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Review

Virological diagnosis of African swine fever—Comparative study of available tests

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ABSTRACT

The rapid and reliable detection of African swine fever virus (ASFV) is essential both for timely implementation of control measures to prevent the spread of disease, and to differentiate African swine fever (ASF) from other pig disease with similar clinical presentations. Many virological tests are currently available for the detection of ASFV (live virus), antigen and genome, including virus isolation, ELISA, fluorescent antibody, polymerase chain reaction (PCR) and isothermal assays. In recent years real-time PCR (rPCR) has become one of the most widely used formats for virological diagnosis providing sensitive, specific and swift detection and quantification of ASFV DNA. The ability to integrate rPCR into automated platforms increases sample throughput and decreases the potential for cross-contamination. In more recent years isothermal assays, which are a lower-cost alternative to PCR more suitable for use in non-specialised or mobile laboratories, have been developed for the detection of ASFV, however these assays have not been fully validated for routine use in the field. The performance of all virological detection assays in ASF diagnostics, as well as prospects for improving diagnostic strategies in the future, are discussed and reviewed in this chapter.

Contents

1. Introduction 00
2. Diagnostic samples for ASFV detection 00
3. Virological diagnosis of ASFV 00
   3.1. Virus isolation 00
   3.2. Antigen-based assays 00
      3.2.1. The fluorescent antibody test (FAT) 00
      3.2.2. Antigen ELISA 00
   3.3. Molecular assays for ASFV detection 00
      3.3.1. PCR (conventional) 00
      3.3.2. PCR (real-time) 00
      3.3.3. PCR assays (multiplex) 00
      3.3.4. Isothermal amplification assays 00
4. Comparison of rPCR, LAMP and antigen ELISA for detection of ASFV in experimental and field tissues 00
5. Detection of ASFV in *Ornithodoros* ticks 00
6. Concluding remarks and prospects for the future 00
   Acknowledgements 00
   References 00

1. Introduction

Diagnosis of African swine fever (ASF) is the identification of animals that are or have previously been infected with African swine fever virus (ASFV). A positive diagnosis involves the detection and identification of ASFV-specific antigens, antibodies or DNA in

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diagnostic samples taken from pigs using virus isolation, serological or molecular assays. Laboratory diagnostic procedures for ASF fall into two groups; virological and serological. The virological tests, including virus isolation and the detection of viral antigens and genomic DNA, will be reviewed in this chapter, while the serological diagnosis of ASF will be reviewed in the following chapter (this issue).

Recognition of the clinical signs of ASF is usually the first sign that the virus is circulating within a pig population, as ASFV usually spreads rapidly becoming quickly established within a susceptible population. Early diagnosis resulting in the rapid implementation of control measures is therefore vital. The diagnosis of ASF is complicated by the similarity of a range of other infections, particularly classical swine fever (CSF), porcine dermatitis and nephropathy syndrome (PDNS), and Porcine Reproductive & Respiratory Syndrome (PRRS). ASF viruses produce a wide range of syndromes varying from per acute, acute to chronic disease and apparently healthy virus carriers. Acute disease, which is most commonly seen in outbreaks, is characterised by a short incubation period (3–5 days) followed by high fever and death in 5–10 days. The clinical features of acute ASFV infection are high fever, haemorrhage and generalised reddening of the skin (Fig. 1). These clinical features, as well as additional post-mortem findings such as congestion and enlargement of the spleen and generalised haemorrhage of the lymph nodes, liver and kidney (Fig. 1) are indistinguishable from those seen in CSF infection, making it extremely important to be able to differentiate these two viral diseases of pigs through laboratory tests. The severity and distribution of lesions varies according to the virulence of the virus with some 'moderately' virulent strains resulting in lower levels of mortality (40–60%). Chronic disease, which is rarely seen in outbreaks, is characterised by emaciation, swollen joints and respiratory problems (Oura, 2010).

The rapid, reliable sensitive and specific detection of ASFV is therefore essential, not only for the implementation of control measures to prevent the spread of ASF, but also in the differential diagnosis of other pig diseases with similar clinical presentations. The World Organisation for Animal Health (OIE) recommended tests for virus detection, including virus isolation and both real-time and conventional PCR assays, are described in its Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2012, Chapter 2.8.1 (http://www.oie.int/).

Prior to confirming a primary outbreak of ASF, when clinical signs or lesions of disease have been detected in pigs, it is recommended that at least two distinct antigen, genome or antibody detection tests have given a positive result on samples taken from the same suspected pig. It is also recommended to send positive samples to an OIE reference lab in order to carry out virus isolation and haemadsorption (HAD), to both confirm the positive nature of the samples and to enable further identification of the ASFV genotype through sequence analysis.

2. Diagnostic samples for ASFV detection

ASFV replicates primarily in cells of the reticuloendothelial system (Anderson, 1986) and consequently, where ASF is suspected, the following samples should be sent to the laboratory: blood in anticoagulant (EDTA), spleen, lymph nodes, liver and tonsil (Wilkinson, 1989). These samples should be kept as cold as possible, without freezing, during transit. After the samples arrive at the laboratory, they should be stored at −80 °C if processing is going to be delayed. As maintaining a cold chain is not always possible, samples can be submitted in glycerosaline, however this may decrease the likelihood of virus isolation. The diagnosis of ASF in tropical environments is often hampered by the lack of suitable clinical material and the necessity to maintain a cold chain for sample preservation up to the laboratory. A recent study has showed that filter papers can be used for sample collection. This study showed that molecular detection and genotyping of ASFV can be carried out on samples collected on filter paper, even after the samples had been stored for long periods of time at elevated temperatures (Michaud et al., 2007).

