African Swine Fever Gap Analysis Workshop
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Diagnostics Report

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INTRODUCTION

During the days 3 through 5 of April of 2013 a group of experts in ASF met to review current diagnostic tests and to evaluate immediate needs and gaps of ASF diagnostics. The main idea behind this encounter was to generate and/or improve tools of diagnostic fit for purpose, counting with the help of the expertise of the people who deals with ASF in regular bases. This group prepared a list of "gaps" toward which global efforts and resources can be directed, maximizing efficiency and harmonizing results. A well-coordinated global effort should result in enough transparency and communication to avoid overlapping or duplication of work, spread benefits evenly and enrich general knowledge.

ASF remains a devastating disease, endemic in most of the countries of sub-Saharan Africa and in Sardinia (Italy), with a shown tendency of expansion since its entrance in the Caucasus Region in 2007, also affecting Russian Federation, and moving towards countries that have been historically free of this disease such as Ukraine (2012) and Belarus (2013).

Understanding the pathogenesis and immune response of ASF is the key step for the correct use of the available diagnostic tools and to design new ones. To date, there is no vaccine available, and laboratory diagnosis is the essential component prevention and control strategies. However, ASF is a highly complex, and yet not very well understood, disease of wild and domestic swine with the possibility to also infect and persist in soft ticks. Therefore, within the currently short inventory of countermeasures to help prevention and control this challenging disease, diagnostics is in the top of the list, whether as surveillance of healthy but in high risk animal populations, or as tool for controlling an outbreak situation.

The incubation period of the disease oscillates between 3-15 days. Animals infected with ASFV usually induce antibodies (Ab) from 7 to 10 days post-infection, or even up to several month later (CISA-INIA, personal communication). Antigen (Ag)/DNA detection can be detected from 2 to 3 days after infection and usually up to several weeks in blood and serum. Therefore, in case of a suspicion of disease it is recommended the use of a combination of virological detection techniques (PCR test is recommended since Ag detection techniques such as DIF and antigen ELISA show very limited sensitivity in chronic cases) simultaneously with the use of serological test (ELISA, and confirmation of positives and doubtful results by IB/IFA/IPT test). Additionally, the characterization of ASFV isolates should be performed by genotyping, using standardized protocols, established at the international level and by the European Union Regional Reference Laboratory.

The "gap analysis" consists in the comparison between the current situation and the desired situation, which is set as a future goal. Tool for identifying the tasks needed to reach the goal. To carry out a gap analysis for ASF diagnosis we should:

- Identify the features that should be considered to create an ideal ASF diagnostic test
- Analyze the current tools for ASF diagnostics
- Describe the mechanisms that lead from the current situation to the desired situation

1. IDEAL ASF DIAGNOSTIC TOOL

The first Working Group (WG) in ASF Countermeasures (2010) thoroughly worked the methodology to carry out the gap analysis and reviewed the principles that should be considered for the ideal diagnostic test. That way, they produced the first list of Criteria and Weights critical for scientifically defining Decision Models, depending on the epidemiological scenario in which it is considered.

The 2013 WG, reviewed and complemented the Criteria and Weights from the previous meeting, from the current perspective of the epidemiological situation of ASF, considering recent scientific advances and responding the following questions:

a. Given the performance characteristics of the assay, is it better suited for:
    - Herd vs. Individual animal test
    - Clinically healthy vs. Clinical signs
    - Detection of classical presentations vs. the possibility of new forms of virus exposure

b. Depending on the reasons for the diagnostic, the test would be better for:
    - Suspicion of disease (i.e.: FAD investigation)
    - Surveillance (i.e.: feral or high risk populations)
    - Movement (i.e.: import/export, quarantine, transport of animals or products),
    - Epidemiological investigation (i.e.: trace-in or trace-out)
    - Disease freedom (i.e.: trade agreements)

c. Considering complexity of the test and its interpretation, should the test be used by:
    - Only National Reference Laboratories
    - Same plus official Regional Surveillance Laboratories
    - Same plus official Basic Local Laboratories
    - Same plus private diagnostic laboratories
    - Same plus officially FAD trained Field State/Federal Veterinarians
    - Same plus all Veterinarians
    - Same and all public (producers and farmers)

d. Finally, what are the critical aspects of the ideal diagnostic tool:
    - Detect all known ASF genotypes and strains
    - Allow clinical, preclinical and subclinical detection of ASF (>95% sensitivity)
    - Accurately identifies ASF (>95% specificity)
    - Serves for control and eradication as well as for post-control monitoring
    - Has been bench and field validated following OIE guidelines
    - Rapid performance
    - Deliver quantifiable results
    - Have pen-side capabilities

Possibility for DIVA Compatible
Requires low to medium skilled operator
Reasonable cost
Scalable (easy to produce in case of high demand)
Adaptable to high throughput requirements

2. CURRENT ASF DIAGNOSTIC TESTS

Currently available diagnostic tests for ASF are mostly based in "in-house" methodologies. This circumstance makes diagnostic more affordable and facilitates issues of accessibility in some ways, but also makes harmonization and standardization more difficult. For that reason, only OIE recommended methods, tests that have been thoroughly validated, and/or methods which are widely used in most reference centers for ASF were taken into consideration. Most of the "in house" tests, which are locally used, were excluded because the lack of enough validation data to support its good performance. Therefore, valuable techniques such as Electron Microscopy, etc…not in use in the diagnostic laboratories will not be included in the following list of ASF diagnostic tests. On the contrary, a number of recently developed though not employed techniques (i.e., MGB probe, rtPCR, Invader assay, Late PCR, etc) ,have been included since they may be the sights of diagnostic laboratories for the near future, if further improvements are performed.

ASF is a complex disease. ASFDiagnosis may be also complex due to many reasons and factors: the epidemiology situation (free, epidemic, endemic), affected breeds, reservoirs, the characteristics of the virus isolate, clinical signs, etc. A wide spectrum of reliable accurate ASF diagnostic tests is available and most of them have been successfully employed in surveillance control and eradication programs. However, as in any other disease, there is not a single test 100%reliable (sensitive and specific). For this reason, final diagnosis should be based in 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. Virus isolation and sequencing should be definitive for ASF diagnosis. In endemic areas where chronic and subclinical-unapparent forms are also present, the diagnosis could be more complicated.

