysis of the C-terminal end of the *B646L* gene, which encodes the p72 protein, allows ASFV to be classified into 24 different genotypes (Quembo *et* al., 2019). This method, used internationally, allows relatively quick and easy typing of ASFV strains and remains the first approach to identify the origin of the virus in case of introduction into new territories. However, the genotyping method based on the *B646L* gene, it does not always provide adequate typing resolution or the ability to discriminate between viruses closely related. Analysis of the tandem repeats sequences (TRS) in the central variable region (CVR) of the *B602L* gene or the intergenic region (IGR) between the *I73R* and *I329L* genes at the right end of the genome (Gallardo et al., 2014) can be used to distinguish closely related ASFV isolates. The *B602L* gene is a particularly discriminative genetic marker whose sequencing has distinguished up to 31 subgroups of viruses with varying tetrameric amino acid repeats (Nix *et* al, 2006). Many other gene regions, such as the *E183L*, *CP204L* and *EP402R* encoding the p54, p30 and CD2v proteins, respectively, have also proved valuable in the analysis of ASFV from various locations to trace its spread (Qu *et* al., 2022).

The complex epidemiological pattern of ASFV is evident in sub-Saharan African that result in greater genetic variability of ASFV isolates from eastern and southern Africa with all 24 genotypes present (Penrith *et* al., 2022). In West African viruses are highly homogeneous, and outbreaks have historically been associated with genotype I, although different studies describe the spread of ASFV genotypes from East Africa to West Africa (Adedeji *et* al., 2021). In 1957, ASF genotype I was identified for the first time in Europe, in Lisbon coming from West Africa (Danzetta *et* al., 2021). In 1960 emerged in Lisbon and after that ASFV spread through the Iberian Peninsula (Spain and other areas of Portugal), and from there to other countries in Europe, the Caribbean and Brazil. Eradication was achieved in the mid-1990s, except in Sardinia, where the disease remains endemic. Genotype I was responsible for this first transcontinental spread.

In 2007, the second jump from the African to the European continent took place, when the ASFV emerged in the Republic of Georgia. The cause of this outbreak was a genotype II, which was circulating in Mozambique, Madagascar and Zambia (Rowlands *et* al, 2008). Subsequently, the disease continued to spread through the Caucasus region and

later through the Russian Federation and Eastern Europe, until reaching the European Union (EU) in 2014. Since then, ASF has been reported by 13 EU countries, including Lithuania, Poland, Latvia and Estonia (2014), the Czech Republic and Romania (2017), Bulgaria and Hungary (2018), Belgium and Slovakia (2019), Greece and Germany in 2020, and most recently in January 2022, in the Piedmont region of north-western Italy. Two European countries have managed to eradicate the disease: Belgium (event resolved in March 2020) and the Czech Republic (event resolved in April 2018). In August 2018, the worst scenario happened when China reported the outbreak of ASF in the Liaoning province caused by a genotype II strain (Ge *et* al., 2018). Since then, the disease continued to spread in China, and by the end of February of 2022, ASFV was detected in 32 China provinces and in 13 Asian countries, being the latest Thailand in January 2022. In September 2019, the first occurrence of ASF in Oceania was reported by Timor-Leste, followed by Papua New Guinea (March 2020). In July 2021 the disease reappeared in the Americas after an absence of almost 40 years, having been introduced in Dominican Republic and later in Haiti (FAO situation update, www.fao.org).

The investigation of virus molecular evolution in combination with spatio-temporal data is an integral part of pathogen tracing and may help in the identification of potential routes of its spreading, therefore in disease prevention and control. With the introduction of ASF into Asia, place with the highest population of domestic pigs in the world, the world is now facing the worst pandemic of an animal disease seen to date, and new ASFV whole-genome sequences from Europe and Asia are being published with increasing frequency. Currently there are available 139 whole genome sequences from 13 out of the 24 genotypes described, and 41 corresponds to genotype II (figure 1).

