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Review

African swine fever (ASF) diagnosis, an essential tool in the epidemiological investigation

Gallardo C.*, Fernández-Pinero J., Arias M.

European Union Reference Laboratory for African Swine Fever (EURL), Centro de Investigación en Sanidad Animal, INIA-CISA, Valdeolmos, 28130, Madrid, Spain

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ABSTRACT

Since there is no vaccine available, prevention, control, and eradication of African swine fever (ASF) is based on the implementation of appropriated surveillance and strict sanitary measures. Success of surveillance activities depends on the availability of the most appropriate diagnostic tests. Although a number of good validated ASF diagnostic techniques are available, the interpretation of the ASF diagnostic results can be complex. The reasons lie in the complexity of the epidemiology with different scenarios, as well as in the characteristics of the viruses circulating giving rise to a wide range of clinical forms of ASF. This review provides guidance for an accurate interpretation of ASF diagnostic results linked to the different clinical presentations ranging from per-acute to chronic disease, including apparently asymptomatic infections.

1. Introduction

African swine fever (ASF) is one of the most complex infectious swine diseases. Its notification to the World Organization for Animal Health (OIE) is mandatory due to the high mortality it causes, its efficient transmission rate and the great sanitary and socioeconomic impact that it produces on international trade of pigs and pork products. The aetiological agent is the African swine fever virus (ASFV), a large, enveloped double-stranded DNA virus, which is the only member of the Asfarviridae family (Dixon et al., 2005). The ASFVs have been divided into 24 different genotypes based on their *B646L* gene, which encodes the capsid protein p72 (Quembo et al., 2018).

Endemic in more than 20 sub-Saharan African countries (Mulumba-Mfumu et al., 2019) and in Sardinia since the last century (Cappai et al., 2018; Jurado et al., 2018a; Laddomada et al., 2019), ASF arrived at a Black Sea harbour in Georgia in 2007 (Rowlands et al., 2008), from where the disease spread quickly to other neighbouring countries, reaching the European Union (EU) in 2014. The first cases of infected wild boar in 2014 were reported from Lithuania and Poland in January and February, followed by Latvia in June and Estonia in September (Gallardo et al., 2014). In the three Baltic States and Poland the disease has become endemic in the wild boar population, whereas the sporadic outbreaks occurring in domestic pigs have been efficiently controlled preventing extensive secondary spread (Cwynar et al., 2019; European Food Safety Authority (EFSA), 2018a, 2018b). The latest countries affected in Europe were Romania and Czech Republic in 2017, although in the latter was declared as resolved in January 2019, and Bulgaria, Hungary and Belgium, in 2018. In contrast to what has been observed in non-EU European countries (i.e., the Russian Federation or Ukraine), in the EU scenario, except in Romania, the number of infected farms has been comparatively lower, with wild boar being the most severely affected host (European Commission (EC), 2019; Iglesias et al., 2017; Jurado et al., 2018b).

In August 2018, ASFV demonstrated its huge capacity for transboundary and transcontinental spread jumping to China, several hundreds of kilometres away from previously known infected regions (World Organisation for Animal Health (OIE), 2019a, 2019b). There, it rapidly spread with 149 ASF outbreaks confirmed and the culling of more than 1 million pigs by 18 July 2019 (Food and Agriculture Organization of Animal Health (FAO), 2019). This represents a new change in the epidemiological situation of ASF worldwide, suggesting that the disease may have reached global proportions. The continuous spread of ASF to other Asian countries, with confirmed detections in Viet Nam, Mongolia, Cambodia and Hong Kong, and recently in North Korea (Food and Agriculture Organization of Animal Health (FAO), 2019) will make controlling the spread even harder.

Although ASF was first described almost a century ago, controlling the disease has proven to be a challenge, in particular because no vaccine or treatment are available. Spread of ASF can only be prevented by early detection and the application of strict compliance of classical disease control methods, including surveillance, epidemiological investigation, tracing of pigs, stamping out in infected holdings, strict

* Corresponding author at: Centro de Investigación en Sanidad Animal (INIA-CISA), Ctra Algete el Casar s/n, 28130, Valdeolmos, Madrid, Spain.
E-mail address: gallardo@inia.es (C. Gallardo).

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quarantine and biosecurity measures and animal movement control. Surveillance, to be successful, must have adequate laboratory support for a rapid diagnosis, which added to the epidemiological data, will allow the early detection of the disease and therefore reduce/prevent ASFV spreading (European Commission(EC), 2013). A wide spectrum of accurate ASF diagnostic tests is available and most of them have been successfully employed in surveillance, control and eradication programs (Arias et al., 2018; Arias and Sánchez-Vizcaíno, 2012; Gallardo et al., 2015a; Oura et al., 2013; Sánchez-Vizcaíno and Mur, 2013). 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 combination with the information coming from disease epidemiology, scenario, and the clinical signs. This requires an updated knowledge about the circulating strains, the disease, its mechanisms of spread and disease presentation on farms and field.

Based on experiences and data gained at the European Union Reference laboratory for ASF (EURL) from the current situation in the EU, this review highlights recent knowledge in the diagnosis of ASF, especially concerning the interpretation of results and its role in the epidemiological investigation of ASF. In this regard, the ASF diagnosis in wild boar and its relevance to understand the ASF clinical evolution are especially discussed.

1.1. ASFV circulating strains versus clinical presentations in affected areas of central-eastern Europe and Asia

The ASFV strains circulating in Europe (except in Sardinia) and in Asia belong to the p72 genotype II (Gallardo et al., 2014, 2018a, 2018b; Garigliany et al., 2019; Ge et al., 2018; Malogolovkin et al., 2012; Rowlands et al., 2008). Further analysis of small genomic regions has allowed the identification of different genetic variants within closely related ASFV genotype II isolates (Fraczyk et al., 2016a; Gallardo et al., 2014, 2018a; Mazur-Panasiuk and Woźniakowski, 2019a; Nieto et al., 2016). These genotyping approaches are used to identify the origin of viruses and can differentiate closely related strains from a genetic point of view. However, the correlation between currently established ASFV genotypes and virulence is not precisely clear (Arias et al., 2018).