Recently a first version of a commercial pen side test for ASF antibody detection have been validated, under the ASFRISK EU project as a valuable technique for ASF antibody detection in both serum and blood samples. Very probably new pen side tests for antigen and antibody detection will emerge soon in the market to offer new possibilities for ASF surveillance and control programs, improving the wide spectrum of ASF diagnostic techniques.

3.1 <u>VIRUS DETECTION TESTS</u>

2.1.1. VI (Virus Isolation) and HA (Haemadsorption) test

<u>Description and references:</u> Virus isolation is based on the inoculation of sample material (blood or tissue suspension from suspect pigs) into susceptible primary leukocyte cultures of porcine origin, either from blood or lung (alveolar) monocytes and macrophages cells

(Malmquist and Hay, 1960). It is the reference virological test for confirmation of positive virus detection techniques results in primary outbreaks.

ASF virus infection induces expression of a protein in the surface of the primary infected cells that attracts and attaches pig erythrocytes producing “rosettes”, what is known as the haemadsorption effect (HA). This HA is observed just before the cytopathic effect (CPE) occurs and is usually definitive for ASF diagnosis allowing identification with highest sensitivity and specificity than any other technique. However, a small number of non-haemoadsorbing field strains have been isolated, some of them still virulent; these non-HA viruses still produce a noticeable CPE.

After VI/HA, it is recommended confirmation of the presumptive agent using PCR or Direct Immunofluorescence (FAT) test on the sediments of the cell cultures. In case of non-HA strains, confirmation is absolutely necessary.

Advantages: This assay is the gold standard due to its high sensitivity and specificity. It is also well suited for confirmation of the disease presence in new outbreaks. It is used as individual diagnostic.

Pitfalls: Requires the use of pig macrophages which is cumbersome and limited to laboratories with tissue culture capabilities. Takes days, is difficult to scale up and cannot be adapted to high throughput, need technical expertise, is complicated QA/QC and not commercially available as a kit. Very importantly, some field strains do not produce HA, only CPE. In any case, the confirmation of ASF and identification of the agent by another viral detection technique like PCR or FAT is necessary.

2.1.2. DNA detection systems.

3.1.2.1. **PCR (Polymerase Chain Reaction): Conventional, real time PCR and UPL.**

Description and references: Polymerase chain reaction (PCR) is a highly sensitive and specific method that allows detecting the virus by amplification of a specific fragment genome in blood, serum, tissues or organ samples. Tick homogenates may also be analyzed by PCR. Several PCR techniques have been developed, using primers of highly conserved regions of the genome, which allow detection of isolates from a wide range of known genetic lineages including non-HA strains and low virulence strains. Even small fragments of viral DNA are amplified by PCR to detectable quantities, making the technique highly sensitive. For every new ASF PCR assay developed, it is necessary to be sure that an extensive validation has been carried out to ensure not cross-reaction with related pig viruses (CSFV, PRRSV, PCV, Aujeszky disease, and others), and to ensure that all known genotypes of ASF are detected.

Some of the PCR and real time PCR tests have been validated (see OIE, 2012 and also see: Agüero et al., 2003; King et al., 2003; Zsak et al., 2005). (). New recently developed real time PCRs (Tignon et al., 2011; Fernandez-Pinero et al., 2013) have proved to show

the highest sensitivity for the detection of chronically infected animals. There is a good real time PCR commercially available based on one of the validated real time PCRs (Zsak el al., 2005), which includes all reagents dried down, rehydration buffer and controls. The MGB real time PCR of McKillen et al., 2010 shows good specificity and sensitivity, though it is not yet validated in the different epidemiological situations.

Conventional Multiplex ASF-CSF PCR (Agüero et al. 2004) is very 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, but ASF diagnostic sensitivity drops slightly than the conventional single assay. Other conventional and real time multiplex techniques include CSFV-ASFV-PCV type II-PRRS-PPV PCR (Giammarioli et al. 2008); and the new recently developed, multiplex real time PCR, ASF-CSF (Haines et al 2013) that could be useful in regions with the presence of several viruses co-circulating at the same time.

The LATE-PCR from Ronish et al 2011, seems highly sensitive but no validation data is available to date.

3.1.2.2. **Isothermal assays**

Description and references: Other molecular approaches for detection of ASFV genome are the Isothermal molecular assays. They could be a cheaper diagnostic alternative to PCR, and very useful in field conditions. Currently its sensitivity is lower than PCR but seems enough for detection of acute cases. Nevertheless they are still in developmental stage respect to optimizing the cut-off point and lacking field validation data (Hjertner et al., 2005; James et al. 2010).

3.1.2.3. **Genotyping**

The current approach for ASFV genotyping is based on the analysis of three independent regions located at the conserved central area of the ASFV genome comprising; i) partially sequence of the C-terminal end of the gene B646L encoding the major protein p72 (Bastos et al., 2003) which allow us to classify the ASFV in 22 major genotypes (Boshoff et al., 2007), ii) to sequence the full E183L-gene encoding the p54 protein (Gallardo et al., 2009) as a valuable additional genotyping method for molecular epidemiological studies of p72 genotype I viruses, particularly in West Africa where this genotype predominates, and iii) the sequencing of the central variable region within B602L-gene (CVR) characterized by the presence of amino acid tandem repeats (Nix et al., 2006; Gallardo et al., 2011). The CVR remains the genome target of choice when attempting to determine the origin and map the spread of closely related virus.

Determining the sequence of specific fragment of the ASF genome or, in certain cases, the whole genome (de Villier et al, 2010; Chapman et al, 2008;) is very useful to trace the source of the outbreaks and to improve the knowledge of the epidemiology of the disease, though it is not used for molecular diagnosis purposes.