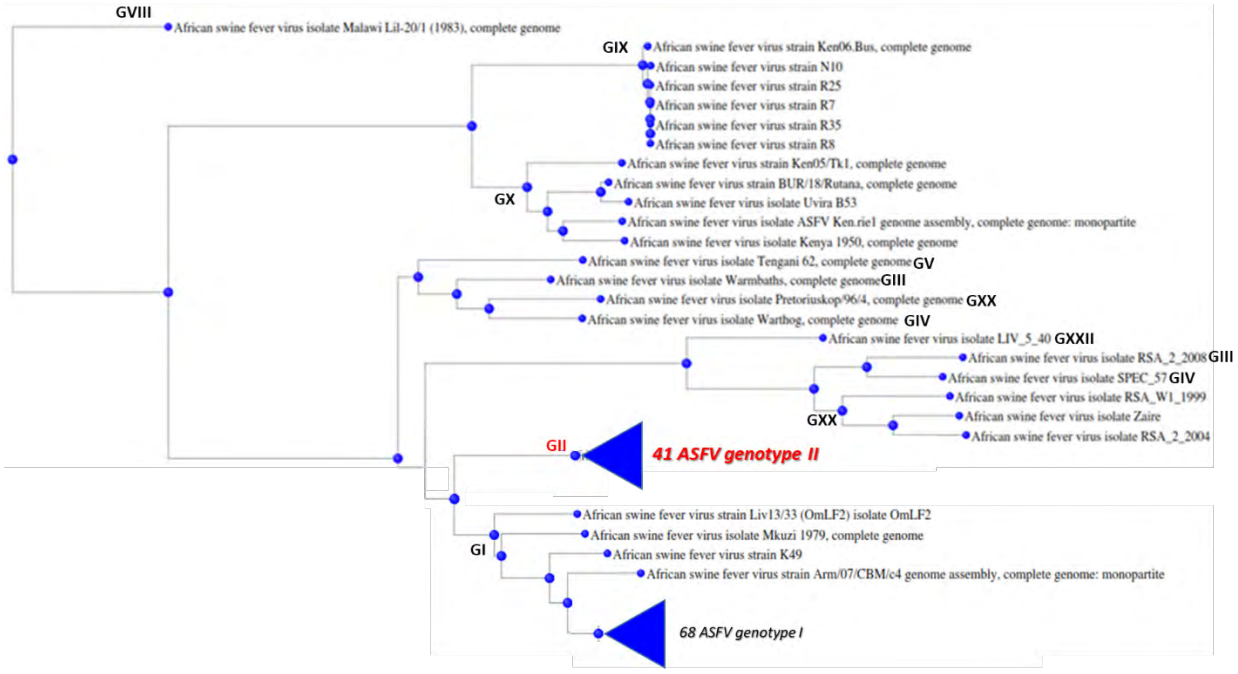


**Figure 1. Phylogenetic tree showing all available ASFV complete genome sequences that share a cover > 90% (n=139).**

Based on the 41 available whole genomic sequences deposited in the GenBank database, a low mutation rate (< 0.3%) up to date have been detected in the genotype II-ASFVs currently circulating Eurasia and all of them place in the same cluster. In general, ASFV is very stable and this leads to low genetic variability in affected regions and even the use of next-generation sequencing does usually not allow molecular tracking of virus strains in a higher resolution e.g. for molecular epidemiology in an epidemic situation. Published data confirmed that genomic regions containing tandem repeats could reveal disease trajectories in space and time. Due to technical issues, these regions are of particular interest in terms of standard genotyping procedures due to the difference in PCR product length, which is convenient to observe during regular agar electrophoresis. Gallardo *et* al. (2014) found that the sequences of ASFV isolates from index cases in the EU (Lithuania and Poland) had an identical TRS insert (IGR2 variant) to that present in ASFV isolates from Belarus and Ukraine, and different from other viruses in Eastern Europe and Russia. These molecular data, together with epidemiological findings, confirmed that the ASFVs detected in Poland and Lithuania originated in Belarus, but probably emerged in 2012 or even earlier in the Russian Federation. The extensive molecular characterization done at the ASF-EU reference laboratory (EURL, Madrid, Spain) of more than 2,600 ASF isolates from both wild boar and domestic pig obtained between 2014 and 2022 in the EU, identified four different IGR

variants (IGR1 to IGR4), with variant IGR2 being the predominant one in the EU and Russia (Mazur-Panasiuk *et* al., 2020). Further analysis of the CVR sequence within the *B602L* gene identified a new ASFV genotype II CVR variant 2 (GII-CVR2) in southern Estonia in 2015 and 2016 within the wild boar population. The CVR2 variant was characterized by the deletion of three TRSs (Vilem *et* al., 2020). Similarly, sequencing of the *O174L* gene detected two variants in Poland, with variant 2 characterized by the additional 14 nt insertion, representing a tandem repeat (Mazur-Panasiuk *et* al, 2020). It is important to note that 95% of reported cases in the EU since 2014 have occurred in wild boar populations, and the virus, depending on the ecological context, may persist in wild boar populations with or without reintroduction of infected domestic animals. Therefore, variations in the TRS could be related to a spontaneous mutation caused by the maintenance of ASFV within the wild boar population in certain regions of the EU. Sequencing of the IGR between the *9R* and *10R* genes of MGF505 supported this hypothesis with seven MGF variants identified from wild boar genotype II viruses circulating in Europe (C. Gallardo personal communication 2021).

The virus causing ASFV outbreaks in China was initially classified as genotype II and the IGR-II variant, which is predominant in Europe (Ge *et* al, 2019). Further molecular characterization studies conducted on genotype II isolates from Asia and Oceania since 2018 up to 2022, have identified the four ASFV IGR variants (IGR1 to IGR4) that circulate in both domestic pigs and wild boar in China, Korea and Vietnam. Additional analysis of a new genome marker located between based on the intergenic region between the *A179L* and *A137R* identified multiple genotype II-variants in Vietnam (Tran *et* al., 2021). But without a doubt the most important fact has been the identification of genotype I of ASFV in the Asian continent in July 2021 from pig farms in Henan and Shandong province (Sun *et* al, 2021a). Phylogenetic analysis of the whole genome sequences suggested that both isolates share high similarity with NH/P68 and OURT88/3, two genotype I attenuated ASFVs isolated in Portugal in the last century. It is important to note that animals infected with this virus developed a chronic disease that could go unnoticed in the field due to its reduced virulence. The source of these viruses and the nature of their introduction into China is unclear. Although they may represent a new introduction of the virus from an African source, the striking degree of genetic similarity to NH/P68 and OURT88/3, two genotype I ASFV isolated in Portugal in the 1960, suggests they may have originated from other source, possibly imported legally or illegally for evaluation as potential African swine fever vaccine candidates in China. The emergence of genotype I ASFVs present more problems and challenges for the control and prevention of ASFV in Asia.

In summary, current genotype II ASFV strains affecting Europe and Asia are closely related and share more than 99% homology when whole genome sequences are compared. Introducing a method of subtyping into routine diagnosis within affected areas worldwide, especially new disease incursions, may help identify potential disease origins and provide a deeper understanding of spatial-specific trajectories disease seasons. Due to the low mutation rate of the ASFV genome and its slow molecular evolution, the utility of a single subtyping method within the same genotype is still limited and allows only moderate discrimination of closely related strains. The use of a standardize protocol using multiple genetic markers should be further investigate and implement at international level that may help determine potential disease trajectories with higher resolution. Additionally, since current genetic characterization approaches are not related to biological properties (Arias *et* al., 2018), more research throughout the full ASFV genome length sequence is needed to identify new genetic markers that could explain the moderate virulence and attenuated phenotypes of genotype II-ASFVs. The genetic characterization of the virulence of multigene family (MGF) genes such as the gene MGF505-7R (Li *et* al., 2021) and the EP402R (CD2v) to cluster/group ASFV isolates based on virulence factors could be a potentially interesting area of research

**Biological properties of genotype II Eurasian ASFV strains and its role in the transmission of the disease.**

Different strains of ASFV have been found to cause variable clinical presentations, ranging from acute and peracute infections with 90 to 100% mortality, to subacute and chronic forms with much lower mortality (Salguero *et* al., 2020). Genotype II ASFV strains circulating in Europe and Asia are generally highly virulent in both domestic pigs and wild boar, causing acute disease with almost 100% lethality in animals. In experimental infections, after intramuscular inoculation with virulent strains, regardless of dose, animals became infected after an average of 4.4 ± 1.2 days and did not survive for more than 11 days. Previous studies have shown that both the intranasal and oronasal routes are equally lethal, although the nasal route resulted in a higher incidence of ASF than the oral route when a lower infective dose was used. Once infected, animals developed acute clinical signs between 3.5 and 14 dpi (mean) and 91 to 100% of infected animals died between 7 and 21 days after the first case. A similar picture has been observed in pigs when they were exposed to the virulent virus through direct contact with infected animals. Exposed animals developed a similar acute clinic that resulted in death between 11 and 25 days after exposure (Pikalo *et* al., 2021). However, one of the biggest concerns in recent years is the continuing trend towards the appearance of clinically milder or completely unapparent forms in areas where the disease is endemic in Europe and Asia that difficult the control of the disease. This attenuated phenotype was initially reported for a strain in Estonia in 2014 in the northeast of the country where mortality was surprisingly low and anti-ASFV antibodies were detected in hunted animals. *In vivo* studies in wild boar and domestic pigs showed that the ASFV isolated in 2014 in north-eastern Estonia (Ida-Viru region) was moderately

virulent in domestic pigs, but remained highly virulent in adult wild boar. Genome sequence analysis revealed a 14.5 kilobase pair deletion at the 5-end of the viral DNA, which is responsible for the attenuated phenotype in domestic pigs (Nurmoja *et* al., 2017; Zani *et* al., 2018). One year later, in 2015, Gallardo *et* al. describes the presence of strains of moderate virulence in southern Estonia circulating among the wild boar population (Gallardo *et* al., 2018; Vilem *e*t al., 2020). The results obtained from the *in vivo* studies indicated that, regardless of the Estonian ASF strain used, infected pigs presented variable clinical and pathological findings ranging from acute, subacute to chronic forms of ASF, characteristic of viruses of moderate virulence. A 33.3% of pigs survived the infection and exhibited mild, nonspecific clinical signs from approximately 14 days to one month post-infection. After a period of apparent recovery, clinical signs reappeared two months later (50-60 days) and were similar to those described in previous studies using moderately virulent ASFV isolates belonging to p72 genotype I. Similar findings were described by Walczak et al., 2020 using an ASFV from Poland isolated from wild boar (Pol18_28298_O1). Following intranasal infection of domestic pigs with Polish virus, the animals developed various forms of the disease (acute, subacute and chronic) and mortality ranged from 80 to 100% depending on the dose. Two pigs survived the infection with nonspecific clinical signs, no fever, and short viremia.