Full genome sequences of 17 genotype II strains have been up to now reported including the ASFV-China strain (Bao et al., 2019; Chapman et al., 2011; Forth et al., 2019; Mazur-Panasiuk et al., 2019b; Olesen et al., 2018; Zani et al., 2018). The alignment of all genotype II isolates shows that all these genomes are nearly identical with identities of more than 99.9% (Forth et al., 2019; Mazur-Panasiuk et al., 2019b). These results suggest that after a decade-long circulation in Europe, the European ASFV genotype II strains show a low mutation rate and high genetic stability that hindered the definition of reliable genetic markers associated to virulence. In this context, Zani et al. (2018) reported that the deletion of 26 genes belonging to the MGF110 and 360 located at the 5′ end of the ASFV genome could be associated to the attenuated phenotype found in the North Eastern Estonian 2014 strain. Despite o this interesting finding, Nurmoja et al. (2017) concluded that oronasal inoculation using the same strain, led to an acute and severe disease in wild boar and only one animal survived the infection. Therefore, the description of genome markers related to the virulence of ASFV isolates needs to be further investigated and is still considered as a gap (Arias et al., 2018).

Thus, current approach to identify changes in virulence and pathogenesis mechanisms is based on classical experimental infections. From the published data, most of the genotype II isolates of the "Georgia 2007 type" that are currently circulating in Eastern and Central Europe and, now in Asia, are highly virulent and cause very high mortality rates of 91–100%. After incubation period of 3 to 14 days (depending of the administration route and dose) domestic and wild boar, both equally infected, develop acute clinical signs and die between 4–7 days after the onset of the clinical signs (Gallardo et al., 2018a; Pikalo et al., 2019; Zhao et al., 2019). However, a percentage of the infected animals, between 2–10%, are recovered from the ASFV acute infection. These survivors may establish a persistent infection in some tissues and, under certain natural or induced conditions (transport, underfeeding, immunosuppression, etc.) may reactivate the virus, thereby facilitating its transmission. Furthermore, these animals are protected to a secondary ASFV infection, remaining sub-clinically infected, acting as a potential source of infection for the environment and for healthy animals as they could show low levels of viremia (wild boar and domestic pigs). This allows the natural evolution of the ASFVs including the emergence of less virulent forms over time, as occurred in different geographic regions where ASF has been present for a long time (Africa, Iberian Peninsula and Sardinia) (Arias and Sánchez-Vizcaíno, 2002, 2012; Arias et al., 2018; Gallardo et al., 2015b, 2018a).

Despite the controversies arising with regard to the evolution of the genotype II ASFVs to less virulent forms, data obtained from the field and from experimental infections have clearly supported this finding. Sargsyan et al. (2018) described the presence of atypical clinical forms of ASF coexisting with acute typical forms in Dilijan municipality in Taush province (Northeast of Armenia) in 2011. Similarly, field epidemiological investigations conducted in Estonia showed two different epidemiological patterns in terms of mortality, suggesting the co-circulation of strains of different virulence in the country (Nurmoja et al., 2017; Zani et al., 2018). The work developed by Gallardo et al. (2018a) confirmed the presence of strains of moderate virulence circulating among the wild boar population in Estonia in 2015. Finally, the first non-haemadsorbing (non-HAD) ASFV genotype II was isolated from a hunted wild boar in Latvia in 2017. Domestic pigs experimentally infected with the non-HAD ASFV developed a non-specific or subclinical form of the disease. (Gallardo et al., 2019).

Nowadays there is important knowledge that provides evidence of the natural evolution of the ASFV in Central-Eastern Europe, where genotype II is circulating. Different ASF clinical forms from acute to subclinical infections coexist in the field, in more or less proportion, depending the affected region, challenging the recognition of the disease and the early detection. The understanding of the clinical presentations and the infection dynamics, including pathogenesis and immune response, is the key step for the correct use of the available diagnostic tools and to design effective control and eradication programs.

2. Available ASF diagnostic tests

Currently, a wide number of validated ASF diagnostic techniques are available for giving a confident diagnosis of ASF in the affected countries. The techniques used to diagnose ASF, their advantages and drawbacks as well as prospects for improving diagnostic strategies in the future, are discussed and reviewed in this section.

2.1. Virus detection tests

Due to the acute characteristics of ASF, passive surveillance is the most effective and efficient method for an early detection in free areas (European Food Safety Authority (EFSA), 2018a). Samples (blood and target organs) taken from sick or dead animals must be tested to negate or confirm the ASFV infection. The virological tests are therefore vital for the rapid implementation of control measures. These encompass detection of viral genome by PCR, detection of viral antigens by antigen ELISA or a Direct immunofluorescence test (DIF), and virus detection using virus isolation (Gallardo et al., 2015a; World Organisation for Animal Health (OIE), 2019a, 2019b; Oura et al., 2013). Table 1 summarizes the current validated diagnostic techniques for ASFV detection.

2.1.1. ASFV genome detection

Currently, the PCR is considered the 'gold standard' test for early detection of the disease due to its superior sensitivity, specificity,

Table 1
African swine fever validated ASFV detection tests.

