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REVIEW ARTICLE

African Swine Fever Diagnosis Adapted to Tropical Conditions by the Use of Dried-blood Filter Papers

T. Randriamparany[1,*], K. V. Kouakou[2,*], V. Michaud[3,4], J. Fernández-Pinero[5], C. Gallardo[5], M.-F. Le Potier[6], R. Rabenarivahiny[1], E. Couacy-Hymann[7], M. Raherimandimby[8] and E. Albina[4,9]

[1] Laboratoire National de Diagnostic Vétérinaire, Antananarivo, Madagascar
[2] Laboratoire National d'Appui au Développement Agricole, Bingerville, Côte-d'Ivoire
[3] CIRAD, UMR CMAEE, Montpellier, France
[4] INRA, UMR1309 CMAEE, Montpellier, France
[5] Centro de Investigacion en Sanidad Animal (CISA-INIA), Valdeolmos, Spain
[6] Anses, Laboratoire de Ploufragan, Unité Virologie Immunologie Porcines, Ploufragan, France
[7] LANADA/Laboratoire Central de Pathologie Animale, Bingerville, Côte-d'Ivoire
[8] Université d'Antananarivo, Antananarivo, Madagascar
[9] CIRAD, UMR CMAEE, Petit-Bourg, Guadeloupe, France

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Correspondence:
E. Albina. CIRAD, UMR CMAEE, Domaine Duclos, 97170 Petit Bourg, Guadeloupe, France. Tel.: +33 59025431;
Fax: +33 590940396;
E-mail: emmanuel.albina@cirad.fr

*These two authors contributed equally to this work.

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Summary

The performance of Whatman 3-MM filter papers for the collection, drying, shipment and long-term storage of blood at ambient temperature, and for the detection of African swine fever virus and antibodies was assessed. Conventional and real-time PCR, viral isolation and antibody detection by ELISA were performed on paired samples (blood/tissue versus dried-blood 3-MM filter papers) collected from experimentally infected pigs and from farm pigs in Madagascar and Côte d'Ivoire. 3-MM filter papers were used directly in the conventional and real-time PCR without previous extraction of nucleic acids. Tests that performed better with 3-MM filter papers were in descending order: virus isolation, real-time UPL PCR and conventional PCR. The analytical sensitivity of real-time UPL PCR on filter papers was similar to conventional testing (virus isolation or conventional PCR) on organs or blood. In addition, blood-dried filter papers were tested in ELISA for antibody detection and the observed sensitivity was very close to conventional detection on serum samples and gave comparable results. Filter papers were stored up to 9 months at 20–25°C and for 2 months at 37°C without significant loss of sensitivity for virus genome detection. All tests on 3-MM filter papers had 100% specificity compared to the gold standards. Whatman 3-MM filter papers have the advantage of being cheap and of preserving virus viability for future virus isolation and characterization. In this study, Whatman 3-MM filter papers proved to be a suitable support for the collection, storage and use of blood in remote areas of tropical countries without the need for a cold chain and thus provide new possibilities for antibody testing and virus isolation.

Introduction

African swine fever virus (ASFV) is a large enveloped DNA virus (Dixon et al., 2005) that belongs to the family *Asfarviridae*, genus *Asfivirus*. It is the only known DNA arbovirus. African swine fever virus infects members of the vertebrate family *Suidae* (domestic and feral pigs, wild boars, bush pigs, warthogs and the giant forest hog) and some Argasid ticks (Ornithodoros complex). It is one of the most fatal diseases of domestic pigs and wild boars and the most serious transboundary pig disease that could spread rapidly and have crippling socio-economic

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consequences (Penrith and Nyakahuma, 2000; Babalobi et al., 2007).

African swine fever was first described in Eastern Africa at the beginning of the 20th century. The disease was restricted to many sub-Saharan countries until 1957 when the first outbreak outside the African continent occurred in Portugal. Subsequently, the virus spread to the Caribbean and South America in 1970s and 1980s (Arias et al., 2002). Meantime, the infection has been eradicated from Caribbean, South America and Europe except in Sardinia, where the disease still persists. However, recent introductions of the virus were reported in Western Africa (1997), Madagascar (1998), trans-Caucasian (2007), Russia (2007), Iran (2009), Ukraine (2012), Belarus (2013) and Estonia (2014) (Rowlands et al., 2008; Rahimi et al., 2010; Dietze et al., 2012., Le Potier and Marcé, 2013). The infection is mainly detected in wild boars but also in domestic pigs (OIE, 2014) in Lithuania, Latvia and Poland, emphasizing the risk for Central and Eastern Europe (Gallardo et al., 2014).