In 2017 was isolated the first non-haemadsorbing (HAD) and attenuated genotype II ASFV strain, Lv17/WB/Rie1, from a hunted wild boar in Latvia (Gallardo *et* al., 2019). The HAD phenomenon consists of the adsorption of red blood cells around monocytes/macrophages that have been infected by ASFV. The sequence analysis of the *EP402R* gene, coding the CD2-like protein responsible for the ASFV distinctive HAD phenomenon, revealed a single adenosine deletion that generates a truncated protein. In Lv17/WB/Rie1 ASFV isolate, the non-functional CD2-like protein is responsible of its non-HAD capacity, a feature shared with other naturally attenuated ASFV strains, such as NH/P68 and OURT88/3, or the recently discovered non-HAD Chinese genotype I ASFVs (Sun *et* al., 2021a). Pigs experimentally infected with Lv/17/WB/Rie1 ASFV developed non-specific clinical signs, and in some cases remained asymptomatic, showing intermittent and weak viremia and a high antibody response. Furthermore, two months following the primary infection with Lv17/WB/Rie1, the two pigs exposed were fully resistant to challenge with a virulent HAD Latvian ASFV. Since the first description in 2017, eleven non-HAD genotype II ASFVs have been isolated from wild boar in the EU, including Lv17/WB/Rie1 (Gallardo. C, unpublished data). The eleven non-HAD ASFV isolates had different types of mutations or deletions in the *EP402R* gene that prevent the viruses from translating intact CD2v protein and result in a non-HAD phenotype. When tested in domestic pigs induced subacute or chronic diseases, or even some pigs remained asymptomatic. Consistent with what was reported in the EU, 11 non-HAD viruses were isolated in China during a surveillance program conducted from June to December 2020 (Sun *et* al., 2021b). Chinese non-HAD viruses had four different types of naturally occurring mutations or deletions in the EP402R gene and showed lower virulence in domestic pigs, but were highly transmissible similar to that seen with non-HAD ASFVs from the EU.

In conclusion, several studies illustrate the natural evolution of ASFV genotype II in Europe and Asia towards less virulent forms over time circulating together with virulent viruses, as has occurred in other geographic regions where ASF has been present for a long time (Arias *et* al., 2018). Regardless of the genotype responsible for the initial outbreaks, in areas where ASF is not efficiently eradicated, the disease becomes endemic and the virus evolves into moderate and attenuated strains with an increase in the number of subacute, chronic, and subclinical infections. In such situations, the clinical manifestations of the disease are more variable and difficult to recognize in the field. The infection can persist for several months with no particular symptoms evident in infected animals, apart from growth retardation or emaciation, or it can even mimic other diseases. Although a long-term carrier state has not yet been experimentally demonstrated, the question is whether these animals have the capacity to infect a naïve population and whether or not they play an important role in the epidemiology of the disease, that is, in the persistence of the virus in endemic areas, the appearance of sporadic outbreaks and the introduction to new regions. Transmission studies with ASFV genotype II of different virulence have shown that animals surviving acute and subacute infections, shed ASFV by the oral secretions up to 22-30 days and from blood up to about 44-60 days, and that an infected animal could play a role as virus-carrier during that period (Blome *et* al., 2020; Gallardo *et* al., 2021; Walczak *et* al., 2020; Zani *et* al., 2018; Sun *et* al., 2021a,b). Pigs infected with attenuated strains can shed infectious virus from the blood up to about 15 to 20 days, but with titers similar to those of the moderate virulence group. On the contrary, the risk of oral transmission, which is the natural route of infection, is much lower than in the case of infections with strains of high or moderate virulence, although this circumstance cannot be excluded. In fact, Gallardo *et* al., (2015) reported that seropositive animals infected with genotype I NH/P68 ASFV were able to transmit the virus to a susceptible population, more than three months after the first virus inoculation, even in the absence of viremia or clinical signs. Since the virus was isolated from the lung and mediastinal lymph nodes four and half months later, the ASFV could be easily transmitted from the respiratory tract through oral excretions.

These results contradict the assertion of some researchers that the probability of a seropositive but virus-negative "survivor animal" shedding infectious virus and playing a role as a carrier is practically zero. It cannot be excluded that a very small number of animals can transmit ASFV even in the absence of virus presence in blood and thus maintain the virus in endemic areas. An example could be found in Estonia. In this country, wild boar that were ASFV-

positive, seropositive, or both, were regularly detected until February 2019. Thereafter and for more than 1 year, only wild boar were found to be seropositive but negative to ASFV and no outbreak had been detected in domestic pigs since 2017. Since August 2020, several ASFV-positive wild boar were reported in the centre and north-east of the country and the virus appeared in a domestic pig farm in 2021. The re-emergence of ASFV-positives wild boar and domestic pigs raised the question of the role of seropositive wild boar in the epidemic situation in the country. Schulz *et* al (2021) hypothesized that the most likely reason for this re-emergence was the reintroduction of ASFV from neighbouring countries. However, the study by Schulz did not exclude that seropositive but virus-negative wild boar are capable of transmitting ASFV and spreading the disease. In addition, an important factor to take into account is the type of samples that are analysed in epidemiological surveillance programs. Both the matrix used and the quality of the samples may influence the probability of detecting low amounts of viral genomes or that the virus is confined in a specific tissue. In this respect, screening of various tissues could aid detection of potential virus-carrier. The experimental *in vivo* studies with ASFV genotype II strains of different virulence, reaffirms this statement. In surviving animals and those that develop chronic or subclinical infections, the virus is cleared more or less rapidly from target organs such as bone marrow, spleen, or kidney. However, it persists for more than two months, even up to four months, in primary replication sites, such as tonsils and lymph nodes, or in secondary replication sites, such as intra-articular tissues (Gallardo *et* al., 2019; 2021; Walczak *et* al., 2020). This long-term detection of virus in animals infected with attenuated and moderately virulent isolates has been described in previous studies with ASFV genotype I viruses and virus was detected in lymph nodes and/or tonsils for long periods of time, even up to 13 weeks after infection (Gallardo *e*t al., 2015). The localized presence of virus in lymphoid tissues, primary replication sites, occurring to some extent in any of the survivor categories, could suggest the likelihood of persistent infection or that pigs have multiple reinfections with the same strain, as virus is usually present where primary viral replication occurs. All together these data suggest that other tissues should be also considered as target samples in the surveillance programs. Furthermore, the question whether the ASFV present in a tissue could be reactivated in seropositive wild boar under immunosuppression, stress or in case of death has to be pursued further. To find scientific evidence regarding these questions, it will be inevitable to conduct long-term experimental studies.