AVAILABLE TESTS		TYPE, In house/ Commercial	Recommended Use	REFERENCE
Virus Isolation		*VI /Haemadsorption (HAD) test (i.h.)	Confirmation of primary outbreak.	Malmquist and Hay (1960)
Antigen detection		*Direct Immuno fluorescence (DIF) (i.h.)	Individual testing	Bool et al. (1969)
		ELISA INgezim PPA DAS, Double Ab Sandwich (C)	Surveillance Herd testing	INGENASA
PCR	Conventional	*Conventional (i.h.)	Surveillance Individual and Herd testing	*Agüero et al. (2003).
		Multiplex ASF-CSF (i.h.)	Co-circulation ASF and CSF	Agüero et al. (2004).
	Real-Time	Taqman Probe (i.h.)	Surveillance Individual and herd testing	*King et al., 2003; * Zsak et al. (2005); Tignon et al., 2011
		UPL Probe (i.h.)	Surveillance Individual and herd testing	*Fernandez-Pinero et al. (2013)
		Multiplex ASF-CSF (i.h.)	Co-circulation ASF and CSF	Haines et al. (2013)
		TETRACORE dried down (C)	Surveillance, Individual and herd testing	TETRACORE
		INgene q PPA (C)	Surveillance, Individual and herd testing	INGENASA
		Virotype ASFV PCR Kit (C)	Surveillance, Individual and herd testing	INDICAL BIOSCIENCE
		LSI VetMAX™ ASF (C)	Surveillance, Individual and herd testing	THERMO FISHER SCIENTIFIC
		IDEXX RealPCR ASFV Mix (C)	Surveillance, Individual and herd testing	IDEXX
		ID Gene® African Swine Fever Duplex – IDVet (C)	Surveillance, Individual and herd testing	IDVet
		[C]ADIAVET ASFV REAL TIME 100R (C)	Surveillance, Individual and herd testing	BIO-X DIAGNOSTICS

*Included in the OIE Terrestrial Manual for Diagnostic Test and Vaccines, 2019.
i.h. (in house methods); C: Commercial Kits currently validated.

robustness and high-throughput application to detect the ASFV genome in any kind of clinical samples from domestic pigs, wild boar and ticks (Gallardo et al., 2015a; Oura et al., 2013).

Over the last twenty years, a variety of PCR tests, including both conventional and real time (rPCR), have been developed and validated to detect a wide range of ASF isolates belonging to different known virus genotypes, non-haemadsorbing strains, and diverse virulence (Agüero et al., 2003; Fernandez-Pinero et al., 2013; King et al., 2003; Tignon et al., 2011; Zsak et al., 2005). All of them have been designed in the VP72-coding region, a highly conserved gene coding the major viral protein, assuring the (potential) detection of any ASFV isolate (Oura et al., 2013; Gallardo et al., 2015a). The OIE rPCR (King et al., 2003; World Organisation for Animal Health (OIE), 2019a, 2019b) and the Universal probe library (UPL) rPCR (Fernandez-Pinero et al., 2013) are the most widely used for routine diagnosis at the EU´s national reference laboratories (NRLs) level (Nieto R., personal communication 2018). Both methods are able to provide a confident ASF diagnosis, although the UPL-PCR has greater diagnostic sensitivity for detecting survivors and allows earlier detection of the disease even when the typical clinical signs are not yet evident (Fernandez-Pinero et al., 2013; Gallardo et al., 2015a).

Since new viral isolates displaying new genetic patterns can emerge at any time, it is important to check periodically the performance of the routine PCR assays. In this regards, the ASF EURL has already announced that the OIE-recommended conventional PCR test (Agüero et al., 2003) shows lower sensitivity than expected in the detection of the current circulating ASFV genotype II strain. This is due to a nucleotide mismatch close to the 3′ end of the reverse primer existing in the circulating viruses. In the same way, the CSFV primers described in the multiplex PCR for ASFV/CSFV detection (Agüero et al., 2004) are not adequate for the detection of recent CSFV isolates from Caribbean region belonging to genotype 1.3 (personal data).

Multiplex PCRs have been developed for the simultaneous and differential detection in a single test of ASFV and other porcine pathogens such Classical swine fever virus (CSFV) (Agüero et al., 2004; Giammarioli et al., 2008; Grau et al., 2015; Haines et al., 2013; Hu et al., 2015). These techniques are useful for surveillance in free areas with high risk of entrance of CSF and/or ASF, and in case of co-circulation of both viruses, though a lower diagnostic sensitivity could be expected compared to the single assays.

Concerning other molecular tests, isothermal assays could be a cheaper diagnostic alternative to PCR, and useful in field conditions (Frączyk et al., 2016b; Hjertner et al., 2005; James et al., 2010). The sensitivity is appropriate in cases whereclinical signs are present and it seems enough for detection of acute cases. However, these tests are not recommended for the detection of recovered or virus carrier animals, since the genome detection level is significantly lower than PCR. These infected animals are capable of disseminating (shedding) the virus, but which itself shows no sign of clinical disease (European Food Safety Authority (EFSA), 2015). Although already developed, these techniques still lack field validation data.

Finally, the number of commercial kits for ASFV genome detection based on published rPCRs, has greatly increased over recent years (Tetracore Inc.; Ingenasa S.A; Indical Bioscience; Thermo Fisher; IDDEX; IdVET; Bio-X Diagnostics). These represent an alternative that can guarantee a certain homogeneity in results, which is important in establishing testing procedures to be adopted by many laboratories. Each of the new ASF-commercial assays must to be evaluated and validated following international guidance to ensure they are specific, sensitive, reproducible, precise, robust and accurate.

In conclusion, PCR is a basic diagnostic tool for surveillance considering the long-term viremia and high viral load that exhibits in the infected animals suffering acute or subacute clinical courses. Besides, only the highest sensitive PCR tests are useful to detect the low viral load during the first days of infection and to diagnose the weak sporadic viremia shown by animals with a chronic or subclinical infection

course. In the latter situations and despite the PCR result, the serological tests will play a key role in the recognition of infected animals.