3. Virological diagnosis of ASFV

Ideally the virological diagnosis of ASF should be carried out through a combination of tests including the detection of viral genome by PCR, the detection of viral antigen by antigen ELISA or a fluorescent antibody test (FAT) and the detection of virus through virus isolation. This is not however possible in many countries in which ASFV is currently circulating, due to a lack of molecular diagnostic tools and the difficulty in carrying out virus isolation. Cheaper assays are available for the detection of ASF antigen, such as the antigen ELISA and FAT, however these assays have a reduced

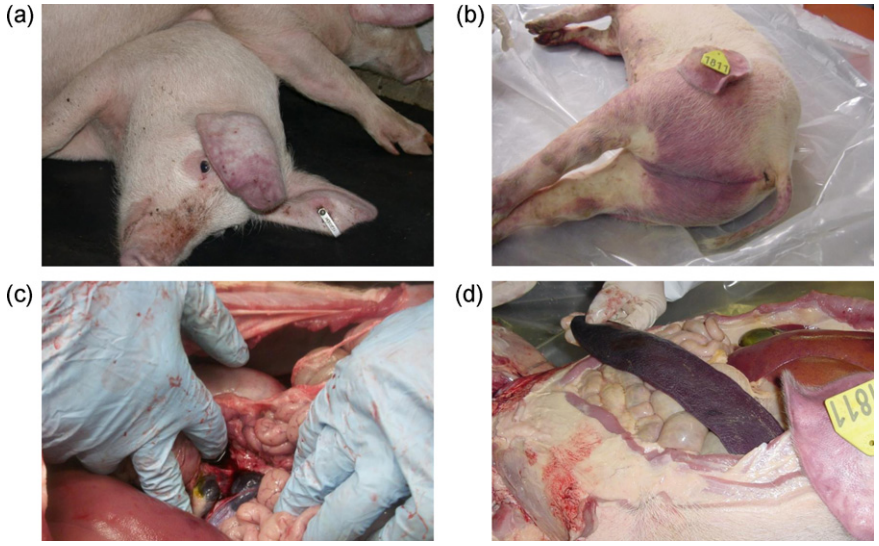

Fig. 1. Typical clinical and pathological signs of ASF infection: (a) reddening of the ears starting at the tips followed by (b) further generalised haemorrhages on the body. Enlargement and haemorrhage of the (c) gastrohepatic lymph node and (d) spleen.

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sensitivity compared to PCR. In many cases where suspected outbreaks of ASF are occurring in endemic countries, ASF is diagnosed on the clinical picture alone and laboratory confirmation in not carried out. In some cases samples from affected pigs are sent to OIE regional reference labs for confirmation through laboratory testing, which is usually carried out through a combination of PCR and virus isolation techniques. In countries free from ASF but suspecting its presence, laboratory diagnosis must be carried out initially through an antigen detection test (ELISA or FAT) or through PCR testing, but must also be directed towards the isolation of the virus by carrying out the inoculation of pig leukocytes or bone marrow cultures. In this way the virus can be further characterised through sequence analysis and molecular epidemiological studies can be carried out in order to understand more about the origin and genotype of the virus.

A variety of laboratory tools can be used for the detection of ASFV. The established haemadsorption virus isolation (VI) method (Malmquist and Hay, 1960) can be sensitive, but it takes several days to obtain a result, and is reliant upon the regular sourcing of fresh pig tissues for the preparation of primary cells. Furthermore, the emergence of virulent non-haemadsorbing, non-cytopathic strains of ASFV (Gonzague et al., 2001) raises the potential of this test to generate false-negative results. In addition to antigen-detection immunoassays, a variety of molecular tests including agarose gel-based (conventional) and real-time PCR (rPCR) assays have also been developed and adopted for routine diagnostic use in reference laboratories around the world.

3.1. Virus isolation

ASFV can be isolated from blood and other tissue samples including spleen, liver, lymph node and tonsil. The haemadsorption (HAD) test (Malmquist & Hay, 1960) is based on the fact that pig erythrocytes will adhere to the surface of pig monocyte or macrophage cells infected with ASFV, and that most virus isolates produce this phenomenon of haemadsorption. A positive result in the HAD test is definitive for ASF diagnosis. A small number of 'non-haemadsorbing' viruses have been isolated, most of which are low virulent, but some do produce typical acute ASF. The test is carried out by inoculating blood or tissue suspensions from suspect pigs into primary porcine bone marrow (PBM) cell cultures, primary leucocyte cultures or into alveolar macrophages cell cultures, and also by preparing leucocyte cultures from the blood of suspect cases. The procedures used are described in the OIE manual (OIE, 2012). A major disadvantage of the HAD test is that it requires primary cell cultures to perform and it takes 6 days to declare a negative result. However, if samples are strongly positive, haemadsorption is usually seen in the cultures within the first 24 h. Virus isolation and identification by HAD are recommended as a reference test for the confirmation of positive results of a prior ELISA, PCR or FAT. They are also recommended when ASF has already been confirmed by other methods, particularly in the case of a primary outbreak or case of ASF.