## ASF diagnosis.
## State of the art, gaps and priorities

Since there is no vaccine available, prevention, control, and eradication of ASF is based on the implementation of appropriated surveillance that detects ASF outbreaks as early as possible, as well as the ability to respond to outbreaks quickly and efficiently so that ASFV spread can be prevented and, ideally, eradicated. A key element of ASF control strategies is the early detection of infected domestic and wild pigs. This is important for any infectious disease, but even more so for ASFV, because the virus survives for extended periods in the environment and in pork products, and because the appearance the presence of ASF of different virulence co-circulating in affected countries regardless the genotype is affecting. Therefore, any onward spread prior to detection will have a major adverse impact on the ability to contain or stop spread. The design of a sufficiently sensitive ASF surveillance system requires a sound understanding of the epidemiology, the virus, and the disease, coupled with adequate diagnostic laboratory infrastructure with qualified personnel, adequate financial resources, and internationally validated techniques. Both passive (observer-initiated) and active (investigator-initiated) surveillance system components may be used, but the passive component is of major importance for early detection in domestic and wild pigs. Passive surveillance is based on farmers, other actors involved in the pork food system, and anyone encountering potentially diseased wild pigs notifying the veterinary authorities of their suspicion. Active surveillance implies actively looking for infected or clinically diseased domestic and wild pigs and sampling legal and illegal live pig and pork imports at border inspection posts.

Success of surveillance activities depends on the availability of the most appropriate diagnostic tests. A wide spectrum of accurate ASF diagnostic tests is available and most of them have been successfully employed in surveillance, control and eradication programs (Gallardo *et* al., 2019b). However, as in any other disease, there is not a single test being 100% reliable (sensitive and specific). For this reason, final diagnosis should be based on the interpretation of the results derived from the use of a number of validated tests in the appropriated samples, in combination with the information coming from disease epidemiology, scenario, and the clinical signs.

### ASF diagnostic workflow

In case of an ASF suspicion, the PCR is by far the most sensitive method for the detection of the agent and the method of choice for first-line laboratory diagnosis. It is a basic diagnostic tool for surveillance, considering the long-term viremia, the high viral load in the infected animals suffering acute or subacute clinical courses. It is quick and can be used for individual as well as pooled samples although with size-limitation (Gallardo *et* al., 2019b). A variety of PCR tests, including both conventional and real time (rtPCR), as well as commercial kits have been developed and validated to detect a wide range of ASF isolates belonging to different known virus genotypes, non-HAD strains, and diverse

virulence (Auer *et* al, 2022; Gallardo *et* al., 2019b; Pikalo *et* al., 2022). Nevertheless, although rare, to avoid any false positive PCR results, (e.g., due to lab contamination or other factors) several procedures are implemented. Thus a primary outbreak (or wild boar case) of ASF should be ideally confirmed by virus isolation of ASFV and the identification by the HAD assay, by the national reference laboratories or at international level, and by genetic typing at the laboratories. A recent described duplex real-time PCR based on ASFV E296R gene for rapid detection and differentiation between genotypes I and II ASFVs (Li et al., 2022) provides an additional powerful tool that can facilitate efficient control of ASFV in regions where both genotypes can be circulating such as China or several African countries (i.e. Nigeria). However, this might not always be possible due to technical limitations, absence or appropriated facilities or the reduced sensitivity, particularly in samples obtained from altered carcasses or hunted wild boar, or in weak positive PCR samples (Gallardo *et* al., 2019b).

Whenever the suspicion is raised that ASFV is circulating in a swine population, a negative PCR result cannot excluded the presence of ASF. Since animals usually develop antibodies within the second week after infection, they can test positive for both ASFV and antibodies simultaneously for at least two months. Samples from animals surviving this period are usually positive for ASFV-specific antibodies, but negative for ASFV and its genome. Therefore, if the PCR gave a negative result but there is a suspicion that ASFV is circulating, serological assays should also be used for the diagnosis. The current recommendations for ASFV antibody detection involve the use of an ELISA for antibody screening, backed up by Immunoblotting (IB), Indirect Immunofluorescence test (IFAT) or the Indirect immunoperoxidase tests (IPT) as confirmatory tests (OIE 2021). The ELISA remains the most useful method for large-scale serological studies in serum samples: it is fast, easy to perform and economical. However, only serum can be analysed, which restricts its application range, especially in case of passive surveillance of wild boar when animals are usually found dead. In addition, hemolysed serum samples could arose either false positive or negative results depending of the ELISA format employed. Therefore, positive ELISA results should always be confirmed by additional methods such as IPT, IFAT or IB tests, as recommended by the OIE (OIE 2021). The IB is a rapid and sensitive assay but, similarly to that described above, only serum samples can be tested. On the contrary, IPT or IFAT can be easily used for analysing all type of porcine samples, including exudates from tissue, whole blood, fluids and even bone marrow. The antibody detection by IPT in exudates tissue samples is a common successful method when wild boar are analysed.

Taken together, sensitive, specific and robust laboratory diagnostic assays are available but, as for any other disease, there is not a single test being 100% reliable (sensitive and specific). For this reason, final diagnosis should be based on the interpretation of the results derived from the use of appropriate samples and validated tests in combination with the information coming from disease epidemiology, the presence of clinical signs and the scenario. A thorough understanding of the viremia and antibody seroconversion timing during ASFV infection is a prerequisite to conclude the dynamic of the infection in the investigated areas, and to support control and eradication programs. Positive results for both virus and antibodies indicate that the tested animal was infected at the time of sampling, whereas a positive ASFV antibody test in absence of virus indicates an ongoing or past infection, where the animal has recovered or could be chronically or sub-clinically infected with attenuated strain. These animals should be detected since they can act as carrier of the virus and, in certain conditions to infect a naive population

On the international level, laboratory methods as well as sampling and shipping guidelines can be found in the World Organisation for Animal Health (OIE) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Chapter 3.9.1, version adopted in May 2021). The selection of which test to use depends on available matrices, the purpose of the testing (surveillance, eradication, diagnosis, confirmation), as well as the ASF epidemiological status of the country (region) or stage of the epidemic in the region.