Laboratory diagnosis of ASF is based on the detection of the virus and specific ASF-antibodies. Virus isolation (VI) (Malmquist and Hay, 1960) in porcine bone marrow cells is a reliable and sensitive method, but it can take several days or even weeks to obtain the final result. In addition to antigen-detection immunoassays (Vidal et al., 1997; Hutchings and Ferris, 2006), several molecular tests including agarose gel-based PCR (Steiger et al., 1992; Agüero et al., 2003, 2004; Basto et al., 2006) and real-time PCR (King et al., 2003; Zsak et al., 2005; Tignon et al., 2011; Fernández-Pinero et al., 2012) have also been developed and adopted for routine diagnostic purposes. The transport of infected materials for initial or confirmatory diagnosis of ASF infection in local, regional or international laboratories requires the use of a cold chain or the addition of preservative agents that do not interfere with the diagnostic procedures. The maintenance of a cold chain for the preservation of biological samples is often impossible in remote areas of tropical regions where the appropriate infrastructure is not available. There is thus a pressing need for a sampling and shipping procedure from the farm to the laboratory that does not rely on a cold chain.

In recent years, studies have demonstrated that samples could be poured and stored on filter papers at room temperature for relatively long periods (Pitcovski et al., 1999; Vilcek et al., 2001; Uttenthal et al., 2013). The use of blood samples dried on filter papers was described as a possible alternative to preserving human and animal samples for serological testing (Behets et al., 1992; De Swart et al., 2001; Helfand et al., 2001; Riddell et al., 2003; Johannessen et al., 2009; Joseph and Melrose, 2010). Filter papers have also been successfully used to store biological materials and to detect the presence of viral nucleic acids by PCR (Steiger et al., 1992; De Swart et al., 2001; Abdelwhab et al., 2011). More recently, filter papers were used for virus isolation (Abdelwhab et al., 2011). Different types of filter papers were used in these studies, but all basically belonged to two main classes. The first class consists of filter papers specifically engineered for nucleic acid preparation and preservation. They contain impregnated matrices that lyse cells, denature proteins and protect nucleic acids from nucleases, thus providing additional useful inactivation of the biological material which theoretically makes it compatible with safe shipment without the need for containment. Whatman FTA cards belong to this category of filter papers. However, in the laboratory, they require extra-preparation such as rinsing and elution before being used for diagnosis and are not suitable for subsequent pathogen isolation. In contrast, other filter papers like Whatman 3-MM filter papers do not contain additives; they can thus preserve infectivity and can theoretically be used for further pathogen amplification. Another advantage is that they do not contain PCR inhibitor and can be directly used in conventional PCR without previous nucleic acid extraction, as has already been demonstrated in the detection of ASFV (Michaud et al., 2007).

Based on these results, this study was designed to assess whether Whatman 3-MM filter papers can be used for ASF diagnosis with a series of currently available tests that were originally designed for the ASF virus and antibody detection in conventional biological samples. These tests included conventional and real-time PCR, viral isolation and antibody detection by ELISA. In addition, the robustness and thermal stability of Whatman 3-MM filter papers were assessed.

Materials and Methods

Samples

The biological samples were obtained from pigs during field surveys or from pigs that were experimentally infected. Domestic pigs in infected areas in Madagascar were randomly sampled at slaughter houses in 2009. A total of 91 paired samples were collected from all pigs. The samples consisted of a spleen specimen and the corresponding blood dried on Whatman 3-MM filter papers (VWR, Fontenay-sous-Bois, France). The filter papers were stored at room temperature (22–25°C) for 9 months. In addition, 346 farm pigs in Côte d'Ivoire were bled for conventional serum collection and on 3-MM filter papers, the samples were tested to assess the specificity of the methods since the country has been free of the disease for at least 10 years until the recent re-introduction of the disease reported in September 2014.