Advantages: PCR systems are highly sensitive and specific, rapid, allowing taking control measures quickly. They are easy to be scaled up and the tests are relatively simple. The systems based on real time PCR can be quantitative, so it is easier for harmonization between laboratories and easy to QA/QC. The majority have shown to be adapted to detect all known genotypes, including non-HA strains and low virulence strains. Since PCR may detect presence of ASF genome even when no infectious virus is present are suitable for problematic diagnostic samples or where the virus is inactivated. This test is recommended for both individual and herd diagnostic.

Pitfalls: The use of primer pairs and probes selected from a highly conserved region of the viral DNA is not guaranty for detection of unknown or new variants with different nucleotide sequence in that regions, hence needs a confirmatory test. The PCR might present problems of false positives due to contamination issues. Might be expensive, and harmonization of results and optimal performance is linked to the use of QA/QC reagents and validated extraction methods.

2.1.3. Antigen Detection Techniques

3.1.3.1. **FAT (Fluorescent Antibody Test)**

Description and references: Direct Immunofluorescence Test (FAT) (Bool et al. 1969) is a common technique used for detection of infectious agents in tissues from suspected animals. It is used for presumptive diagnosis when there are symptoms and lesions associated with ASF or to confirm VI of non-HA strains. This technique has been used since 1968, but is important to consider that its sensitivity drops significantly for detection of Antigen (Ag) from sub-acute and chronic forms of the disease. It is able to detect viral antigens in smears or thin cryosections of organs from suspected or infected animals. it should be used with caution and not employed as a sole virus detection test after the second week post infection, when ASF specific antibodies appears inducing the antigen-antibody complex formation, since it could produces false negative reactions.

ASFV specific antibodies FITC conjugate is required.

Advantages: Fast, economic, validated, good specificity and very sensitive for HA and non-HA strains of ASF in peracute and acute forms of the disease. This test is well suited for herd diagnostics.

Pitfalls: Reading the results might be subjective and needs a well-trained operator. In sub-acute and chronic ASF it shows only a 40% of sensitivity. It is difficult to scale up or to be adapted to high throughput. ASFV specific antibodies FITC conjugate are required, which might or might not be easy to obtain within the expected QA/QC conditions.

2.1.4. Antigen-ELISA (Antigen Enzyme Linked Immuno Sorbent Assay)

Description and references: A number of “in house” direct Antigen ELISA and sandwich ELISA employing monoclonal antibodies produced against ASF (Wardley et al. 1979,

Vidal et al., 1997). Other indirect sandwich ELISA using polyclonal antibodies or a combination of monoclonal antibodies (Hutchings and Ferris, 2006) has been shown to detect antigen of most representative field strains, including phylogenetically distinct groups of ASF virus. The latest could detect viral antigen in crude tissue sample suspensions. The most frequently used is the sole commercially available to date, antigen ELISA kit (Ingezim K2), that has the advantage of the use of serum samples for the analysis.The results are quickly obtained and are highly specific, but sensitivity is limited, therefore only recommended for herd diagnostic in acute and sub-acute forms of the disease, in combination with antibody detection techniques.

Advantages: fast, easy to perform, very specific but not very sensitive, unless for acute forms of ASF. It is easy to scale up and to be adapted to high throughput. Is well suited for herd diagnostics

Pitfalls: Need for the presence of a significant amount of virus in the sample. There is not data about validation studies. It requires confirmation by a second technique. It is recommended to be used as a herd assay in combination with some other virological and serological tests.

2.2. ANTIBODY DETECTION TESTS:

ASF-IgG antibodies persist for long periods of time in infected pigs allowing to be used as a tool for surveillance and detection of ASFV infection, especially in the sub-acute and chronic forms as well as surviving carrier pigs which is essential for ASF control and eradication programs. Due to the absence of vaccines, the presence of ASFV antibodies is an excellent indicator of infection. Antibodies are usually not detected in serum, in pigs infected with virulent strains as they die in the first week, before immune response is produced. However, some animals infected with virulent virus could survive more days, and even become carrier pigs, On the contrary, in endemic situations and in case of low virulent infections serological detection is the best way of detecting infected animals, since some of these ASFV strains produces low and intermittent virus shedding in a limited period of time, and therefore the virus detection by DNA/antigen detection techniques.
Antibody detection is a cost effective tool for surveillance screening and detection of the sub-acute and chronic forms of ASF (Bech-Nielsen et al. 1993; Arias and Sanchez-Vizcaino, 2002)

2.2.1. Antibody-ELISA (Enzyme Linked Immuno Sorbent Assay)

Description and references: Detection of specific antibodies against ASFV by ELISA is the OIE prescribed test for international trade. The most commonly used ELISAs are suitable for examining serum. Currently there is a number of ASF ELISA variants including recombinant ELISAs (Gallardo et al. 2006, 2009; Pérez-Filgueira et al 2006), commercial ELISAs, (see table 1), and a number of (OIE) *"in house"* versions of the test. Confirmatory testing of ELISA positive and doubtful samples should be performed by immunoblotting, imunofluorescence or immunoperoxidase assays.

Advantages: is the most useful method for large-scale serological studies; it is fast, easy to perform, and economic. The procedure of an “in house” OIE ELISA as well as a standardized/validated soluble antigen for OIE ELISA test could be also provided by the EURL previous a request. It is well suited for herd diagnostics

Pitfalls: the Commercial ELISA tests might be expensive and not easily available for all geographic locations. Some recently new commercial tests are not fully validated.

3.2.2 Immunoblot (IB) test

Description and references: IB (western blotting) is a rapid and sensitive assay for the detection of specific antibodies and provides a better recognition of weak positive 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.

Advantages: Highly specific and sensitive showing the specific characteristic pattern of reacting ASFV proteins. This test is recommended as confirmatory test for ELISA suspected positive or inconclusive/doubtful results. The method has been validated throughout field studies performed during control and eradication programs.

Pitfalls: This is an *In house* method, not commercially available, with limited production for large scale survey. European Union RL can provide it, though production is limited. In ASF-endemic areas, where chronically infected animals are present, non-specific characteristic pattern could be visualized in certain cases, with a difficulty the interpretation of the results. Therefore, the lecture of the results could be subjective and in this situation and in case of limit samples an accurate evaluation of the results should be performed taking into consideration alternative diagnostic tests.