**Sampling: classical versus alternative sampling methods**

The starting point for any laboratory investigation on ASF is sample collection. An important consideration is the purpose of the investigation, for example disease diagnosis, disease surveillance, or health certification. Which animals to sample will depend on the objective of the sampling. For example, when investigating an outbreak (passive surveillance), sick and dead animals should be targeted, while the oldest animals should be sampled when checking if animals have been exposed to the disease (active surveillance). To be effective, appropriate samples combined with the selection of diagnostic methods are of fundamental importance in order to make a rapid and reliable diagnosis. Samples collected from live pigs should include anti-coagulated whole blood for the detection of virus or viral nucleic acid and serum for the detection of antibodies, whereas samples collected from dead pigs or wild boar should comprise tissues for both virus and antibody detection (table 1)

**Table 1**: target samples for ASF virus and antibody detection, the classical ASF diagnostic tests and their recommended use.

| DETECTION | TECHIQUE | TARGET SAMPLES | RECOMMENDED USE |
|---|---|---|---|
| Nucleic acid detection | PCR tests (i.h. conventional and real time PCR tests and commercial tests) | **Organs:** spleen, lymph nodes, liver, tonsil, heart, lung, kidney, bone marrow (wild boar) and intra-articular cartilage.<br>Anticoagulated **blood***<br>**Ticks** | Early detection: suspicion, outbreak investigation, surveillance.<br>Individual and herd testing.<br>Movements from restricted zones |
| Virus detection | Virus isolation and identification by haemadsorption (HAD) test (i.h) | **Organs:** spleen, lymph nodes, liver, tonsil, heart, lung, kidney, bone marrow (wild boar) and intra-articular cartilage.<br>Anticoagulated **blood***<br>**Ticks** | Confirmation of primary outbreak. |
| Antigen detection | Direct Immunofluorescence (DIF) (i.h) | **Organs:** spleen, lymph nodes and tonsil. | Individual and herd testing (in case of clinical signs), early detection.<br>*It is recommended its use in parallel with antibody detection tests.* |
| | Antigen ELISA commercial kit INgezim PPA DAS, Double Ab Sandwich. | **Organs:** spleen and lymph nodes.<br>**Plasma** from anticoagulated blood | Surveillance<br>Herd testing (in case of clinical signs). |
| Antibody detection | ELISA (i.h ELISA tests and commercial methods) | **Sera** | Individual and herd testing when deemed appropriate.<br>Surveillance |
| | Immunoblot (IB) (i.h) | **Sera** | Confirmatory test<br>Individual and herd testing when deemed appropriate. |
| | Indirect Immunoperoxidase test (IPT) (i.h) | **Sera**<br>**Plasma** from anticoagulated blood<br>**Exudates from tissues**<br>**Corporal fluids** (pericardial, intraarticular, thoracic, etc) | Confirmatory test<br>Individual and herd testing when deemed appropriate.<br>Surveillance; epidemiological studies (time of the infection) |
| | Immunofluorescence Antibody (IFAT) test (i.h) | **Sera**<br>**Plasma** from anticoagulated blood<br>**Exudates from tissues**<br>**Corporal fluids** (pericardial, intraarticular, thoracic, etc) | Confirmatory test<br>Individual and herd testing when deemed appropriate.<br>Surveillance; epidemiological studies (time of the infection) |

*Source: webpage of the ASF-EU reference laboratory (EURL);* https://asf-referencelab.info/asf/en/

The sample matrices described above are routine for veterinary practitioners or pathologists. However, these sample types may not always be available and alternative samples may be better suited, especially for wild boar carcasses and for active ASF surveillance in large pig farms. Different types of alternative samples have been tested that meet the objective of providing a reliable diagnosis. Among the published options are dry blood swabs, dried filter papers and FTA cards, fecal samples, oral, nasal and rectal swabs, meat-juice, different rope-based options, ear punches or dry-Sponges (manufactured by 3M) (Flannery *et* al., 2020; Kosowska *et* al. 2021; Onyilagha *et* al., 2021; Pikalo *et* al., 2021). This review summarizes the latter approaches that has provided reliable results.

While shedding will depend on the virulence of the isolate most secretions and excretions will be positive for ASFV genomes in the clinical phase, although some considerations should be taken into account. (Gallardo *et* al., 2021). The ASFV through the feces occurs only in the acute phase of the infection caused by virulent strains and two or even four days later than in blood, therefore, the use of feces or rectal swabs seems to be limited in the diagnosis of ASF. 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). In contrast, the ASFV genome could be then easily detected by PCR from oropharyngeal swabs earlier than in blood, independently of the strain virulence. This is due to primary replication in the tonsil and retropharyngeal lymph nodes in the normal route of infection in the field. However, during the course of the infection, important differences are detected depending on the virulence of the strain and the clinical form of the disease. When samples are collected within the first three weeks of infection (≈ 3-20 days), the highest proportion of PCR-positive samples is obtained from oropharyngeal swabs, regardless of strain. On the contrary, in the blood samples there are significant differences. While in acute and subacute

infections the virus is detected in similar proportions in blood and in oropharyngeal swabs, in animals with chronic or subclinical disease, viremia peaks are intermittent, even at the beginning of the infection. As of day 20, the ASFV genome is only detected sporadically in these animals and in a clearly lower percentage than that detected in oropharyngeal samples (Gallardo *e*t al., 2021). However, in animals that survive infection caused by moderately virulent isolates, the ASFV genome can be detected in blood for a period of about two months or even up to 100 days (Blome *et* al., 2020), while in oropharyngeal samples the detection range is usually lower at one month (Gallardo et al. 2021). Therefore, the oropharyngeal swabs should not be used as a substitute for blood in active surveillance, as it would decrease the detection of animals that survived to the primary infection with either virulence or moderate virulence ASFVs, as they would possibly not be showing any clinical signs. But together with blood, the oropharyngeal swabs samples could allow to detect ASFV infection for a longer period and could be a useful an alternative sample in the passive surveillance programs for the early detection prior to onset of obvious clinical signs, mainly in large pig farms (Gallardo *et* al., 2021; Pikalo *et* al., 2021). Regarding the detection of antibodies, more studies are needed since, compared to serum, the sensitivity percentages are below the adequate limits to give a reliable diagnosis (Gallardo C. personal communication 2021).

Sampling individual pigs on commercial pig farms is a cornerstone of current surveillance for ASFV, but it is labour-intensive and expensive. Rope-based oral fluid collection is a non-invasive method that is widely used in industry as a diagnostic and surveillance sample to detect various endemic swine pathogens. Collection of oral fluids with ropes in a pen can be performed by non-veterinary personnel with minimal resources and discomfort to the animals. In recent experimental studies using domestic pigs inoculated with highly virulent ASFV Georgia 2007/1 or moderately virulent ASFV Malta' 78, the ASFV genome was detected in oral fluid before the animals developed noticeable clinical signs and the pigs continued to chew the ropes daily until severe clinical signs developed (Goonewardene *et* al., 2021). However, the ASFV genome was detected in oral fluids at low to moderate levels (Ct > 30) and between 2 and 4 days later than in blood. These results, consistent with that described by Lee *et* al., 2021 using a virulent strain from Vietnam, suggest that the use of oral fluids to supplement the use of traditional samples for rapid detection during ASF surveillance should be carefully considered and requires more field validation studies. When it comes to antibody detection, oral fluids were shown to work with a slight delay in detection and depends of the type of antibody test used (Mur *et* al., 2013).