3.2. Antigen-based assays

3.2.1. The fluorescent antibody test (FAT)

The FAT can be used to detect ASFV antigen in tissues of suspect pigs (Bool et al., 1969). The principle of the test is the microscopic detection of viral antigens on impression smears or thin cryosections of organ material from pigs suspected of being infected with ASFV. Intracellular antigens are detected using FIT-conjugated specific antibodies. Fluorescent inclusion bodies or granules appear in the cytoplasm of infected cells. FAT can also be used to detect ASFV antigen in leucocyte cultures in which no HAD is observed, and can thus identify non haemadsorbing strains of virus. It also distinguishes between the cytopathic effect (CPE) produced by ASFV and that produced by other viruses, such as Aujeszky's disease virus or a cytotoxic inoculum. However, although the FAT is a highly sensitive test in cases of acute ASF, it is important to note that in subacute and chronic disease, FAT has a significantly decreased sensitivity. This may be related to the formation of antigen–antibody complexes in the tissues of infected pigs which block the interaction between the ASFV antigen and ASF conjugate.

3.2.2. Antigen ELISA

Viral antigens can also be detected using ELISA, but it is only recommended for acute forms of disease. The sensitivity of the ASF antigen ELISA is not thought to be as high as PCR (Steiger et al., 1992). The first direct ELISA for the detection of ASFV could detect antigen concentrations of 50–500 HAD_{50}/ml (Wardley et al., 1979). A sandwich ELISA, based on monoclonal antibodies raised against VP72, was later shown to be highly sensitive for the detection of homologous antigen, but results were not presented using field samples (Vidal et al., 1997). Two indirect sandwich ELISAs have been described; the first assay using polyclonal serum and the second using a combination of monoclonal and polyclonal serum. Both assays detected antigen from a diverse range of field isolates, however the assay using the polyclonal antisera was found to be slightly more sensitive than the assay using the monoclonal antibody (Hutchings and Ferris, 2006). A commercially produced antigen ELISA kit is currently available (ELISA INGEZIM K3, Ingenasa, Spain), although very little data on the sensitivity and specificity of this assay has been published. This antigen ELISA assay has the advantage in that it is cheaper to set up than PCR and can be used in labs across the world which have ELISA technology in place, but cannot afford to purchase the high priced thermocycling machines and related equipment needed to carry out PCR. Due to the severe nature of the disease, with the majority of cases resulting in mortality with high levels of virus present in all tissues, it is highly likely that virus would be detected through the antigen ELISA in a high proportion of samples taken from dead pigs during an acute outbreak of disease. It is however important to note, as in the case of the FAT, that in subacute and chronic disease, the antigen ELISA has a significantly decreased sensitivity. This is likely to be due to antigen–antibody complexes in the tissues of infected pigs blocking the interaction between the ASFV antigen and ASF conjugate. It is therefore recommended to use the antigen ELISA only as a 'herd' tests and in conjunction with other virological tests.

3.3. Molecular assays for ASFV detection

The ASFV genome is made up of double-stranded DNA of between 170 and 190 kb and contains at least 150 genes (Dixon et al., 2000). The numbers of genes differ slightly between different isolates of the virus and there are currently thought to be at least 22 virus genotypes (Boshoff et al., 2007). When designing primers for molecular assays it is important to select areas of the ASFV genome that are sufficiently conserved to enable the detection of all the ASFV genotypes, but also sufficiently divergent from those of the members of other closely related viral species, so there are no cross-reactions. It is therefore necessary to carry out extensive validation of all newly developed PCR assays in order to ensure that they do not cross react with related pig viruses (for example CSFV, PRRSV and porcine circoviruses), and to ensure that they detect all the known genotypes of ASFV that are currently circulating. Currently rPCR is considered to be the 'gold standard' test for the detection of ASFV genome and this assay format is used in all the OIE regional reference labs. Isothermal molecular assays, which offer a cheaper alternative to PCR, are also being developed and have the potential to be used in the future in less developed laboratories around

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the world, however these assays have not been fully validated for routine use in the field.

The PCR is applied to detect the ASFV genome in blood, serum or organ samples. Small fragments of viral DNA are amplified by PCR to detectable quantities. Since the PCR detects viral genome it may be positive even when no infectious virus is detected by virus isolation. The PCR provides a sensitive, specific and rapid alternative to virus isolation for the detection of ASFV, providing a much higher sensitivity and specificity than alternative methods for antigen detection, such as antigen ELISA and FAT assays (Steiger et al., 1992). PCR enables the diagnosis of ASF to be made within hours of sample receipt so that control measures and restrictions can be implemented or lifted in a much shorter time scale than if virus isolation was employed as the sole diagnostic method. A wide range of isolates belonging to all the 22 known virus genotypes (Boshoff et al., 2007), including both non-haemadsorbing viruses and isolates of low virulence, can be detected with PCR assays, even in inactivated or degraded samples. However the extreme sensitivity of PCR assays can also make them prone to cross-contamination, potentially resulting in false positive results. False negative PCR results, due to the presence of inhibitors or damaged nucleic acids, also cannot be excluded, particularly when working with degraded samples (Belák and Thorén, 2001). The detection of genomic DNA by PCR is the most sensitive technique for detecting the presence of the agent in persistently infected animals and is particularly useful if samples submitted are unsuitable for virus isolation and antigen detection because they have undergone degradation. ASFV can be detected by PCR from a very early stage of infection in tissues, EDTA blood and, at a lower level, in serum samples. Pigs recovered from acute or chronic infections usually exhibit a viraemia for several weeks making the PCR test a very useful tool for the detection of ASFV DNA in pigs infected with low or moderately virulent strains.