2.1.2. Virus isolation (VI) and haemadsorption (HAD) test

Growing ASFV isolates obtained mainly from the field is a critical step for diagnosis. In theory, all of the ASFVs collected from natural outbreaks can be isolated in susceptible primary leukocyte cultures of swine origin, either from blood or lung (alveolar) monocytes or from macrophages cells. If the ASFV is present in the porcine sample, it will replicate in the cells and will produce the cytopathic effect (CPE) and the haemadsorption reaction (HAD), a characteristic feature of the ASFV-infected cell, widely used for diagnostic purposes (Carrascosa et al., 2011; Enjuanes et al., 1976; Malmquist and Hay, 1960; Malquimst, 1962). No other pig viruses are capable of haemoadsorbing in leukocyte cultures making the HAD assay the confirmatory test in case of primary outbreak (European Commission (EC), 2003a; Gallardo et al., 2015a). However, attempts to isolate infectious virus from field-derived samples provide irregular results. Gallardo et al. (2015a) reported the low effectiveness (30.7%) to isolate the virus when testing wild boar field-derived samples collected from affected regions in Europe, despite the high values of viral DNA that were present in the samples. Further studies done at the EURL have confirmed these findings. From 1719 PCR positive field samples subjected to virus isolation, infectious virus was only isolated in 404 cases (23%). Samples that exhibited unsuccessful results were mainly derived from wild boar and resulted in 233 ASF viruses isolated (18%) from 1302 samples tested. The reason lies in the poor state of samples received, which affects the virus viability, especially taking into account that the highest percentage of them were obtained from dead or hunted animals (unpublished data at the EURL). Additionally, some field strains do not produce HAD, but only CPE (Boinas et al., 2004; Leitão et al., 2001; Gallardo et al., 2019); these non-HAD viruses are not easily isolated and require further confirmation using PCR or DIF test on the sediments of the cell cultures (Oura et al., 2013).

Even though VI and identification by HAD tests are recommended as a reference confirmatory test in the event of a primary outbreak or a case of ASF (European Commission (EC), 2003a, 2003b), it is not likely to be the most fruitful approach for an effective ASF diagnosis at the NRLs. It is more expensive than other techniques, requires both specialized facilities and training, is time consuming and cannot be adapted to high throughput. However, despite these constraints, virus isolation is essential to obtain virus stocks for future molecular and biological characterization studies. These problems were initially overcome by using African green monkey kidney established cell lines, such as VERO or Monkey Stable (MS) cells, in which some ASFV isolates were adapted (Parker and Plowright, 1968; Enjuanes et al., 1976), but only for those cell-culture-adapted isolates. Hurtado et al., 2010 described the COS-1 cells as an established cell line susceptible to all of the ASFV isolates tested allowing the amplification of any virus sample for diagnosis, detection, and production. Additionally, the possibility to infect cell lines like IPAM or wild boar lung cells (WSL), derived from swine alveolar macrophages, can facilitate studies in which a more natural environment (swine, macrophage) is required to mimic more precisely the course of *in vivo* ASFV infection (Carrascosa et al., 2011). However, the use of these cells is not without its disadvantages. Moreover, it has been recently published that none of the porcine cell lines IPAM, WSL show a mature macrophage phenotype, and among them, only WSL are able to sustain productive ASFV infection, although it is strain-dependent (Sánchez-Cordón et al., 2017; Sánchez et al., 2019 ; Sánchez et al., 2017).

Finally, despite these cell lines have many well-known advantages compared to primary cells, they are not always suitable for the ASFV isolation from field samples without a little apparent adaptation (Carrascosa et al., 2011; Gallardo et al., 2013). Therefore, further evaluation studies are required for the potential use of established cell lines in ASF diagnosis.

2.1.3. Antigen detection techniques

Antigen detection techniques have been widely employed for presumptive diagnosis in the past. Among these, the DIF is an *"in house"* technique, to detect viral antigens in smears or thin cryosections of organs, and is useful for ASFV identification from VI of non-HAD strains (Bool et al., 1969; Oura et al., 2013). The DIF is a rapid test and provides good specificity and sensitivity enough for HAD and non-HAD strains of ASFV in the peracute and acute forms of the disease. However, the sensitivity of this technique drops significantly when the antibody response is developed after the first week post-infection due to antigen-antibody complex, giving a high proportion of false negative results (World Organisation for Animal Health (OIE), 2019a, 2019b; Arias and Sánchez-Vizcaíno, 2012). In addition, it is difficult to adapt to high throughput and the results might be subjective, so skilled staff are required. On the other hand, the required ASFV specific antibodies fluorescein conjugate could be difficult to get within the expected quality standards conditions.

A number of *"in house"* antigen ELISAs, including direct, indirect and sandwich ELISA formats, which employ monoclonal and polyclonal antibodies, were developed in the past although are not currently in use (Hutchings and Ferris, 2006; Vidal et al., 1997; Wardley et al., 1979). A commercially produced antigen ELISA kit is the only currently available (ELISA INgezim PPA DAS, Ingenasa, Spain), which allows the use of tissue and serum samples for the analysis. It is a rapid test and easy to scale up. However, comparative testing of 277 samples from experimentally infected pigs and field samples from wild boar and domestic pigs, showed a poor sensitivity (77.2%) of the commercially antigen ELISA test when compare to the UPL-PCR, above all in the case of field-derived samples, even when there was a high virus load (Gallardo et al., 2015a, 2015b, 2015c; Oura et al., 2013: Sastre et al., 2016a, 2016b; Gallardo, C. presented at the "Workshop on laboratory diagnosis of African and Classical swine fever (ASF and CSF)", Madrid, Spain, 2–3 June 2014).

Consequently, the use of DIF or antigen ELISA is only recommended as a herd assay and should be combined with some other virological and serological tests.