TABLE 1: LIST OF CURRENTLY FULLY DEVELOPED ASF DIAGNOSTIC TESTS

DETECTION	AVAILABLE TESTS		TYPE, In house/ Commercial	Recommended Use	REFERENCE
Virus Detection Techniques	Virus Isolation		*VI /Haemadsorption (HAD) test (i.h.)	Confirmation of primary outbreak.	Malmquist and Hay, 1960
	Antigen detection		*Direct Immuno fluorescence (FAT) (i.h.)	Individual testing	Bool et al., 1969
			ELISAIngezim-K2, Double AbSandwich/ Commercial	Surveillance Herd testing	INGENASA
			ELISA (i.h.)	Not in use	Pastor et al.1990; Hutchings and Ferris, 2006;
	PCR	Conventional	Conventional (i.h.)	Surveillance Individual and Herd testing	Aguero et al. 2003.
			Multiplex ASF-CSF (i.h.)	Co-circulation ASF and CSF	Aguero et al. 2004.
		Real Time	Taqman Probe (i.h.)	Surveillance Individual and herd testing	*King et al., 2003; *Zsack et al. 2005; Tignon et al. 2011
			UPL Probe (i.h.)	Surveillance Individual and herd testing	Fernandez-Pinero et al. 2013
			MGB Probe (i.h.)	Not in use	McKillen et al., 2010
			TETRACORE dried down (Commercial)	Individual testing	TETRACORE
			Multiplex ASF-CSF	Surveillance Individual and herd testing	Haines et al.2013
	Isothermal Tests		Invader Assay	Not in use	Hjertner et al., 2005
			LAMP assay	Not in use	James et al., 2010
Antibody Detection Techniques	ELISA Tests		*OIE Indirect ELISA (i.h.)	Surveillance Herd testing	Sánchez-Vizcaíno et al.1982; Pastor et al., 1990.
			Recombinant proteins (rp)-ELISA (i.h.)	Surveillance Herd testing	Gallardo et al. 2006,2009, Pérez-Filgueira et al,, 2006
			ELISA *Ingezim-K3*, Bloking/Commercial,	Surveillance Herd testing	INGENASA
			ELISA *ID-VET* Indirect/Commercial	Surveillance Herd testing	Not available
			ELISA-*Svanova* Indirect/Commercial	Surveillance Herd testing	Not available
	Pen side Tests		Ingezim PPA-CROM Commercial	Surveillance Individual Testing	INGENASA
			Dot Blot (i.h.)	Surveillance Individual Testing	Pastor et al. 1992
	Confirmatory Antibody tests		*Immunoblot (IB) Test (i.h.)	Confirmatory Herd testing	Pastor et al. 1989
			*Immunofluorescence Antibody (IFA) test (i.h.)	Confirmatory Herd testing	Pan et al., 1974
			Indirect Immunoperoxidase test (IPT)	Confirmatory Herd testing	Gallardo et al.2013

(i.h.) means in house preparation of the test; *Included in the OIE Terrestrial Manual for Diagnostic Test and Vaccines, 2012.

3.2.3 IFA (Immuno Fluorescence Antibody Test)

Description and references: It is an immune-cytochemistry technique based on the use of fixed ASF infected cultured cells. Monolayers of VERO or MS cells are infected with adapted ASF isolates; at the very early stage of CPE the infected cells are fixed with formalin and stored at -70 ^{0}C until used .After incubating the cells with the suspected serum, the immune-complexes are detected at the microscope using a species specific anti-serum labeled with fluorescein (Pan et al., 1974).

Advantages: Highly sensitive and specific. This test is recommended as confirmatory test for ELISA results.

Pitfalls: No commercially available, this is only an in-house test which requires preparation of monolayer of cell lines infected with adapted ASF virus. The lecture of the results is subjective in limit samples and an accurate evaluation of the results should be performed taking into consideration alternative diagnostic tests.

3.2.4 IPT (Immunoperoxidase Test)

Description and references: Based in the same principle that is described above for IFA, it works on the detection of ASF antibodies in swine sera through the interaction with the ASF antigens expressed in the surface of infected cells. The technique makes use of VERO or MS cell cultures, infected with strains of ASFV adapted to grow in these cell cultures and fixed. The antibody-antigen complex formation is developed using a peroxidase enzymatic reaction.

Advantages: Highly sensitive and specific. It is recommended as confirmatory test for doubtful and/or positive ELISA results. This technique has been validated by the European Union RL for different scenarios.

Pitfalls: No commercially available, this is an in-house test. The lecture of the results is subjective in limit samples and an accurate evaluation of the results should be performed taking into consideration alternative diagnostic tests. Requires preparation of monolayer of cell lines infected with adapted ASF virus

3.3 VECTOR DETECTION TESTS:

3.3.1. Information of the presence of ASFV in natural reservoirs is important when planning countermeasures and control strategies. A recent study (Boinas et al. 2011) has confirmed the particularly long survival time of infectious ASFV in ticks to be over 5 years after been removed from the infectious hosts. Notice that not all detection methods previously described can be applied for ticks; only a few of them have been optimized to be used.

3.3.2 Most studies of detection of ASFV in ticks have used virus isolation (VI) in porcine macrophage cultures.This method detects the presence of infectious, live virus in

the ticks.). Virus replication is detected by cytopathic effect (CPE) and haemadsorption.

3.3.3 A nested PCR assay (Basto et al., 2006a and 2006b) has been prove sensitive for ASF genome detection in tick samples. This nested PCR includes an internal control of amplification that avoids false negative results related to PCR inhibitors in the tick homogenate. OIE conventional PCR technique (Agüero et al 2003) has proved to be an alternative method with appropriated analytical sensitivity and specificity . Alternatively, a more informative duplex one-step PCR (Bastos et al., 2009) is also available.