3.3.1. PCR (conventional)

Various conventional PCR assays for ASFV detection have been described (Agüero et al., 2003; Steiger et al., 1992; Wilkinson, 2000; Bastos et al., 2003). These have now mostly been superseded by rPCR assays, although they are useful in less developed labs that do not have rPCR equipment.

3.3.2. PCR (real-time)

The rPCR approach, which detect amplification of target sequences by fluorescence signals from target-specific 'oligonucleotide probes', has several advantages over gel-based conventional PCR methods. These include increased speed, sensitivity, reduced chances of cross contamination (as it is a closed system), and provision of a quantitative result. Real-time PCRs can use a 96-well format, making them well suited to high throughput diagnostic systems, and potentially for process automation. Portable rPCR machines are now also becoming available, making it possible to use these molecular technologies in the "field", with prospects for radical changes in future diagnostic approaches. Several rPCR assays have been described for the detection of ASFV (King et al., 2003; McKillen et al., 2007, 2010; Zsak et al., 2005; Tignon et al., 2011; Fernández-Pinero et al., 2012). The main characteristics of these assays are summarised in Table 1.

The first reported real-time (quantitative) TaqMan PCR assay for the detection of ASFV was developed by King et al. (2003). This assay is currently described in the OIE manual (OIE, 2012). The assay targets VP72 and includes an artificial mimic demonstrating the absence of inhibitory substances to PCR, thereby validating negative results. The assay was found to be sensitive and specific with an analytical sensitivity of between 10 and 100 molecules and was validated against 25 diverse ASFV isolates and 16 African and European tick isolates. The assay did not cross react with related pig viruses. Zsak et al. (2005) described an alternative real-time TaqMan PCR assay, also based on VP72, which was performed in a single tube containing dried down PCR reagents. Test results were obtained by using a portable detection instrument in real-time, thus simplifying all pre- and post-PCR operating procedures. The assay was found to be both sensitive and specific, with a higher analytical sensitivity of 1.4–8.4 copies compared to the King et al. assay, and was validated

Table 1
Real-time PCR, Linear-After-The-Exponential-PCR (LATE-PCR) and isothermal assays for the detection of African swine fever virus.

Reference	Detection method	Target	Internal control	Analytical Sensitivity (no. of copies)	Validation (no. of diverse ASFV isolates)	Validation (no. of experimental and field samples)
King et al. (2003)	Real-time PCR (TaqMan)	VP72	Artificial template (mimic)	10–100	41	None
Zsak et al. (2005)	Real-time PCR (TaqMan)	VP72	None	1.4 to 8.4	48	Samples from 6 experimentally infected pigs
McKillen et al. (2010)	MGB probe PCR	9GL gene	None	20	15	Samples from 6 experimentally infected pigs
Tignon et al. (2011)	Real-time PCR (TaqMan)	VP72	β-Actin	5.7–57	44	170 field samples 111 experimental samples
Fernández-Pinero et al. (2012)	UPL probe PCR	VP72	β-Actin	18	46	260 field samples
Ronish et al. (2011)	LATE-PCR assay	VP72	None	1–10	19	Tissue samples from experimentally infected pigs
Hjertner et al. (2005)	InvaderR isothermal assay	VP72	No control	2500	1	None
James et al. (2010)	LAMP isothermal assay	Topoisomerase II gene	No control	330	38	Samples from 7 experimentally infected pigs

MGB, minor groove binder; UPL, Universal Probe Library; LATE, Linear-After-The-Exponential; LAMP, loop-mediated isothermal amplification.

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against a larger panel of 48 diverse ASFV field isolates as well as against samples from experimentally infected pigs and related pig viruses.

A third rPCR assay for the detection of ASFV was reported by McKillen et al. (2010). They utilised minor groove binder (MGB) probes (Afonina et al., 2002; Belousov et al., 2004), which are short oligonucleotides with a minor groove binder molecule and a quencher conjugated to the 5′ end, and a fluorophore to the 3′ end. This assay was designed against the 9GL gene of the ASFV genome, and was found to be sensitive and specific with an analytical sensitivity of 20 copies. The assay was validated against 15 diverse ASFV isolates, samples from 6 experimentally infected pigs and related pig viruses.