2.2. Antibody detection tests

Serological assays are the most commonly used diagnostic tests due to their simplicity, relatively low cost and need for slight specialized devices or few facilities. For ASF diagnosis, the antibody detection is particularly relevant given that no vaccine is available against ASFV, which means that the presence of anti-ASFV antibodies always indicates infection. Furthermore, anti-ASFV antibodies appear soon after infection and persist for up to several months or even years (Arias and Sánchez-Vizcaíno, 2002, 2012). Antibody-based surveillance is therefore essential for the detection of surviving animals, to elucidate the epidemiological characteristics of the epidemics, i.e., time since the virus introduction into a farm, and for detecting incursions involving low virulence ASFV isolates (Arias et al., 2018; Gallardo et al., 2015a, b; Laddomada et al., 2019; Mannelli et al., 1997; Pérez et al., 1998). The use of antibody detection assays was also crucial for successful eradication programs in the past (Arias and Sánchez-Vizcaíno, 2002, 2012).

Current ASFV antibody-based tests approved by the OIE involve the use of an ELISA for antibody screening, backed up by Immunoblotting (IB), Indirect Immunofluorescence (IIF) or the Indirect immunoperoxidase tests (IPT) as confirmatory tests (Gallardo et al., 2015a; World Organisation for Animal Health (OIE), 2019a, 2019b) (see Table 2).

2.2.1. Antibody-ELISA (enzyme linked immuno sorbent assay)

Detection of specific antibodies against ASFV by ELISA is the OIE prescribed test for international trade so far. Currently there is a number of ASF ELISA variants including recombinant ELISAs (Gallardo et al., 2006, 2009; Pérez-Filgueira et al., 2006), and several (OIE) "in

Table 2
African swine fever validated ASFV antibody detection tests.

AVAILABLE TESTS	TYPE, In house/ Commercial	Recommended Use	REFERENCE
ELISA	*OIE Indirect ELISA (i.h.)	Surveillance Herd testing	Sanchez-Vizcaino et al. (1982)
	Recombinant proteins (rp)-ELISA (i.h.)	Surveillance Herd testing	Gallardo et al. (2006), (2009) and Pérez-Filgueira et al (2006)
	INgezim PPA COMPAC competition-ELISA. (C)	Surveillance Herd testing	INGENASA
	ID Screen® ASF Indirect ELISA. (C) ID Screen® ASF Competition-ELISA. (C)	Surveillance Herd testing	IDVET
	SVANOVIR® ASFV Indirect-ELISA.(C)	Surveillance Herd testing	SVANOVA
Confirmatory Antibody tests	*Immunoblot (IB) Test (i.h.)	Confirmatory Herd testing	Pastor et al. (1989)
	*Immunofluorescence Antibody (IIF) test (i.h.)	Confirmatory Herd testing	Lawman and Caie (1979)
	*Indirect Immunoperoxidase test (IPT) (i.h.)	Confirmatory Herd testing	Pan et al. (1982) and Gallardo et al. (2015a); (2015b), c

*Included in the OIE Terrestrial Manual for Diagnostic Test and Vaccines, 2019.
i.h. (in house methods); C: Commercial Kits currently validated.

house" versions of the test based on the use of live virus as antigen. Three commercial ELISA kits are also available and validated for the detection of ASF antibodies based on the most antigenic proteins so far described such as p72, p32, pp62 and p54 (INGENASA, IDVET and SVANOVIR), of which the INGEZIM PPA COMPAC, K3 from INGENASA is the most widely used at EU level (Nieto R., personal communication, 2018).

It has been reported the ELISA exhibit lower sensitivity for the detection of antibodies from days 7–12 dpi, when compared to the confirmatory serological assays (Gallardo et al., 2015a). Nevertheless it is a very confident test for the detection of specific antibodies from 12 to 14 dpi, and therefore the ELISA test remains as the most useful method for large-scale serological studies; It is fast, easy to perform and economical. However, only serum can be analysed, which restricts its application range. The accuracy of either "in house" or commercial ELISA formats is around 80% when tissues are tested due to the lack of specificity (Gallardo et al., 2015a). This is a deficiency in the current epidemic situation in Europe, where the diagnosis of ASF in wild boar is of major importance. Samples that are usually obtained from hunted / captured animals or animals found dead are sent to the laboratory to determine the presence of the disease. Therefore, the type of samples may limit the performance of a complete diagnosis of the disease, mainly in endemic areas where moderate and low virulence strains may be circulating. This issue is nowadays surpassed by some NRLs by the use of IPT test, which can easily analyse all type of exudates from tissue samples, including bone marrow. Nevertheless, a priority should be to develop standardized ELISAs for the detection of specific antibodies of ASF virus in tissue extracts for an easy and more reliable evaluation of epidemiological situation in affected areas.

2.2.2. Confirmatory antibody detection tests

Positive ELISA results should always be confirmed by additional methods such as IPT, IIF or IB tests, as is recommended by the World Organisation for Animal Health (OIE) (2019a); (2019b). The IB is a rapid and sensitive assay for the detection of specific antibodies and provides good recognition of weak seropositive samples by specific reaction of the antibodies against the antigen-proteins (IP 12, IP 23, IP 25, IP 25.5, IP 30, IP 31, IP 34 and IP 35). These polypeptides begin to positively react by IB with sera obtained at just 7–9 days post infection, and the positive reaction of most of them is maintained in sera obtained several months after infection (Pastor et al., 1989). Despite this method being highly sensitive, similarly to that described above, only serum samples can be used in the IB test. In addition, in ASF-endemic areas, where chronically infected animals of subclinical infections are present, non-specific characteristic pattern could be visualized, hindering the interpretation of the results. In this situation, an accurate evaluation of the results should be performed taking into consideration alternative confirmatory serological diagnostic tests such as IIF (Lawman and Caie, 1979) or IPT (Gallardo et al., 2015a; Pan et al., 1982). Both are based on the same principle and require the use of fixed cultured VERO or MS monolayer cell lines infected with adapted ASFV. These tests have high specificity and sensitivity, although the interpretation of the results can be subjective and well-trained staff are required. Despite this limitation, the IPT has been proved as the best test for ASF serological diagnosis due its superior sensitivity, but moreover, its performance to test any kind of porcine material such as blood, exudate tissues or body fluids (Gallardo et al., 2015a). This is particularly relevant for wild boar surveillance and control programs. Currently the IPT technique is the selected confirmatory test at the EU NRLs (Nieto R., personal communication 2018).