3.3.4 Finally, an ELISA for detection of specific antibodies in pigs infested with the tick *Ornitodoros erraticus* can also help in epidemiological studies (Canals et al. 1990)

TABLE 2: EXAMPLE FOR A CONTEXT IN WHICH ASF VALIDATED DIAGNOSTIC TESTS COULD BE USED. This table summarizes a hypothetical general situation of ASFV infection in pigs with acute/sub-acute/subclinical virus isolates, and the total or partial tools that may be used.

			***ASF ENDEMIC**	****ASF FREE**
Virus detection	**Surveillance**	active	PCR (not recommended)	PCR (not recommended)
		passive	PCR	PCR,VI/HA
	Outbreak		PCR, VI/HA	PCR, VI/HA, FAT,ELISA
	Recovery		PCR	PCR, VI/HA,
	Post-recovery		PCR,	PCR,VI/HA,
Antibody detection	**Surveillance**	active	ELISA	ELISA-IB/IFA
		passive	ELISA	ELISA-IB/IFA
	Outbreak		Penside test,ELISA-IB/IFA	Penside test, ELISA-IB/IFA
	Recovery		ELISA ***Penside test	ELISA-IB/ IFA ***Penside test,
	Post-recovery		ELISA-IB/IFA	ELISA-IB/IFA

*Depending on the strategy in use, characteristics of the pig production system, law in place, resources, etc. the use of diagnostic techniques could vary along the control/eradication programs.

** Depending on the epidemiological situation, outbreak confirmation, characteristics of the pig production system, law in place, resources, etc.the use of diagnostic techniques could vary along the control/eradication programs.

***Particularly useful in case of remote zones or with difficult access.

4 BRIDGING THE GAP

Once identified the best and the available tools of diagnostics, it is necessary to apply specific value to each test. The goal is to provide guidance and information about how much “fitted for purpose” is each tool, in the context of the main epidemiological scenarios:

- ASF Free
- ASF Endemic
- ASF Outbreak

This analysis will provide information and support for those working on ASF, and will be the starting point in considering ASF diagnostic needs.

Comparing products that involves such different components and characteristics as are the diagnostic tools will require the design of some factors or coefficients that allow pondering the variables. Hence, we will first define the criteria that compose every diagnostic tool, and assign weights to each of them in the context of every possible epidemiological scenario. That will allow us to define the gaps that keep us apart from the ideal situation in ASF diagnostics.

4.1 CRITERIA

Being nourished from the body of the biology sciences, diagnostics is a live discipline influenced by new technical and scientific discoveries. Thus were recognized the need to periodically review the criteria and challenge its validity. From the previous document we found that most of the criteria were still prevalent, however, few of them required a reformulation of its meaning and some new criteria needed to be added to the list.

For instance, even though is a very advantageous feature, DIVA cannot be a criterion yet, since vaccination is far from being defined. Instead, the low cost criterion was separated as cheap to establish and cheap to run (for example, the cost of ELISA is excellent once the initial invest in equipment has been done). Also, we found very valuable that the test has clear cut-off that homologates interpretation everywhere, or the fact that can be used for a variety of sample types, and so on.

The new completed list of criteria to define the ideal diagnostic tool depending on the scenario is as follows:

- Sensitivity
- Specificity
- Validation to purpose
- Speed of Scale-up
- Throughput
- Pen-Side Test
- Rapid Result
- Definitive results
- Interpretation/cut-off
- Easy to perform
- Low Training needs
- Herd testing
- Versatility for sample
- Cheap to establish
- Cheap to run

4.2 WEIGHT OF THE CRITERIA:

To quantitatively compare the impact of each test it is necessary to change the weight given to each criterion depending on the scenario that is considered. Assigning different numeric values to each criterion in the test, depending on the purpose of use was a rational way to make

comparison between very disparate tests as objective as possible (see Tables 3, 4 and 5 in Annex).

4.3. GAPS:

The following blanks in tools and /or knowledge need to be fulfilled to improve diagnostics:

a. Virus isolation techniques need to find cell lines that replace primary cultures.
b. Field validation data need to expand for all tests
c. Pen side tests need to be developed and validated
d. Need to determine serotypes and pathotypes of current ASF strains
e. There is not enough genetic information to develop new good molecular based technologies and nanotechnologies
f. To improve detection it is necessary wider knowledge of clinical presentations
g. Enhance the use of diagnostic tests through the exploration of new source of samples (oral fluids, meat juice, support for transporting samples, etc…)
h. Development of multiplexed tests for syndromic diagnostics
i. Intensify and potentiate communication and training

5. RECOMMENDATIONS

What assays should we have in place in FADDL?

Recommendations for research:

While some direct and indirect methods of diagnosis, including nucleic acid amplification techniques, are very sensitive and rapid, viral culture is particularly useful. It provides the possibility of identifying other viral pathogens, and supports amplification of the agent for further characterization using not so sensitive techniques. However the use of primary PBMC cultures is restricted to few specialized facilities, is cumbersome and complex. New cell line cultures derived from monocyte/macrophage lineages need to be explored as possible substrates for ASF isolation and growth.

With the ever-increasing number of ASF diagnostic tests implemented worldwide, trusting results is becoming more difficult. Substantiation of absence of disease, certification of permits between countries and control policies based on surveillance data require confidence in the veracity of the data. Only the use of tests that have passed through the whole process of validation under OIE guidelines can be harmonized and trusted. Hence, it is justified to dedicate resources for developing new and easier validation parameters, and for making validation a requirement.

Pen-side tests, point of care or lateral flow devices for rapid detection of both antigen and antibodies and irrefutable support for awareness and response in case of disease suspicion. Therefore, there is no need to explain how important is its research and development from the perception of diagnostics for control and emergency response.

The possibility of monovalent vaccines as tools for control and eradication in defined geographical areas is increasing consideration, and should be kept as an element to take into account for developing differential diagnosis in the future.

Research of immunological/serological response typing and genetic profiles relevant to protection and pathotypes would clarify our understanding of viral strain and cross-protection, but will also propel development of diagnostic methods to help in epidemiology, in clinical prognosis, definition of homologous and heterologous viral strains and the immediate selection of correct control strategies.