Tignon et al. (2011) reported the development and inter-laboratory validation of a 4th rPCR assay for ASFV detection, based on the VP72 gene. This assay included a β-actin endogenous control and presented an improved analytical and diagnostic sensitivity compared to the OIE-prescribed assays (Agüero et al., 2004; King et al., 2003), with an analytical sensitivity of 5.7–57 copies. The assay was validated against a large panel of samples (44 diverse isolates, 170 field samples, 111 experimental samples and related pig viruses) as well as through an inter-laboratory proficiency test involving four national reference laboratories within the European Union. Recently Fernández-Pinero et al. (2012) reported on the development of a rPCR assay using a commercial Universal Probe Library (UPL) probe combined with a specifically designed primer set to amplify an ASFV DNA fragment within the VP72 coding region. UPL comprises a collection of 165 pre-synthesized fluorogenic hydrolysis locked nucleic acid probes labelled with FAM dye at the 5′ end and a dark quencher dye at the 3′ end. This assay had a high analytical sensitivity (18 copies), included a β-actin endogenous control and was validated against a large panel of 46 diverse ASFV isolates and 260 field samples. The UPL, comprising a collection of 165 pre-synthesised fluorogenic hydrolysis locked nucleic acid probes, is available commercially (Roche Diagnostics).

A diagnostic assay based on the Linear-After-The-Exponential-PCR (LATE-PCR) principle was developed to detect ASFV (Ronish et al., 2011). LATE-PCR is an advanced form of asymmetric PCR which results in direct amplification of large amount of single-stranded DNA. Fluorescent readings are acquired using endpoint analysis after PCR amplification and amplification of the correct product is verified by melting curve analysis. The assay was designed to amplify the VP72 gene of the ASFV genome and was validated using 19 ASFV DNA cell culture virus strains and three tissue samples (spleen, tonsil, and liver) from experimentally infected pigs. The assay had a high analytical sensitivity (1 and 10 copies) and was designed to be used in either a laboratory settings or in a portable PCR machine (Bio-Seeq Portable Veterinary Diagnostics Laboratory; Smiths Detection, Watford, UK), thus providing a tool for the diagnosis of ASF both in the laboratory and in the field.

Currently only one commercially available rPCR kit is being produced for the detection of ASFV genome (Tetracore®, USA), however other commercial companies are in the process of developing rPCR assays for sale as commercial kits.

3.3.3. PCR assays (multiplex)

Surprisingly, up to the present time, multiplex rPCR assays have not been developed for the simultaneous detection of ASFV and CSFV. A conventional multiplex PCR assay has however been developed for the simultaneous detection of both viruses (Agüero et al., 2004). This method uses two primer sets, each one specific for the corresponding virus, amplifying DNA fragments of differing length, allowing a gel-based differential detection of the PCR products. The sensitivity of the multiplex test was 10-fold lower than the corresponding uniplex PCR (Agüero et al., 2003). A second hot-start multiplex PCR for simultaneous detection of CSFV, ASFV, porcine circovirus type 2, porcine reproductive and respiratory syndrome virus (PRRSV) and porcine parvovirus has also been developed (Giammarioli et al., 2008). The analytical sensitivity of this assay was not reported, however the sensitivity of this multiplex assay was found to be 2 logs lower than the conventional uniplex PCR assay developed by Agüero et al. (2003) and one log lower than the multiplex CSFV/ASFV PCR (Agüero et al., 2004).

3.3.4. Isothermal amplification assays

Although PCR is a highly sensitive method for the detection of ASFV, it relies upon precision specialist thermocycling requiring instrumentation which is expensive. Isothermal assays provide an alternative to PCR-based detection assays for the detection of ASFV and may be more suitable for use in non-specialised or mobile laboratories. Isothermal technologies should prove to be a valuable tool in the laboratory diagnosis of ASF and will complement existing molecular methods to provide rapid differential diagnosis in cases of suspected swine fever. In contrast to other molecular formats, such as PCR, isothermal assays can be undertaken at a single temperature using an inexpensive water bath, without the need for expensive thermocycling equipment. Two isothermal methods have been designed and evaluated for use in the diagnosis of ASF. The main characteristics of these assays are summarised and compared to PCR assays in Table 1.

The first isothermal assay to be developed was an ASFV specific InvaderR assay (Hjertner et al., 2005). The Invader® assay is a linear, isothermal (63 °C) signal amplification system able to accurately quantify DNA and RNA targets with high sensitivity and specificity (Lyamichev et al., 1998; Kwiatkowski et al., 1999). The assay is based on the association of target specific Invader oligonucleotide and Signal probe with the target of interest. Compared to PCR-based diagnosis, the main advantages of an Invader® assay are ease of use, availability of dried down formats and the possibility to read the assay with simple and inexpensive equipment such as ordinary fluorescent plate readers. The Invader® assay showed a sensitivity of around 2500 copies and exhibited no cross reactivity with CSFV RNA. The analytical sensitivity of the assay was considerably lower than other molecular assays (Table 1), although it is likely to be sensitive enough to detect ASFV DNA in samples from pigs which had died from ASFV, due to the high amounts of virus present in the samples.