Over the last years, the blood swabs as an alternative matrix for passive surveillance, especially in wild boar, has been widely used, even for field detection (Sauter-Louis *et* al., 2021). However, sensitivity is dependent of several factors such as the circulating strains and the type of swabs and buffers used (Gallardo C. personal communication 2021; Pikalo *et* al., 2021). In a recent study conducted at EURL-ASF (Madrid, Spain), a total of four hundred and sixteen EDTA-blood samples from experimental and field infections were used for routine PCR testing and dried blood swab generation using cotton devices. Animals were infected with ASFV genotype II strains of different virulence, including the attenuated ASFV Lv17/WB/Rie1. Dried blood swabs were tested in parallel using the OIE real-time PCR developed by Fernandez Pinero *et* al., 2013 (OIE 2021) and the IPT for virus and antibody detection (OIE 2021). Using the PCR test, the percentage of agreement was 90% between blood and swabs when samples had Ct <30. However, the percentages decreased dramatically (<35%) when testing samples with Ct >30. This was related to the time of infection and the clinical form. False negative results in swabs were obtained early in the infection but especially in animals that had a chronic or subclinical type of infection where viremia is usually weak. The paired swabs and blood were then tested by IPT for antibody detection. Sensitivity was 78% with 32 false negative results in swabs. Combining the results of PCR and IPT, the percentage of positives in blood was 91% compared to 81% in swabs, depicting almost a perfect agreement [$\kappa$ =0.9 $_{95\%CI}$] between both type of samples. In summary, of the 416 samples from diverse source, only 18 cases (4.3%) would not have been identified as infected using the blood swabs. Other studies reported even increased sensitivity when Genotubes (Carlson *et* al., 2018) or PrimeSwab are used (Pikalo *et* al., 2021), so fast-drying swabs could be an alternative for ASF detection. They have several advantages from easy handling to long-term storage and the ability to cut and use one swab for multiple diagnostic tests. Another key feature of this swab is the diversity of samples it may be used with, including organs and bone marrow from dead wild boar. In conclusion, swabs are a practical, inexpensive and straightforward approach for passive surveillance of ASFV, mainly in the deceased wild boar. Whether it is worth using this approach instead of classical sampling system, remains the choice of users based on risk assessment, integration into surveillance strategies, and financial resources.

The use of blood samples dried on filter papers has been also described as a possible alternative to for serological and virological testing. Whatman FTA cards consists of filter papers specifically engineered for nucleic acid preparation and preservation. They contain impregnated matrices that lyse cells, denature proteins and protect nucleic acids from nucleases, thus providing additional useful inactivation of the biological material which theoretically makes it compatible with safe shipment without the need for containment. However, in the laboratory, they require extra-preparation such as rinsing and elution before being used for diagnosis and are not suitable for subsequent pathogen isolation nor by antibody detection. Other filter papers like Whatman 3-MM filter papers do not contain additives; they can thus preserve infectivity and can be used for both virus and antibody detection. However, dried blood samples

on filter paper provide variable results depending on the diagnostic method used and are not versatile samples that can be analyzed in all laboratories (Randriamparany *et* al., 2016). Finally, although with certain limitations, ear tissue samples and meat juice, especially the diaphragmatic muscle, has proven to be a good matrix to the detection of ASFV and/or ASFV specific antibodies (Onyilagha *et* al., 2021)

In summary, the comparative studies of alternative samples *vs* classical samples confirmed that EDTA blood is the most suitable option for ASFV genome and antibody detection, together with sera, both in the initial phase of infection as in late. Alternative samples, such as oropharyngeal or blood swabs, have shown to be the most promising alternative samples and could detect ASFV and/or antibodies to some extent, although sensitivity results depend on virulence of the strain. In conclusion, alternative approaches are feasible, but should be integrated into control strategies by selecting test methods and sample materials following a "fit for purpose" approach.

## ASF diagnosis; gaps and future trends

A large number of validated ASF diagnostic techniques are now available to provide a reliable diagnosis of ASF in affected countries (Gallardo *et* al., 2019b). However, there are some gaps that have begun to be filled in the last five years. This section will discuss some of the advances made in the development of rapid, reliable, sensitive, and convenient diagnostic tests with the potential to overcome the limitations of currently available assays.

### Molecular tests

There is a strong demand for accurate, rapid, and simple detection methods especially for on-site application. Nucleic acid testing is the most commonly used method for ASFV detection. However, traditional nucleic acid purification step is time- and labor-consuming. The nucleic acid purification, amplification and amplicons detection rely on laboratory settings which limits the on-site detection. Point of care (PoC) molecular detection methods have been adapted for ASFV genome detection and preliminary validation has been achieved although with limit number of samples (Gaudreault *et* al., 2020; Zurita *et* al., 2021). Other affordable diagnostic solutions include the isothermal assays that could be a cheaper diagnostic alternative to PCR, and useful in field conditions. Numerous studies from recent years have described new diagnostic tests based on this technology, such as recombinase polymerase amplification (RPA), loop-mediated isothermal amplification (LAMP) and cross-priming amplification (CPA). Moreover, those isothermal amplification assays in combination with immunochromatographic strips have also been developed for application in the field. The main drawback of these techniques is the lack of high sensitivity which limits their application in the detection of ASFV. Recently, nucleic acid detection techniques based on the clustered regularly interspaced short palindromic repeats (CRISPR)-associated endonucleases (CRISPR/Cas) systems have been developed. The detection relies on the cleavage preferences of Cas12 or Cas13 in a nonspecific way after binding to a specific target DNA or RNA via programmable guide RNAs. Combined with isothermal amplification RPA assay, the CRISPR system has been used for detecting ASFV. However, the high cost of RPA assay limits its application in the field. A recent study (Yang *et* al., 2021) describes a LAMP assay coupled with the CRISPR)/Cas12a system established in one tube for the detection of the ASFV p72 gene. The performance of the LAMP–CRISPR assay was compared with the OIE real time PCR test (OIE 2021) testing 41 clinical samples including nasal swab, spleen, liver, lung, submandibular lymph node and kidney. The result showed that these two assays had 96.6% consistency, which supports the fact that the LAMP–CRISPR could be regarded as a novel diagnostic assay for the detection of ASFV. The method shed a light on the convenient, portable, low cost, demonstrating a great application potential for monitoring on-site ASFV in the field. However, although depicting rapid result and good specificity and sensitivity, the low number of samples tested in this study limits its use for giving a reliable and confident diagnosis and further validation is still required to better understand the reliability and utility of the test as ASF diagnostic method.

In conclusion the are several approaches for rapid nucleic acid detection, including molecular platforms now available that could allow sensitive ASFV DNA detection in infected pigs, mainly at the early stages of disease. These tests can also be used to detect contaminated carcasses, and pork and environmental samples at the point-of-need (e.g. abattoir, airport or wild boar/feral pig habitats). However, these platforms are technically more complex than rapid antibody or antigen tests and require further field validation studies and a much higher level of training and competency for accurate testing. Molecular field tests also require expensive equipment for amplification and, in many cases, for extracting viral DNA. An update of the current knowledge of the OIE ASF Reference Laboratory Network on commercially available molecular point of care (PoC) tests, including a range of technical details, cost, as well as advantages and disadvantages of each is available at https://www.oie.int/app/uploads/2022/02/2022-02-09-final-oie-asf-tests-guide.pdf.