Full genome sequences database is an urgent diagnostic necessity for both molecular epidemiology and design of highly efficient diagnostic tools based on detection of genetic material. Currently 22 genotypes have been described based on the sequence of a single gene, p72. Addition of p54 and CVR sequence has been confirmed as valuable, but still the possibility of other genomic regions with enhanced discriminatory value has not been explored. The PCR based genotyping might be a tool in endemic areas like sub-Saharan Africa and to determine the origin in the event of outbreaks in new geographical areas. To complete the sequencing of the viral genome will provide essential information not only about the potential origin of the virus but possible homologies to other strains.

Multiplexed tests for Syndromic diagnostics: because clinical signs of ASF are sometimes the only available immediate diagnostic tool, and because clinical manifestations vary and may be not specific, a multiplexed diagnostic test including as many differential diseases as possible is greatly desirable. Additionally, these kinds of tests will boost regional labs in endemic countries, which very often lack the infrastructure and/or expertise for routine diagnostic services.

Finally, it is imperative to explore more sample types for diagnostics (oral fluids, meat juice, support for transporting samples, etc…). The more we expand the catalogue of sample choices the better, including the list of biological samples to use (which would be of great help in many cases, and especially when there are antibody presence but no detection of the virus, i.e. in the carrier domestic pigs).

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ANNEX

TABLE 2

SURVEILLANCE (USA and free) Commercial and reference Diagnostics for African Swine

Rank each Intervention (2,4,6,8, or 10) as to its importance to making a decision, only one "10" rankings allowed

Weight	Critical Criteria	ELISA K3	ELISA OIE	IB test	IIF test	rtimePCR-King	PCR AGÜERO	VI	DIF	Antigen ELISA K2	Pen side	Histo Pathology	Isothermal	Tetracore	UPL PCR	Tetracore/ARS	ELISA ID-VET	ELISA-Svanova	IPT	HAI	I-neg stain	PCR Multiplex	PCR Tignon
10	Sensitivity	8	6	8	4	8	8	6	4	4	8	6	8	8	8	8	4	8	8	6	4	8	8
8	Specificity	8	8	8	6	8	8	4	4	6	8	6	8	8	8	8	8	4	8	8	8	8	8
10	Validation to purpose	8	8	8	8	8	8	8	8	4	8	4	2	8	8	8	6	6	8	8	6	8	8
6	Speed of Scaleup	6	6	2	4	6	6	4	4	6	6	4	6	6	6	6	6	6	4	4	4	8	8
6	Throughput	8	8	4	4	8	8	2	4	8	6	4	8	8	8	8	8	8	4	2	2	8	8
6	Pen-Side Test	2	2	2	4	2	2	2	4	2	10	2	8	8	2	2	2	2	2	2	2	2	2
4	Rapid Result	6	6	6	6	8	6	2	8	6	10	4	8	8	8	8	6	6	6	2	8	8	8
6	Definitive results	6	6	8	6	8	8	6	6	6	6	8	8	8	8	8	6	6	8	10	6	8	8
8	Interpretation/cut-off	8	8	6	6	8	8	6	6	8	6	6	8	8	8	8	8	8	6	8	6	8	8
6	Easy to perform	6	4	4	6	6	6	4	6	6	10	2	8	8	6	6	6	6	4	4	8	6	6
6	Low Training needs	6	4	4	6	4	4	2	6	4	10	4	6	6	4	4	6	6	4	4	6	4	4
6	Herd testing	8	8	6	6	8	8	4	4	8	4	2	6	8	8	8	8	8	4	2	2	8	8
8	Versatility for sample	4	4	4	4	8	8	8	4	4	6	2	6	8	8	8	4	4	4	8	8	8	8
2	Cheap to establish	6	6	4	6	2	6	2	6	6	10	2	6	2	2	2	6	6	6	2	2	2	2
6	Cheap to run	8	10	4	8	6	6	2	8	6	6	8	8	4	4	4	8	8	8	2	8	6	6

Rank each Criteria 2,4,6,8 or10 on each criterion -- no more than two "10" rankings allowed

								1			2		3				4	4		1	4		
	Critical Criteria	ELISA K3	ELISA OIE	IB test	IIF test	rtimePCR-King	PCR AGÜERO	VI	DIF	Antigen ELISA K2	Pen side	Histo Pathology	Isothermal	Tetracore	UPL PCR	Tetracore/ARS	ELISA ID-VET	ELISA-Svanova	IPT	HAI	EM	PCR Multiplex	PCR Tignon
	Sensitivity	80	60	80	40	80	80	60	40	40	80	60	80	80	80	80	40	80	80	60	40	80	80
	Specificity	64	64	64	48	64	64	32	32	48	64	48	64	64	64	64	64	32	64	64	64	64	64
	Validation to purpose	80	80	80	80	80	80	80	80	40	80	40	20	80	80	80	60	60	80	80	60	80	80
	Speed of Scaleup	36	36	12	24	36	36	24	24	36	36	24	36	36	36	36	36	36	24	24	24	48	48
	Throughput	48	48	24	24	48	48	12	24	48	36	24	48	48	48	48	48	48	24	12	12	48	48
	Pen-Side Test	12	12	12	24	12	12	12	24	12	60	12	48	48	12	12	12	12	12	12	12	12	12
	Rapid Result	24	24	24	24	32	24	8	32	24	40	16	32	32	32	32	24	24	24	8	32	32	32
	Definitive results	36	36	48	36	48	48	36	36	36	36	48	48	48	48	48	36	36	48	60	36	48	48
	Interpretation/cut-off	64	64	48	48	64	64	48	48	64	48	48	64	64	64	64	64	64	48	64	48	64	64
	Easy to perform	36	24	24	36	36	36	24	36	36	60	12	48	48	36	36	36	36	24	24	48	36	36
	Low Training needs	36	24	24	36	24	24	12	36	24	60	24	36	36	24	24	36	36	24	24	36	24	24
	Herd testing	48	48	36	36	48	48	24	24	48	24	12	36	48	48	48	48	48	24	12	12	48	48
	Versatility for sample	32	32	32	32	64	64	64	32	32	48	16	48	64	64	64	32	32	32	64	64	64	64
	Ceap to establish	12	12	8	12	4	12	4	12	12	20	4	12	4	4	4	12	12	12	4	4	4	4
	Cheap to run	48	60	24	48	36	36	12	48	36	36	48	48	24	24	24	48	48	48	12	48	36	36
	Value	656	624	540	548	676	676	452	528	536	728	436	668	724	664	664	596	604	568	524	540	688	688