Three experiments in contained facilities designed for vaccine testing, or intended to study the virulence of the isolate, generated paired EDTA blood, dried-blood 3-MM filter paper and serum samples. These samples 'trios' were used to determine the analytical sensitivity of the different

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methods of diagnosis using 3-MM filter papers compared to the other biological materials. In the first experiment, four Landrace × Large White pigs were inoculated intramuscularly with 10 HAD50/ml of the haemadsorbing ASFV Arm07 isolate. Two untreated pigs were maintained in contact, housed in the same pen as the inoculated animals. Paired samples were collected from these pigs on different days. The second experiment included two challenge groups of three pigs each, infected with ASF viruses either the virus strain Benin 97/1 or the Spain E75 isolate. The last experiment included two trials. Trial 1 involved six pigs that were first challenged with the non-virulent OURT88/3 isolate from Portugal and 3 weeks later, with the closely related virulent Portuguese isolate OURT88/1, and after a further 3 weeks, with the Benin 97/1 strain. Trial 2 included 16 pigs challenged with the Benin 97/1 isolate. Samples were collected on the day of challenge and thereafter at different intervals post-challenge, as described in the results section. The virus strains we used belong to genotype II (first experiment) and genotype I (second and third experiments), the only two genotypes that have spread beyond the African continent so far. All experimental challenges and use of animals were carried out in agreement with the European Directive 86/609 on the protection of animals used in scientific procedures and approved by the local ethical committee under the numbers 2010.05 for the two-first experiments and 22–17 for the last experiment.

Samples preparation

Conventional samples

For tissues and blood samples, viral nucleic acids were prepared using the QIAamp viral DNA mini kit (QIAGEN, Venlo, the Netherlands) as previously described (King et al., 2003) and stored at −80°C until subsequent testing by PCR or real-time PCR assay.

Filter paper preparation

Whatman 3-MM filter papers, which are often used for the storage and detection of genetic or protein materials, were selected for this study. The 3-MM filter papers were cut into 5 × 0.5 cm strips. The strips were soaked in whole blood collected from slaughtered pigs and allowed to dry. Once dried, the filter papers were stored at room temperature (22–25°C) or at 37°C as previously described (Michaud et al., 2004) until use.

Diagnosis procedures

Direct conventional PCR

The highly conserved region of genome coding for p72 protein was amplified by PCR using the 5 prime Mastermix (Eppendorf, Montesson, France). 3-MM filter papers with dried blood from infected pigs were directly placed in PCR tubes without previous extraction of nucleic acid. One piece of filter paper measuring 5 mm^2 was placed in each 0.2-ml PCR tube. Reaction mix was added to a final volume of 50 μl to allow proper immersion of the filter papers. The reaction mix contained 0.4 μM of forward primer: 5′-T C G G A G A TG T T C C A G G T A G G-3′ and reverse primer: 5′-G C A AAA G G A T TT G G T G A A T-3′. The PCR was run as follows: (i) 5 min at 95°C; (ii) 35 cycles for 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C; (iii) 7 min at 72°C. A PCR fragment of 346 base pairs was visualized on agarose gel. A negative control from an uninfected pig was included. Fragment size was defined by comparison with DNA ladders.

Using the same master mix and primers, 15 μl of DNA extracted from blood and tissues was run in final volumes of 50 μl. The reaction mixture was treated as follows: (i) 5 min at 95°C; (ii) 35 cycles, 30 s at 95°C, 30 s at 60°C and 30 s at 72°C; (iii) 10 min at 72°C.