The major drawback with the confirmatory serological tests is that none of these techniques are produced commercially by companies, which constrains their use in laboratories, especially those with limited resources. The results obtained in a survey conducted by EURL in 2016 aimed to review the strengths and critical points for the diagnosis of ASF at EU level, revealed that 25% of the NRLs considered the implementation of validated antibody confirmatory tests as major critical point (de la Torre A. personal communication 2016). The availability of a commercial confirmatory serological assay should be a priority for future work.

3. Some considerations on ASF diagnosis

Although for ASF there are well-proven diagnostic tests available, the current epidemiological situation worldwide has highlighted the necessity to improve existing tools, in order to more rapidly recognise new cases and to shorten the time interval between introduction of ASFV in free areas and control measures being taken. This section makes a review about the latest developments on ASF diagnostic research focusing on the use of new matrices as alternative samples considering the epidemiological situation in Europe. To be effective, proper samples combined with the selection of diagnostic methods, is of fundamental importance in order to make a rapid and reliable diagnosis.

3.1. Pen-side tests

The time elapsed between the clinical suspicion and laboratory confirmation used to be relatively long due to the logistics of sending samples to official laboratories. On the other hand, in most cases, regional laboratories do not have the expertise, equipment and/or facilities to diagnose exotic diseases such as ASF. This can be sorted out by the use of pen-side tests for a first front-line diagnosis under field conditions, giving real-time data on the animal's infection status. Two different lateral flow devices (LFDs) for the detection of antibodies or the viral antigen in blood are commercially available by INGENASA so far, and it is expected several others in the near future. The pen-side test for antigen detection (INgezim ASF CROM Ag) described by Sastre et al.

(2016a) is based on the use of a monoclonal antibody against VP72 protein of ASFV and provides similar sensitivity to that of the commercial antigen-ELISA when samples from experimental infections are tested. The LFD was demonstrated to be positive for animals with circulating virus levels exceeding 10^4 haemadsorbing units (HAU). When testing field samples, the LFD exceeded the values obtained with the antigen-ELISA, showing 60% positivity versus 48% for the antigen-ELISA, although LFD exhibited lower sensitivity when compared to the rPCR as the gold standard method. About the pen-side assay for antibody detection (INgezim PPA CROM), recent studies under field conditions in hunted wild boar in Sardinia (Cappai et al., 2017), showed a sensitivity of 82% and a specificity of 96% when compared with the commercial antibody INGENASA ELISA and the IPT (better performance under laboratory conditions). Apart from the single LFD for ASF, multiplexing has been reported with CSF, which would facilitate surveillance for both diseases in the field (Sastre et al., 2016b).

The use of the pen-side tests offers a first-line diagnosis that can be useful for rapid application in case of sanitary emergency. However, from the published data, the LFDs should not be used alone due to a very limited sensitivity compared to the gold standard methods, overall in the case of the LFD for the detection of antigens. Test sensitivity needs to be high, whereas specificity is less critical, since any positive result will need to be verified by the competent NRL. The use of pen-side tests for on-farm or field screening requires to be restricted to official veterinarians or regional laboratories with limited resources and should be used taking into consideration a specific circumstances (European Commission (EC), 2003b).

3.2. Alternative samples for ASF diagnosis

Wild boar sampling has been proved as a bottleneck in both passive and active surveillance within the EU. Non-invasive sampling strategies could mean an optimization for wildlife surveillance by circumventing the necessity of fitness-biased hunting/capture sampling schemes. Recently, different approaches for the in-life sampling have been evaluated both under experimental and field conditions.

The collection of unpreserved field faecal samples has been reported as an alternative non-invasive surveillance method of wild boar and free-ranging pigs for ASF virus (de Carvalho Ferreira et al., 2014) and antibody detection (Nieto-Pelegrín et al., 2016). However, the short-term survival of viable ASFV, the temperature-dependence, and the differences found among ASFV strains regarding its virulence (Davies et al., 2017; de Carvalho Ferreira et al., 2014) suggests that the analysis of faecal samples would not be a method sensitive enough to be used in the wild boar surveillance. A recent research at the EURL for ASF (INIA-CISA) confirmed this finding. Faeces were taken from domestic pigs experimentally infected with ASFVs of genotype II of diverse virulence. The analysis by PCR demonstrated that only 8% of the faeces from animals with subacute or chronic infections contained the virus. In contrast, the ASFV was easily detected in faeces taken from animals with acute disease, although this finding took place two or even four days later than in the blood, coincident with the acute-phase of the disease (EURL unpublished data). Moreover, it should be kept in mind that the average life of the virus in the field is strongly affected by enzymes (proteases and lipases) produced by bacteria colonizing faeces and also urine, thus the exact survival time in the forest where ASF is actively circulating is not fully comparable to the estimates obtained in laboratory conditions (European Food Safety Authority (EFSA), 2018b).

The use of dried blood-spots (DBS) on filter papers and swabs have been described for both antibody and ASFV genome detection with good specificity and relatively appropriate sensitivity (Blome et al., 2014; Braae et al., 2015; Michaud et al., 2007; Petrov et al., 2014; Randriamparany et al., 2016). These matrixes obviate the need for a cold chain to preserve specimens during the transport to the laboratories, are generally cheap (although some of the treated papers, such as FTA, are very expensive), requires only a small sample volume, and needs minimal technical expertise for collecting. These factors are likely to make sample collection more acceptable to the hunters and may be an alternative to the classical bleeding in the wild boar surveillance programs.