Major Assumptions in surveillance:

Diagnostic Test Profile

1. Detect all ASFV isolates.
2. Direct and indirect tests.
3. >95% specificity
4. >95% sensitivity
5. Validated
6. Rapid test.
7. Easy to perform
8. Scalable
9. Reasonable cost
10. Pen-side test
11. Expertise

1 VI is the gold standard despite the low rate we think should be considered critical as reference technique

2 Penside is a very promising tool but expensive, thus recommended for field (passive surveillance). Should be accompanied by a penside for Antigen detection

3 Isothermal methods are promising but not yet validated

4 Needs fully validation

TABLE 3

OUTBREAK Commercial and reference Diagnostics for African Swine Fever

Rank each Intervention (2,4,6,8, or 10) as to its importance to making a decision, only one "10" rankings allowed

Weight	Critical Criteria	ELISA K3	ELISA OIE	IB test	IIF test	rtimePCR-King	PCR AGÜERO	VI	DIF	Antigen ELISA K2	Pen side	Histo Pathology	Isothermal	Tetracore	UPL PCR	Tetracore/ARS	ELISA ID-VET	ELISA-Svanova	IPT	HAI	I-neg stain	PCR Multiplex	PCR Tignon
8	Sensitivity	8	6	8	4	8	8	6	4	4	8	6	8	8	8	8	4	8	8	6	4	8	8
10	Specificity	8	8	8	6	8	8	4	4	6	8	6	8	8	8	8	8	4	8	8	8	8	8
10	Validation to purpose	8	8	8	8	8	8	8	8	4	8	4	2	8	8	8	6	6	8	8	6	8	8
6	Speed of Scaleup	6	6	2	4	6	6	4	4	6	6	4	6	6	6	6	6	6	4	4	4	8	8
6	Throughput	8	8	4	4	8	8	2	4	8	6	4	8	8	8	8	8	8	4	2	2	8	8
8	Pen-Side Test	2	2	2	4	2	2	2	4	2	10	2	8	8	2	2	2	2	2	2	2	2	2
8	Rapid Result	6	6	6	6	8	6	2	8	6	10	4	8	8	8	8	6	6	6	2	8	8	8
6	Definitive results	6	6	8	6	8	8	6	6	6	6	8	8	8	8	8	6	6	8	10	6	8	8
8	Interpretation/cut-off	8	8	6	6	8	8	6	6	8	6	6	8	8	8	8	8	8	6	8	6	8	8
8	Easy to perform	6	4	4	6	6	6	4	6	6	10	2	8	8	6	6	6	6	4	4	8	6	6
8	Low Training needs	6	4	4	6	4	4	2	6	4	10	4	6	6	4	4	6	6	4	4	6	4	4
8	Herd testing	8	8	6	6	8	8	4	4	8	4	2	6	8	8	8	8	8	4	2	2	8	8
6	Versatility for sample	4	4	4	4	8	8	8	4	4	6	2	6	8	8	8	4	4	4	8	8	8	8
2	Cheap to establish	6	6	4	6	2	6	2	6	6	10	2	6	2	2	2	6	6	6	2	2	2	2
6	Cheap to run	8	10	4	8	6	6	2	8	6	6	8	8	4	4	4	8	8	8	2	8	6	6

Rank each Criteria 2,4,6,8 or10 on each criterion -- no more than two "10" rankings allowed

								1			2		3				4	4		1	4		
	Critical Criteria	ELISA K3	ELISA OIE	IB test	IIF test	rtimePCR-King	PCR AGÜERO	VI	DIF	Antigen ELISA K2	Pen side	Histo Pathology	Isothermal	Tetracore	UPL PCR	Tetracore/ARS	ELISA ID-VET	ELISA-Svanova	IPT	HAI	EM	PCR Multiplex	PCR Tignon
	Sensitivity	64	48	64	32	64	64	48	32	32	64	48	64	64	64	64	32	64	64	48	32	64	64
	Specificity	80	80	80	60	80	80	40	40	60	80	60	80	80	80	80	80	40	80	80	80	80	80
	Validation to purpose	80	80	80	80	80	80	80	80	40	80	40	20	80	80	80	60	60	80	80	60	80	80
	Speed of Scaleup	36	36	12	24	36	36	24	24	36	36	24	36	36	36	36	36	36	24	24	24	48	48
	Throughput	48	48	24	24	48	48	12	24	48	36	24	48	48	48	48	48	48	24	12	12	48	48
	Pen-Side Test	16	16	16	32	16	16	16	32	16	80	16	64	64	16	16	16	16	16	16	16	16	16
	Rapid Result	48	48	48	48	64	48	16	64	48	80	32	64	64	64	64	48	48	48	16	64	64	64
	Definitive results	36	36	48	36	48	48	36	36	36	36	48	48	48	48	48	36	36	48	60	36	48	48
	Interpretation/cut-off	48	32	32	48	48	48	32	48	48	80	16	64	64	48	48	48	48	32	32	64	48	48
	Easy to perform	48	32	32	48	32	32	16	48	32	80	32	48	48	32	32	48	48	32	32	48	32	32
	Low Training needs	48	32	32	48	32	32	16	48	32	80	32	48	48	32	32	48	48	32	32	48	32	32
	Herd testing	64	64	48	48	64	64	32	32	64	32	16	48	64	64	64	64	64	32	16	16	64	64
	Versatility for sample	24	24	24	24	48	48	48	24	24	36	12	36	48	48	48	24	24	24	48	48	48	48
	Cheap to establish	12	12	8	12	4	12	4	12	12	20	4	12	4	4	4	12	12	12	4	4	4	4
	Cheap to run	48	60	24	48	36	36	12	48	36	36	48	48	24	24	24	48	48	48	12	48	36	36
	Value	700	648	572	612	700	692	432	592	564	856	452	728	784	688	688	648	640	596	512	600	712	712