Real-time PCR

Two real-time PCRs were evaluated. The first was the OIE reference method derived from King et al. (2003), which was carried out using 2× Gene Expression Master Mix (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. Primers at a final concentration of 0.4 μM (5′-C T G C T C A T G G T A T C A A TC T T A T C G A-3′, 5′-G A T A C C A C A A G A T C R G C C G T-3′) and a TaqMan probe at a final concentration of 0.25 μM (5′-[6-carboxy-fluorescein(FAM)]-C C A C G GG A G GA A T A C C A A C CC A G T G-3′-[6-carboxytetramethyl- rhodamine (TAMRA)]) were added to 20 μl of the appropriate master mix. The reactions were made up in final volumes of 40 μl consisting of either one 1.25-mm^2 piece of 3-MM filter paper or 2 μl of DNA template, 20 μl of the appropriate master mix, 0.4 μM of each primer, 0.25 μM of the probe and 18 or 16 μl of water for 3-MM filter paper and DNA, respectively. Water instead of master mix was added to allow correct immersion of 3-MM filter paper without increasing the reagent cost of the PCR. The cycle consisted of 3 min at 95°C, and 45 cycles for 10 s at 95°C and 30 s at 58°C.

The second real-time PCR was based on the use of a Universal Probe Library (UPL, Fernández-Pinero et al., 2012). One 1.25-mm^2 piece of 3-MM filter paper or 2 μl of DNA template were added to a reaction mixture using Light Cycler 480 Probe Master (2×) (Applied Biosystems), consisting of 0.4 μM sense primer, 0.4 μM anti-sense primer and 0.1 μM probe UPL. The reaction mixture was the same as the King reaction for filter papers and DNA.

For the two real-time PCRs, amplification was run on an Mx3005P real-time PCR machine (Stratagene; Agilent technologies, Courtaboeuf, France). During the preliminary tests that were conducted, the King real-time PCR was less

performing than the UPL real-time PCR. The King real-time PCR was consequently progressively abandoned, and only, the UPL real-time PCR was used for all assessments.

Virus isolation on macrophages

Alveolar macrophages were collected from the lungs of piglets according to Baron et al. (1992). This procedure was also approved by the ethical committee under the number 12ANI01. Briefly, piglets were anesthetized, exsanguinated and their lungs collected. The lungs were washed with PBS, and the collected cells were rinsed and cultured in tissue flasks containing MEM medium supplemented with 10% foetal bovine serum, penicillin–streptomycin and amphotericin B. Individual 0.5-cm^2 pieces of 3-MM filter paper were ground in MEM medium and the supernatant used to infect the macrophages. The cells were kept for 5–7 days at 37°C, and haemadsorption and cytopathic effects were checked daily.

Elution of antibodies from 3-MM filter papers for antibody detection by ELISA and evaluation of test performances

Paired samples (3-MM filter papers containing dried blood and sera) were collected from experimentally infected pigs. A 40-mm^2 piece of 3-MM filter paper containing dried blood was removed and added to a volume of 100 μl of the ELISA buffer (Ingezim PPA Compac, Ingenasa, Spain). After incubation for 2 h, the eluate was collected and tested in the ELISA kit specifically designed by the company for this purpose. Sera were tested in the commercial version of this kit according to the manufacturer's instructions.

Detection limit according to the size of 3-MM filter papers and thermal stability of the material

The effect of the size of the piece of 3-MM filter paper on the sensitivity of the PCR diagnosis was evaluated. Seven threefold serial dilutions (1 : 3 to 1 : 2187) of the virus strain E70 titrating $10^{5.8}$ TCID50/ml on porcine alveolar macrophages were prepared in porcine blood. 3-MM filter papers were cut into 50 × 2.5 mm strips, and the strips were soaked in each dilution. The 3-MM filter papers were then dried and stored at room temperature (22–25°C) until use. 3-MM filter papers were cut into different sizes ranging from 0.3 to 6.25 mm^2 and finally added in the PCR tubes containing the mix. The conventional and UPL PCRs were then run directly.

The stability of the 3-MM filter papers containing the dried blood was determined on laboratory and field materials. Virus suspension in EDTA blood was reconstituted in the laboratory and spotted on 3-MM filter papers. After desiccation, the 3-MM filter papers were stored at 4 or 37°C and tested once a month with the real-time UPL PCR. The 3-MM filter papers containing positive dried blood from Madagascar were stored for 9 months at room temperature (22–25°C) and then tested using conventional and King and UPL real-time PCRs. They were tested just after collection and then 9 months later after storage at ambient temperature.