Finally, recent strategies proved the use of (bait) ropes for the collection of oral fluid as suitable strategy for antibody (Giménez-Lirola et al., 2016; Mur et al., 2013) and ASFV genome detection (Davies et al., 2017; Grau et al., 2015).

In conclusion, samples obtained through non-invasive sampling methods such as oral fluid or dried blood on filter papers could be a suitable approach for ASF detection in the wild boar surveillance programs. However, although showing promising results, robust standardized protocols for sampling, storage, processing and testing ASF on such matrices need still to be validated using a wide range of samples from domestic pig and wild swine populations.

3.3. Effect of pooling samples on ASF virus detection

Current epidemic situation in the EU makes the pooling of EDTA blood samples a common practice at the NRLs to reduce the PCR analysis cost and increase throughput. This means that the samples are diluted, which can affect the ability of the rPCR assays to detect ASFV DNA. The EURL carried out a study to evaluate the effect of pooling samples in the detection of a single ASFV-positive animal,using the UPL rPCR as reference method due to its superior sensitivity. A collection of 101 EDTA-bloods from genotype II ASFV infected pigs was selected based on their cycle threshold (Ct) value. Samples were diluted at a range of 1:3, 1:5, 1:10, and 1:20 in ASFV negative porcine blood (simulating the presence of 1 positive sample in groups of 3, 5, 10, and 20 samples), resulting in a 99%, 89%, 82%, and 72% of detection by UPL rPCR, respectively. The impact of pooling on the sensitivity of the rPCR was higher in samples taken at the beginning or at the end of the viraemia period when low viral load is present (Ct > 35). These results indicated that if a blood sample taken from an animal at the onset of the viraemia is pooled 1:5 (or a higher proportion), there would be the possibility of not detecting the viral DNA in the pooled sample, whereas it could be detected in the single specimen. The individual testing of animals by rPCR presents the most sensitive means to detect active ASFV infection in animals. Nevertheless, pooling of EDTA blood samples can be used to detect ASFV in animals arising from ASF-endemic areas. In this instance, the most appropriate and confident strategy would be a pooling ratio 1:3 to animals arising from a single geographic location or herd, since the possibility of a single ASF-infected animal existing within a unit would be unlikely (Gallardo C., personal communication 2016, 2017).

4. Final remarks: the ASF diagnostic interpretation

Diagnosis of ASF means the identification of animals that are, or have previously been, infected with ASFV. An appropriate diagnosis therefore involves the detection and identification of ASFV-specific antigens or DNA and antibodies, to obtain relevant information to support control and eradication programmes.

To achieve a correct diagnosis it is important to consider the laboratory tests results together with the epidemiological findings. Studies on the pathogenesis of highly virulent ASFVs in pigs show that primary viremia can be identified as early as 8 h post-infection and secondary viremia between 15th and 24th hours post infection. Spleen, lymph nodes, liver, and lungs were shown to be the sites of secondary viral growth and after 30 h, all tissues contained the virus, reaching the maximum titers at 72 h post-inoculation (Colgrove et al., 1969). The animals can die within the first four days after the infection even without clinical manifestations. The ASFV is easily detected in any kind of porcine sample by rPCR, VI and even using the antigen detection techniques (DIF or ELISA). No antibodies are developed.