### Antigen detection tests

The only commercially available ELISA test is the INgezim PPA DAS 2.0 (INGEGASA, Eurofins Technologie), but has low sensitivity and is only recommended for herd tests and always together with other virus or antibody detection techniques (SauterLouis *et* al 2021; Gallardo *et* al. 2019b). Despite not being included in the Register of Diagnostic Kits certified by the OIE as validated as fit for purpose, there are several PoC tests, that are available commercially for field testing, including basic rapid test kits for detecting antigens using lateral flow devices (LFD). These tests are simple to use, require minimal training and can provide a result within approximately 20 minutes. Rapid antigen tests are typically less sensitive than molecular techniques for virus detection, but some can have comparable levels of specificity. Antigen tests are recommended for use on symptomatic and terminally ill pigs that have high levels of viraemia, rather than on pigs in the early stages of clinical infection that may not have high enough viraemia to allow detection. It is recommended that samples from more than one sick pig are tested to increase the chances of detecting infection (table 2).

**Table 2:** comparison of four major PoC test methods for rapid ASF virus antigen detection

| **Test** | **Ingenasa** | **Bionote** | **PenCheck™** | **Shenzhen Lvshiyuan Biotechnology Co.** |
|---|---|---|---|---|
| **Catalogue no.** | INgezim ASF CROM Ag (11.ASFV.K.42) | Anigen ASFV Ag rapid test (RG1407DD) | Rapid Screening Test for ASFV (PC-888) | SLB ASF antigen detection RDT |
| **Specimen Type(s)** | Whole blood | Serum, plasma or whole blood | Whole blood | Whole blood |
| **Format** | Lateral flow | Lateral flow | Dipstick | Lateral flow |
| **Level of assessment** | Peer-reviewed published journal article<br>Independent assessment at reference laboratories | Independent assessment at reference laboratories | Independent assessment at reference laboratory | Peer-reviewed published journal article<br>Independent laboratory assessment |
| **Sensitivity** | Low to moderate (~68%) | Low to moderate* | Low* | Low to moderate (~65%) |
| **Specificity** | High (98%) | Moderate* | Moderate to high* | Moderate (~76%) |
| **Training** | Low | Low | Low | Low |
| **Testing Time** | 15 min | 20 min | 25-30 min | 15-20 min |
| **Cost/test (USD)** | $5.80 to $10.45 (depending on pack size) | $14 | $2,50 | $3,50 |
| **Cost of equipment** | None | None | None | None |
| **Advantages** | Rapid (early detection at POC)<br>Easy (anyone can perform)<br>Inexpensive<br>No equipment costs<br>High specificity | Rapid (early detection at POC)<br>Easy (anyone can perform)<br>Inexpensive<br>No equipment costs | Rapid (early detection at POC)<br>Minimal training (e.g. pipette use)<br>Inexpensive<br>Minimal equipment required (pipette and tips for aliquotting test reagent)<br>Moderate to high specificity | Rapid (early detection at POC)<br>Easy (anyone can perform)<br>Inexpensive<br>No equipment costs |
| **Disadvantages** | Sensitivity low to moderate, but high enough for testing very sick and dying animals | Sensitivity low to moderate, but high enough for testing very sick and dying animals; moderate specificity (--> false positives) | Low sensitivity | Sensitivity low to moderate, but high enough for testing very sick and dying animals; moderate specificity (--> false positives) |
| **References** | Sastre et al. (2016a) | Peer-reviewed publication not yet available | Peer-reviewed publication not yet available | Matsumato et al. (2020) |

*Source: The OIE ASF Reference Laboratory Network's overview of African swine fever diagnostic tests for field application. Authors Ken Inui, Carmina Gallardo, Raquel Portugal, Linda Dixon, Carrie Baton & David Williams (https://www.oie.int/app/uploads/2022/02/2022-02-09-final-oie-asf-tests-guide.pdf )*

Virus isolation and identification by the HAD assay

Several established cell lines, such as IPAM, COS-1, and WSL, have been used to propagate and titrate limited strains of ASFV, but are not suitable for isolation of ASFV from field samples without little prior adaptation (Gallardo *et* al., 2019b). Currently, virus isolation from field samples relies on primary cell cultures such as porcine lung alveolar macrophages (PAM) or peripheral blood monocytes (PBM). This procedure is more expensive than other techniques,

requires both specialized facilities and training, is time consuming and cannot be adapted to high throughput. Therefore, to find an established cell line with potential use in ASF diagnosis is strongly needed. One such cell line was identified in 2020, when Rai et al. reported the successful use of MA-104 cells (a commercially available African green monkey kidney epithelial cell line) for the isolation of several infectious strains of ASFV (Rai *et* al. 2020). The sensitivity of this test was found to be ~10-fold lower than with primary porcine macrophages, but ~10-fold higher than that of a qPCR assay. Importantly, MA-104 cells infected with HAD isolates were also found to exhibit HAD in the presence of porcine erythrocytes, and cells infected with non-HAD isolates were identified by immunostaining. Preliminary studies also showed that ASFV was isolated from infected blood samples, indicating that MA-104 is also a good substrate for direct isolation from field samples without the need of prior passage in primary cells (Rai *et* al. 2020). Ray et al. later published a detailed protocol describing infection of MA-104 cells for detection and quantification of infectious isolates by HAD assays or immunostaining (Rai *et* al. 2021). Since ASFV primarily infects macrophages and monocytes, numerous efforts have been made to establish immortalized cell lines of these lineages to avoid genetic changes that may arise after passage in monkey cells. It has recently been shown that different immortalized primary porcine macrophage cell lines such as IPKM (Masujin *et* al, 2021) or ZMAC-4 (Portugal *et* al, 2020) are capable of effectively replicating different ASFV isolates. However, these cells require further investigation to verify whether the virus is isolated directly from clinical samples, without adaptation process, and maintain a productive viral replication. Therefore, although great progress has been made in recent years on this topic, further studies are needed to achieve the goal of having an established cell line for ASFV diagnostic purposes. These studies should be aimed at validating the recently described cell lines with clinical samples and their suitability for isolating ASFV without inducing genetic and/or phenotypic changes.