A second isothermal assay, based on loop-mediated isothermal amplification (LAMP) originally described by Notomi et al. (2000), was developed for ASFV detection (James et al., 2010). After optimisation, the performance of this assay was compared with other laboratory tests used for ASF diagnosis using a panel of 38 diverse ASFV isolates and samples from 7 experimentally infected pigs. The analytical sensitivity of 330 copies was a little lower than other PCR-based assays, however the assay was likely to be sensitive enough to detect the large amounts of virus present in tissues from pigs that had died from ASF. The LAMP assay appeared to be able to detect a lower concentration of virus and was more rapid than the Invader® assay described by Hjertner et al. (2005). Furthermore, this study also demonstrated that the LAMP amplicons can be detected reliably using Lateral Flow Devices (LFDs). In addition to the benefits due to the isothermal nature of the test, LAMP has been reported to be less sensitive than PCR to inhibitors that may be carried over into nucleic acid samples from extraction procedures (Blomström et al., 2008; Poon et al., 2006). Together, these properties of LAMP make it ideally suited for deployment to non-specialised laboratories where equipment is limited, or for incorporation into a simple-to-use "penside" test for use in the field in ASF endemic countries.

Currently one of main challenges to the successful development and validation of LAMP technology revolves around optimising the

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Table 2
Comparison of results of real-time PCR, LAMP and antigen ELISA, for tissues collected from pigs 5 days post-infection with ASFV isolate OURT88/1.

Sample	Real-time PCR (King et al., 2003) Ct value	LAMP (James et al., 2010) Tp value	Antigen ELISA (Ingenasa)
Pig 18 Spleen	26.0	28.4	Inconclusive
Pig 19 GHLN	20.4	23.8	Positive
Pig 19 MLN	20.5	24.4	Inconclusive
Pig 19 SMLN	19.7	22.7	Positive
Pig 19 Spleen	17.7	22.1	Positive
Pig 19 Blood	18.1	22.5	Positive
Pig 20 GHLN	19.6	24.0	Positive
Pig 20 MLN	21.4	24.5	Inconclusive
Pig 20 SMLN	20.5	23.3	Positive
Pig 20 Spleen	18.5	22.7	Positive
Pig 20 Tonsil	19.4	22.8	Positive
Pig 20 Blood	18.4	23.7	Positive
Pig 21 GHLN	20.6	23.8	Positive
Pig 21 MLN	23.7	28.1	Negative
Pig 21 SMLN	21.9	23.3	Positive
Pig 21 Spleen	20.1	23.4	Positive
Pig 21 Tonsil	23.9	24.4	Positive
Pig 21 Blood	18.4	22.7	Positive

GHLN, gastrohepatic lymph node; MLN, mesenteric lymph node; SMLN, submandibular lymph node; Ct, cycle threshold; Tp, time to positivity.

cut-off for the assay. Before this technology can be recommended for use as a front-line test it is essential to optimise the sensitivity and specificity of the assay through setting an optimised cut-off. In order to do this further field-based validation of the assays are required. In many recent LAMP development studies the fluorescent output of the LAMP assay is read using a rPCR machine. It is however unlikely that such a machine would be used for this purpose in laboratories from the developing world, where the LAMP assay, being a low-cost alternative to PCR, is designed for use. Further validation of the LAMP assay for ASFV detection is therefore necessary and is ongoing using specific low-cost LAMP-designed fluorescent readers and lateral flow devises (LFDs), which reflect better how results will be read and interpreted in less developed field laboratories within the developing world. Up to now LAMP assays have been developed and partially validated largely in laboratories from developed countries. The next important step is to transfer these assays to laboratories in developing countries, and to carry out further field-based validation in order to prove their worth in these settings.

4. Comparison of rPCR, LAMP and antigen ELISA for detection of ASFV in experimental and field tissues

A recent study carried out at the OIE reference laboratory at IAH-Pirbright compared the performance of a LAMP assay (James et al., 2010), the OIE-approved rPCR (King et al., 2003) and a commercially available antigen ELISA (INGEZIM K3, Ingenasa, Spain) using samples collected from experimentally infected pigs (Table 2) and suspected naturally infected pigs (Table 3). Good correlation was observed between the LAMP and the rPCR assays when used to test a selection of freshly collected tissue samples from four experimentally infected pigs at 5 dpi with a virulent ASFV isolate (OURT88/1) (Table 2). All 18 tissue samples tested positive with low Ct values (high levels of viral DNA) with the rPCR. The 18 samples also tested positive with the LAMP assay, all becoming positive in less than 30 min (Tp < 30). Negative control samples that were run in parallel tested negative. When the samples were tested with the antigen ELISA 14 samples tested positive, 3 were inconclusive and one sample was negative, according to the cut-off recommended in the ELISA kit protocol. This data indicates that the rPCR and LAMP are good methods to use for the detection of ASFV in fresh samples taken from pigs acutely infected with ASFV. The antigen ELISA showed a lower sensitivity, however it successfully detected ASFV antigen in the majority of samples tested. Only one of the 18 tissue samples, that were all strongly positive in the rPCR, testing negative in the antigen ELISA. These results revealed that, when used as a 'herd' test on multiple freshly collected samples from pigs taken during an acute infection with ASFV, the antigen detection ELISA is sensitive enough to detect ASFV in the affected pigs.

Table 3
Comparison of real-time PCR, LAMP and antigen ELISA results for the detection of ASFV in tissues collected from pigs in Ghana.