In acute infections, caused by virulent strains, clinical signs can start

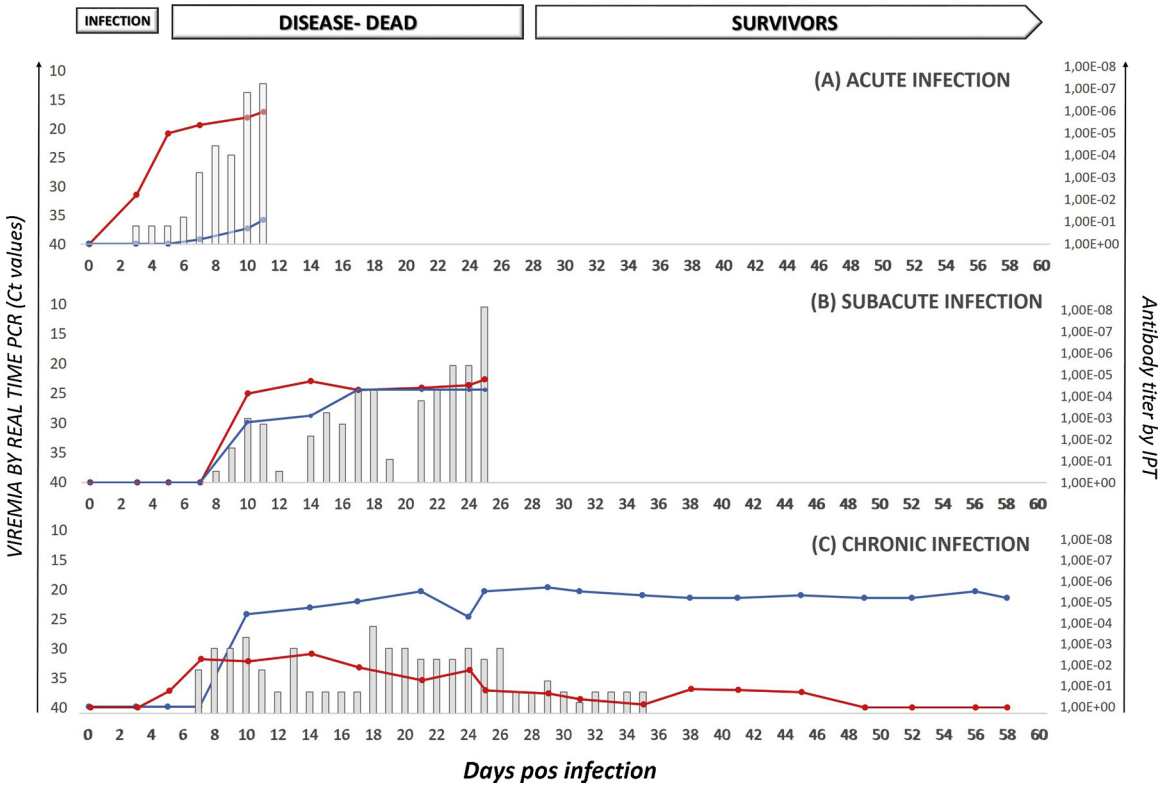

Fig. 1. Viremia (measured by real-time PCR) and antibody response (determined by IPT) over time and in relation to the stage of ASF virus infection, as observed in European domestic pigs infected with genotype II ASFV isolates circulating in the EU (2014–2018). Clinical score, expressed in bars, overlapped with viremia and antibody response.

varying from 4 to 19 days depending on the dose and the route of infection, with mortality 5–12 days post infection (Blome et al., 2013; Gallardo et al., 2018a; Pikalo et al., 2019). Once the animals have been infected, the ASFV genome is usually detected in blood on average 3.75 ± 1.4 days, two days before the onset of the clinical signs. Pigs develop acute-lethal disease and usually die within the two first weeks after the first case (Gallardo et al., 2015b, 2018a; Guinat et al., 2014, 2016; Pikalo et al., 2019). The ASFV is present in all porcine samples showing high viral load mainly in the bone marrow, spleen, liver, lymph nodes, lung and tonsils. A weak antibody response can be detected as early as 7–8 days by IPT (Fig. 1a), 2–3 days before the ELISA, although with the latter it is rarely detected.

Moderately virulent strains are involved in the appearance of acute (pigs dead 11–15 dpi) and subacute (animals die after 20 dpi) forms. In clinical terms, acute ASF develops over a 7-day period, compared with 10–20 days for the subacute form of the disease. The mortality rate ranged in the latter from 30 to 70% (Arias and Sánchez-Vizcaíno, 2012; Beltrán-Alcrudo et al., 2017). Viremia can be detected by rPCR as early as 3 dpi in acute infections and at on average of 8.5 ± 3.6 days in domestic pigs with subacute infections. IPT usually detect antibodies between 8 to 10 days, respectively, reaching mean antibody titres of 1:20,000 from the third week (Fig. 1b). All tissues obtained from animals that succumbed within the first month to the infection originated by moderate virulence strains were positive by rPCR and VI (Gallardo et al., 2018a, b).

In recovered pigs surviving acute or subacute infections, the viral DNA can persist in the blood for up to 78 days (Gallardo et al., 2018b), although VI positive results are mainly obtained within the first month with occasionally isolation up to day 66 in blood (de Carvalho Ferreira et al., 2012). The presence of this second excretion peak might be associated with a new cycle of virus replication in persistently infected tissues (de Carvalho Ferreira et al., 2012; Wilkinson, 1984). Gallardo et al. (2018b) showed that at 78 days after the infection it was possible to recover infectious virus from the tonsil of pigs experimentally infected with a genotype II moderate virulence strain from Estonia.

Chronic ASF has been associated with infection by moderate-to-low virulence isolates. Animals developing chronic infections show non-specific clinical signs, and in some cases remained asymptomatic (Gallardo et al., 2015c; Beltrán-Alcrudo et al., 2017; Moulton et al., 1975; Moulton and Coggins, 1968; Mebus and Dardiri, 1980). Pigs developing chronic-type ASF lesions have recurring cycles of pyrexia and weak and/or intermittent viremia (Cycle threshold Ct > 30) from the first week after the infection which can persist over two months. In contrast, pigs remaining asymptomatic are usually non-viraemic. Despite the absence of viremia and clinical signs, specific antibodies are easily detected in all animals after the first week using IPT and ELISA, reaching antibody levels > 1:160,000 after one month which are maintained over the time (Fig. 1c) (Gallardo et al., 2015c, 2018a, 2018b, 2019; Leitão et al., 2001; Sánchez-Cordón et al., 2017; Sánchez-Vizcaíno et al., 2015). Virus replication in the tissues showed evidence of virus at up to 99 days in lung and thoracic lymph nodes (Gallardo et al., 2015c) and at 101 from the retropharyngeal and submandibular lymph nodes (Gallardo et al., 2019).

These data emphasize the fact that early detection based only on clinical signs and ASFV genome detection is not an efficient approach for the control of ASF in the current epidemiological situation in Europe. It is likely that the European wild boar is getting endemically infected in certain regions within the EU becoming a recurrent source of infection to other wild boar but also, to domestic pigs. Since each animal could be at a different stage of the disease, both virus and antibody detection tests, for confirming transient viremia and the presence of anti-ASFV-specific antibodies, could make sub-clinically ASFV infected wild boar or domestic pigs detectable. A positive test for the presence of the virus indicates that the tested animal was undergoing infection at the time of sampling. On the other hand, a positive ASFV antibody test indicates an ongoing or past infection, where the animals have

recovered (and may remain seropositive for life). The titer of these antibody can give information about the survival time of the infected animal. Antibody detection techniques are therefore essential to obtain complete information in support of control and eradication programmes.

Control-eradication programs in areas with a clear endemic tendency should be reviewed and updated and include parallel routine laboratory monitoring, together with the regular clinical inspection. The use of the most fitting diagnostic tools combining both ASF virus and antibody detection will improve the efficacy of disease-control measures, regardless of the nature of the circulating ASFV strains (Arias and Sánchez-Vizcaíno, 2002, 2012; Gallardo et al., 2015a).

The author(s) declare no conflict of interests.

Declaration of Competing Interest

The author(s) declare no conflict of interests.

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