Antibody detection techniques

The ELISA test is the most widely used test to detect antibodies on a large scale. The available ELISAs against anti-ASFV antibodies, although generally very specific and sensitive, are only suitable for serum samples, which limits their applicability, especially in endemic areas that lack standardized wild boar sample collection programs (Gallardo, *et* al., 2019b). This issue is nowadays surpassed by the use of IPT test, which can easily analyze all type of samples such as blood and exudates from tissue samples, including bone marrow. However, the IPT requires the use of fixed cultured VERO or MS monolayer cell lines infected with adapted ASFV, therefore needs special biosafety conditions and the interpretation of the results can be subjective and well-trained staff is required. The major drawback is that this technique is not produced commercially by companies, which constrains its use in laboratories, especially those with limited resources. In this context, standardized ELISAs are needed for the detection of specific antibodies in tissue extracts or blood for an easy and more reliable evaluation of epidemiological situation in affected areas. Different ELISAs has been validated for the use of dried blood-spots (DBS) on filter papers with good specificity and relatively appropriate sensitivity (Giménez-Lirola *et* al. 2016; Randriamparany *et* al., 2016). This matrix obviates the need for a cold chain to preserve specimens during the transport to the laboratories and requires only a small sample volume, and needs minimal technical expertise for collecting. Commercial kits, evaluated/validated for use with meat exudate, can be used to detect antibodies to ASFV-proteins in meat exudate samples in order to obtain epidemiological information related to low and moderately virulent ASFV strains circulating in wild boars and domestic pigs, thereby facilitating ASF control and business continuity. Different studies have evaluate the suitability of meat, blood and/or tissue exudate as an alternative sample type for ASF serological detection (Gallardo *et* al., 2021; Onyilagha *et* al., 2021). The INgezim® ASFV-R ELISA technique, which uses a monoclonal antibody (Mab) specific for porcine IgG and recombinant proteins cp312 and p30 of ASFV, has been validated for the detection of specific antibodies in serum, blood (fresh or on paper) and spleen exudate samples from pigs and wild boars, with blood being the best target sample. Due to the lower sensitivity detected in acute infections when the antibody titer is ≤1:2560, the test should be used in parallel with an antigen detection test, to complement surveillance programs in endemic areas. But, in general, these results indicate that anti-ASFV antibodies can be detected in tissue and blood samples using the ELISA format, when sera will not be available in case of dead animals. This is especially interesting in endemic areas where strains of low virulence circulate and viruses in organs are not easily detected. These animals have a high titer of antibodies in the tissues (Gallardo *et* al., 2018, 2019a, 2021) and therefore could be easily detected by the ELISA test, providing a more detailed overview of the epidemiological situation in endemic areas.

For detection in the field, developed rapid antibody tests (LFDs) generally have comparable levels of sensitivity and specificity to laboratory ELISAs, although they exhibit lower sensitivity compared to reference tests such as IPT. LFDs can be used to detect antibodies in pigs that have survived infection although, as with LFD antigen, both types of tests should be used in parallel to avoid losing infected animals (table 3).

**Table 3:** reviews three major PoC test methods for rapid ASF virus antibody detection.

| Test | Ingenasa (ASFV/CSFV duplex) | Ingenasa (ASFV) | Global Dx |
|---|---|---|---|
| **Catalogue no.** | INgezim ASFV-CSFV CROM Ab (11.SFV.K41) | INGEZIM PPA CROM (11.PPA.K41/25) | GDX70-2 Herdscreen® ASF Antibody |
| **Specimen Type(s)** | Whole blood and porcine serum samples | Whole blood, plasma, and porcine serum samples | Swine whole blood, plasma or serum |
| **Format** | Lateral flow | Lateral flow | Lateral flow |
| **Level of assessment** | Peer-reviewed published journal article<br>Independent assessment at reference laboratories | Peer-reviewed published journal article<br>Independent assessment at reference laboratories | Independent assessment at reference laboratories |
| **Sensitivity** | Moderate to high (CSFV-92%/ASFV-87%) | Moderate to high (82% sensitivity with respect to the immunoperoxidase monolayer assay [IPMA] in wild boar; 99% correspondence to ELISA) | Moderate to high analytical Ss. (Correspondence with IPMA is 86.2%. Equivalent or higher sensitivity than the commercial ELISAs.) |
| **Specificity** | High (98.4%-CSFV/ASFV-100%) | High (99.9% correspondence with ELISAs. 96% specificity respect IPMA [wild boar]) | High (100% correspondence with reference technique IPMA) |
| **Training** | Low | Low | Low |
| **Testing Time** | 15 to 30 min | 15 to 30 min | 15 to 30 min |
| **Cost/test (USD)** | $16,38 | $5.43 to (depending on pack size) | $4,80 |
| **Cost of equipment** | None | None | None |
| **Advantages** | Rapid (early detection at POC)<br>Easy (anyone can perform)<br>Inexpensive<br>No equipment costs<br>Differential diagnosis of CSFV-ASFV | Rapid (early detection at POC)<br>Easy (anyone can perform)<br>Inexpensive<br>No equipment costs | Rapid (early detection at POC)<br>Easy (anyone can perform)<br>Inexpensive<br>No equipment costs |
| **Disadvantages** | Moderate diagnostic sensitivity for ASFV antibody detection. It is recomended to use in parallel with the Ag LFA | Moderate diagnostic sensitivity for ASFV antibody detection. It is recomended to use in parallel with the Ag LFA | Requires further field validation |
| **References** | Sastre et al. (2016b). | Cappai et al. (2017). | Peer-reviewed publication not yet available |

*Source: The OIE ASF Reference Laboratory Network's overview of African swine fever diagnostic tests for field application. Authors Ken Inui, Carmina Gallardo, Raquel Portugal, Linda Dixon, Carrie Baton & David Williams (https://www.oie.int/app/uploads/2022/02/2022-02-09-final-oie-asf-tests-guide.pdf )*

## Concluding remarks

ASFV is a complex DNA virus that has a significant impact on the global swine industry. The lack of a safe and effective vaccine and the reliance on herd culling to prevent the spread of the disease has resulted in significant economic losses. Therefore, improved early detection remains a significant priority. Despite the great effort made in the last five years, there are still some gaps to fill. The low rate of variation of the African swine fever virus genome and its enormous size make it difficult to properly type newly emerging African swine fever virus isolates and thus make it difficult to trace outbreaks. An international effort should be made to develop a standardized genotyping method based on multiple loci of the ASFV genome to identify the origin of outbreaks. The trend towards endemicity of ASFV in the affected regions of Europe and Asia increases the presence of less virulent strains that induce non-specific clinical signs and make it difficult to recognize the disease in the field. It is necessary to increase knowledge about the mechanisms of spread and persistence of ASF in endemic areas and elucidate the role of animals that survive the disease, that is, the role of seropositive animals as potential carriers based on diagnostic data. These data are essential to determine the dynamics of the infection in the affected countries and support control and eradication programs. The use of alternative samples, such as blood or oropharyngeal swabs, can support these programs. While these alternative approaches are feasible, they should be integrated into control strategies through the selection of test methods and sample materials following a "fit for purpose" approach. Finally, since there is a strong demand for the

development of accurate, fast and simple detection methods, especially for in situ application, significant efforts should be made to validate them in the field at an international level.

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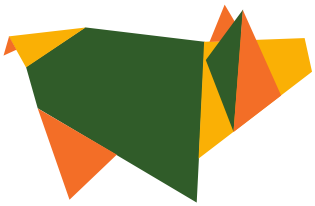

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