Sample	Real time PCR (King et al., 2003) Ct value	LAMP (James et al., 2010) Tp value	Antigen ELISA (Ingenasa)
Ghana 1	No Ct	No Tp	Negative
Ghana 2	26.4	40.6	Negative
Ghana 3	36.2	No Tp	Inconclusive
Ghana 4	25.7	37.9	Inconclusive
Ghana 5	27.2	47.2	Inconclusive
Ghana 6	26.5	43.2	Negative
Ghana 7	42.0	37.0	Negative
Ghana 8	No Ct	No Tp	Negative
Ghana 9	No Ct	No Tp	Negative
Ghana 10	21.2	31.6	Inconclusive
Ghana 11	28.3	37.2	Positive
Ghana 12	No Ct	No Tp	Negative
Ghana 13	No Ct	No Tp	Negative
Ghana 14	No Ct	No Tp	Negative
Ghana 15	25.8	51.3	Positive
Ghana 16	No Ct	No Tp	Negative
Ghana 17	26.6	42.1	Positive
Ghana 18	No Ct	No Tp	Negative
Ghana 19	No Ct	No Tp	Negative
Ghana 20	25.1	39.0	Positive
Ghana 21	No Ct	No Tp	Negative
Ghana 22	27.4	45.6	Inconclusive
Ghana 23	26.1	49.8	Positive
Ghana 24	22.5	35.7	Positive
Ghana 25	30.6	46.5	Positive
Ghana 26	31.5	57.0	Positive
Ghana 27	30.8	42.0	Inconclusive
Ghana 28	24.5	38.3	Negative
Ghana 29	27.5	39.9	Negative
Ghana 30	22.2	32.7	Positive
Ghana 31	21.6	33.7	Inconclusive
Ghana 32	No Ct	No Tp	Negative
Ghana 33	24.9	45.2	Inconclusive
Ghana 34	31.1	42.9	Positive
Ghana 35	29.5	53.7	Negative
Ghana 36	No Ct	No Tp	Negative
Ghana 37	27.4	41.1	Inconclusive
Ghana 38	20.4	30.7	Positive
Ghana 39	29.3	50.1	Negative
Ghana 40	26.4	37.1	Inconclusive
Ghana 41	21.5	33.1	Positive
Ghana 42	28.9	42.9	Negative
Ghana 43	20.1	29.2	Positive
Ghana 44	28.4	53.0	Negative
Ghana 45	21.1	31.9	Positive
Ghana 46	35.2	41.1	Negative

Ct, cycle threshold; Tp, time to positivity.

A second group of 46 tissue samples that were collected from suspected cases of ASF across Ghana from 2004 to 2009 were tested by rPCR, LAMP and an antigen ELISA (Table 3). These samples had been kept for varying periods of time at −20 °C in the laboratory in Ghana. Due to periodic power cuts, the sample may have freeze-thawed on several occasions during storage and therefore may have undergone some degradation. Thirty-four of the 46 samples tested positive with the rPCR (Ct range 20–42) and 33 of the 34 rPCR positive samples also tested positive in the LAMP, if the cut-off for the assay was set at a time to positivity (Tp) of 60 min (Table 3). The

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Tp in the LAMP was however quite variable in these samples ranging from 29 to 57 and did not correlate well with the Ct values in the rPCR. If the cut-off for the LAMP assay was set at a Tp value of 60 min then the two assays (rPCR and LAMP) correlate very well, with only one out of the 34 rPCR positive samples (with a high Ct value of 36.2) testing negative in the LAMP. If however the cut-off was set at a shorter Tp such as 40, then the sensitivity of the LAMP would be severely compromised compared to the rPCR. This shows the importance of setting the correct cut-off Tp value in the LAMP.

When these samples were tested with the antigen ELISA only 14 were positive, 10 were inconclusive and 22 were negative (Table 3). The specificity of the antigen ELISA was high with all samples that were negative in the rPCR and LAMP also testing negative in the antigen ELISA. The sensitivity of the antigen ELISA however was very poor compared to the rPCR and LAMP. This low sensitivity of the antigen ELISA may have been due to the poor nature of the samples, which may have affected the integrity of the antigen. These results taken together indicated that the antigen ELISA is not as sensitive as the rPCR and LAMP, however when used on a 'herd' basis to test multiple fresh samples from an acute outbreak of ASF in multiple pigs, it is capable of successfully detecting ASFV antigen in the herd. When samples are not fresh, the cold-chain has broken down between the field and the lab or the samples have been kept for extended periods at −20 °C, the sensitivity of the antigen ELISA may be reduced. ELISA results on degraded samples should therefore be treated with caution and it is essential in these cases that results are backed up with further alternative testing. Virus isolation was also attempted from 6 of the field collected tissue samples from Ghana with low Cts (high levels of viral RNA). Virus was only successfully isolated from one of the samples. This confirmed that the long term storage of the samples at −20 °C was likely to have affected the viability of the virus.