Major Assumptions in surveillance:

Diagnostic Test Profile

1. Detect all ASFV isolates.
2. Direct and indirect tests.
3. >95% specificity
4. >95% sensitivity
5. Validated
6. Rapid test.
7. Easy to perform
8. Scalable
9. Reasonable cost
10. Pen-side test
11. Expertise

1 VI is the gold standard despite the low rate we think should be considered critical as reference technique

2 Penside is a very promising tool but expensive, thus recommended for field (passive surveillance). Should be accompanied by a penside for Antigen detection

3 Isothermal methods are promising but not yet validated

4 Needs fully validation

TABLE 4

SURVEILLANCE (Endemic country) Commercial and reference Diagnostics for African Sv

Rank each Intervention (2,4,6,8, or 10) as to its importance to making a decision, only one "10" rankings allowed

Weight	Critical Criteria	ELISA K3	ELISA OIE	IB test	IIF test	rtimePCR-King	PCR AGÜERO	VI	DIF	Antigen ELISA K2	Pen side	Histo Pathology	Isothermal	Tetracore	UPL PCR	Tetracore/ARS	ELISA ID-VET	ELISA-Svanova	IPT	HAI	I-neg stain	PCR Multiplex	PCR Tignon
8	Sensitivity	8	6	8	4	8	8	6	4	4	8	6	8	8	8	8	4	8	8	6	4	8	8
10	Specificity	8	8	8	6	8	8	4	4	6	8	6	8	8	8	8	8	4	8	8	8	8	8
10	Validation to purpose	8	8	8	8	8	8	8	8	4	8	4	2	8	8	8	6	6	8	8	6	8	8
6	Speed of Scaleup	6	6	2	4	6	6	4	4	6	6	4	6	6	6	6	6	6	4	4	4	8	8
6	Throughput	8	8	4	4	8	8	2	4	8	6	4	8	8	8	8	8	8	4	2	2	8	8
4	Pen-Side Test	2	2	2	4	2	2	2	4	2	10	2	8	8	2	2	2	2	2	2	2	2	2
4	Rapid Result	6	6	6	6	8	6	2	8	6	10	4	8	8	8	8	6	6	6	2	8	8	8
6	Definitive results	6	6	8	6	8	8	6	6	6	6	8	8	8	8	8	6	6	8	10	6	8	8
8	Interpretation/cut-off	8	8	6	6	8	8	6	6	8	6	6	8	8	8	8	8	8	6	8	6	8	8
6	Easy to perform	6	4	4	6	6	6	4	6	6	10	2	8	8	6	6	6	6	4	4	8	6	6
8	Low Training needs	6	4	4	6	4	4	2	6	4	10	4	6	6	4	4	6	6	4	4	6	4	4
8	Herd testing	8	8	6	6	8	8	4	4	8	4	2	6	8	8	8	8	8	4	2	2	8	8
4	Versatility for sample	4	4	4	4	8	8	8	4	4	6	2	6	8	8	8	4	4	4	8	8	8	8
8	Cheap to establish	6	6	4	6	2	6	2	6	6	10	2	6	2	2	2	6	6	6	2	2	2	2
8	Cheap to run	8	10	4	8	6	6	2	8	6	6	8	8	4	4	4	8	8	8	2	8	6	6

Rank each Criteria 2,4,6,8 or10 on each criterion -- no more than two "10" rankings allowed

								1			2		3				4	4		1	4		
	Critical Criteria	ELISA K3	ELISA OIE	IB test	IIF test	rtimePCR-King	PCR AGÜERO	VI	DIF	Antigen ELISA K2	Pen side	Histo Pathology	Isothermal	Tetracore	UPL PCR	Tetracore/ARS	ELISA ID-VET	ELISA-Svanova	IPT	HAI	EM	PCR Multiplex	PCR Tignon
	Sensitivity	64	48	64	32	64	64	48	32	32	64	48	64	64	64	64	32	64	64	48	32	64	64
	Specificity	80	80	80	60	80	80	40	40	60	80	60	80	80	80	80	80	40	80	80	80	80	80
	Validation to purpose	80	80	80	80	80	80	80	80	40	80	40	20	80	80	80	60	60	80	80	60	80	80
	Speed of Scaleup	36	36	12	24	36	36	24	24	36	36	24	36	36	36	36	36	36	24	24	24	48	48
	Throughput	48	48	24	24	48	48	12	24	48	36	24	48	48	48	48	48	48	24	12	12	48	48
	Pen-Side Test	8	8	8	16	8	8	8	16	8	40	8	32	32	8	8	8	8	8	8	8	8	8
	Rapid Result	24	24	24	24	32	24	8	32	24	40	16	32	32	32	32	24	24	24	8	32	32	32
	Definitive results	36	36	48	36	48	48	36	36	36	36	48	48	48	48	48	36	36	48	60	36	48	48
	Interpretation/cut-off	64	64	48	48	64	64	48	48	64	48	48	64	64	64	64	64	64	48	64	48	64	64
	Easy to perform	36	24	24	36	36	36	24	36	36	60	12	48	48	36	36	36	36	24	24	48	36	36
	Low Training needs	48	32	32	48	32	32	16	48	32	80	32	48	48	32	32	48	48	32	32	48	32	32
	Herd testing	64	64	48	48	64	64	32	32	64	32	16	48	64	64	64	64	64	32	16	16	64	64
	Versatility for sample	16	16	16	16	32	32	32	16	16	24	8	24	32	32	32	16	16	16	32	32	32	32
	Ceap to establish	48	48	32	48	16	48	16	48	48	80	16	48	16	16	16	48	48	48	16	16	16	16
	Cheap to run	64	80	32	64	48	48	16	64	48	48	64	64	32	32	32	64	64	64	16	64	48	48
	Value	716	688	572	604	688	712	440	576	592	784	464	704	724	672	672	664	656	616	520	556	700	700

Major Assumptions in surveillance:

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