5. Detection of ASFV in *Ornithodoros* ticks

The ability to easily and reliably detect ASFV in ticks is essential for studies which aim to understand the potential threat ticks represent in the field. This information is important for planning disease control strategies. Most previous studies have used virus isolation (VI) in porcine macrophage cultures to assay for the presence of ASFV in ticks. This has the advantage over PCR in that live virus can be confirmed to be present in the ticks, rather than viral genome which may not be infectious. Testing tick homogenates using this method however is difficult since they may be cytotoxic (Plowright et al., 1969, 1970), and the procedure is time-consuming. Furthermore, processing of tick samples can inactivate viral particles thus affecting virus detection by this method. Two studies have recently shown the strength of using VI to detect ASFV in ticks. ASFV (Georgia 2007/1) isolate was shown to replicate efficiently in *Ornitohodoros* ticks, highlighting the importance of clarifying the distribution of *Ornithodoros* species ticks in the Russian Federation and Caucasus region and the relationship of these ticks to species susceptible to ASFV (Diaz et al., 2012). A second study used VI in ticks to explore the period for which the European soft tick species *Ornithodoros erraticus* is able to act as a reservoir of ASFV after infected hosts are removed. This study showed that ticks from previously infected farms may contain infectious virus for at least 5 years and 3 months after the removal of infectious hosts (Boinas et al., 2011).

The use of PCR to detect viral DNA in ticks has potential advantages in terms of rapidity, throughput and sensitivity. A nested PCR assay, with an internal control, was developed to detect ASFV DNA in *O. erraticus* ticks (Basto et al., 2006a). All ticks collected from the field, which were positive by virus isolation, were also positive by PCR. Viral DNA was detected in a further 19 out of 60 ticks from which no virus was isolated, inferring a higher sensitivity of the PCR over VI. Since tick homogenates may contain PCR inhibitors, which may result in false negative results, an internal control (IC) was constructed. The same author then went on to investigate the kinetics of ASFV infection in *O. erraticus* ticks through the testing of specimens collected in the field at different times following an outbreak of the disease. Initial screening of the ticks was carried out by PCR, followed by unsuccessful attempts to isolate the virus (Basto et al., 2006b).

A duplex PCR approach providing information on the invertebrate host species and infecting virus has been developed (Bastos et al., 2009). This duplex one-step PCR approach enables the detection of ASFV (VP72) in *Ornithodoros* ticks and, through sequencing of the VP72 PCR product, enables further molecular characterisation of the virus. Also, the sequencing of a dual amplified 313 bp fragment of the 16S rRNA gene, enables the differentiation of 3 geographically discrete *Ornithodoros porcinus* lineages. False negatives were precluded by the inclusion of the host species-informative primers that ensured the DNA integrity of cytoplasmically located genome extracts. The risk of false positives arising from carry-over contamination when performing a two-step nested PCR described above (Basto et al., 2006a) was reduced by using a one-step approach. This assay was successfully used to assess ASFV infection levels in adult *O. porcinus* ticks collected from warthog burrows in southern and East Africa (Bastos et al., 2009).

6. Concluding remarks and prospects for the future

There have been many advances and developments in the virological diagnosis of ASFV in recent years driven by the need for better/faster assays. The most important recent advance is the advent of rPCR which has provided a fast, sensitive, quantitative diagnostic platform with reduced chances of cross contamination (as it is a closed system). Real-time PCR can also use a 96-well format, making it well suited to high throughput diagnostic systems and process automation, which is vitally important for reference labs in order to reach the levels of testing that are required during a disease outbreak. Real-time PCR assays for the detection of ASFV are now commercially available and portable rPCR machines are becoming available, making it possible to use these molecular technologies in the "field", with prospects for radical changes in future diagnostic approaches.

Although ASFV has recently been introduced into Russia and the Caucasus region and has entered Ukraine for the first time, it is still mostly confined to sub-Saharan Africa. Here it continues to cause severe economic hardship to local pig farmers who are severely affected by regular outbreaks of disease that wipe out their stock. One of the main problems in Africa is that national reference laboratories do not have the funding to enable the effective virological diagnosis of ASFV, so the disease often goes undiagnosed in the laboratory.

The effective control and possible eradication of an ASFV incursion, such as the recent outbreak in Russia and the Caucasus region, is dependent on rapid and accurate diagnosis of disease, which will enable timely control measures to be implemented. Data presented in this paper shows that rPCR remains the most reliable 'gold standard' method for the diagnosis of ASFV infections, although it is important to remember that PCR only detects viral DNA and not live virus. It is therefore highly recommended to isolate virus from infected samples prior to the confirmation of an outbreak. Antigen ELISA can be used as a 'herd-based' test to detect ASFV antigen, but does lack sensitivity when compared to PCR. Isothermal technology such as LAMP assays, which is a lower cost alternative to PCR, offer some hope to less developed laboratories as long as this technology is transferrable. Isothermal LAMP assays that have been

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developed for the detection of ASFV show good sensitivity, however they require more 'field' validation before they can be used as front-line diagnostic tests.

The development of robust and reliable pen-side technology for ASFV detection, based on antigen or genome detection, could result in significant time-saving in diagnosis. Future development of molecular technologies (isothermal and/or PCR-based) may allow for diagnosis to be carried out at the pen-side, outside the laboratory setting, using simple equipment or lateral flow devises to interpret the result. Lateral flow devices for ASFV antigen detection are being developed in several labs and, if successfully developed, would enable the on-farm diagnosis of ASF, however the sensitivity of these assays needs to be improved before they can be used as front-line field-based tests. The future development of probe-based diagnostic systems (microarray technologies) could enable the simultaneous detection of ASFV, along with other important pathogens that cause similar clinical signs in pigs (using syndrome-based arrays), as well as the identification of ASFV down to a genotype level.

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