Comparison and evaluation of the performances of PCR and virus isolation on 3-MM filter papers

The analytical sensitivity of the PCR was tested by conventional and UPL real-time PCRs on the seven serial dilutions described above, either spotted on 3-MM filter papers or stored in liquid form. The results of these tests were directly compared with the results of virus isolation.

To assess the sensitivity, specificity, positive and negative predictive values of the different tests carried out on 3-MM filter papers, paired samples (3-MM filter papers containing dried blood and EDTA blood) collected during three different challenge experiments were used and tested by the King and UPL real-time PCR. Next, the same approach was used on paired samples collected from pigs in an endemic region (Madagascar) or single 3-MM filter paper samples from pigs collected in an infection free area (Côte d'Ivoire).

The relative sensitivity and specificity of the different tests were estimated by TP/(TP+FN)% and TN/(TN+FP)%, respectively, where TP, TN, FP and FN are the numbers of true positives, true negatives, false positives and false negatives in the reference test, respectively. The positive and negative predicted values for a given test were calculated with TP/(TP+FP)% and TN/(TN+FN)%, respectively. For sensitivity, specificity and predictive values, a 95% confidence interval was calculated. Gold standard procedure for the calculation of these test performances was the UPL real-time PCR (Fernández-Pinero et al., 2012) using blood and tissues samples: this test was formerly identified as the most sensitive in our hands compared to conventional and King PCR. The agreement between two tests was determined by the kappa test where k values higher than 0.81 were considered as indicative of very good agreement (Altman, 1991).

Results

Effect of the size of the 3-MM filter paper on the analytical sensitivity of the PCR methods

To test the impact of the size of the piece of 3-MM filter papers on the performances of the test, different sized pieces of 3-MM filter papers containing dried blood were prepared and included in either the conventional or UPL PCRs. The results showed that the conventional PCR is not sensitive to differences in the size of the 3-MM filter paper within the limits of 0.3 and 5 mm^2 (Fig. 1). With the UPL PCR, C_t values were still detectable on 0.3–6.25 mm^2 pieces of 3-MM filter paper (data not shown).

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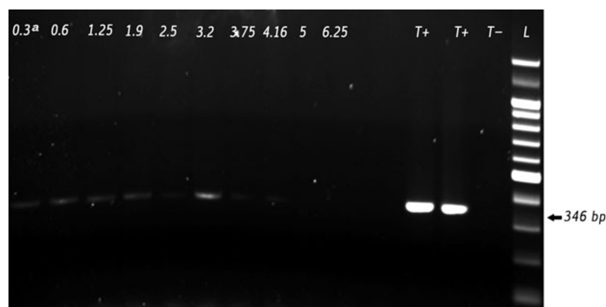

Fig. 1. Direct conventional PCR for ASFV detection according to the FP size. PCR products are shown by gel electrophoresis. An expected product of 346 bp was detected with FPs from 0.3 to 5 mm^2 in size. a : size of Whatman filter paper 0.3 to 6.25 mm^2; T+ :positive control; T− : negative control; L: Ladder.

Detection limit of PCR and virus isolation on 3-MM filter papers containing dried blood

We then determined the end point dilution of the threefold serial dilutions to give a positive PCR amplification signal or virus isolation. The results (summarized in Table 1) showed only one log reduction in analytical sensitivity when 1.25-mm^2 3-MM filter papers were used compared to standard biological materials in the two different PCR methods tested for ASFV detection.

Performance of ASFV detection methods on 3-MM filter papers containing dried blood from experimentally infected animals

ASFV detection was performed on 3-MM filter papers, and the results are summarized in Table 2. The observed sensitivity was 65.2% for King real-time PCR, 78.3% for conventional PCR, 95.7% for UPL PCR and 95.7% for virus isolation. The observed specificity for all tests on filter papers containing dried blood was excellent (100%). The positive predictive value (PPV) was 100% while the negative predictive values (NPV) ranged from 87.5% to 98.2%. When considering these parameters and the values of Kappa test, the best 'fit for purpose' tests were in decreasing order: virus isolation, real-time UPL PCR, conventional PCR and real-time King PCR (see Table 2). In addition, the 3-MM filter papers used in direct conventional or real-time UPL PCRs proved to be able to detect circulation of the virus genome as early after infection as conventional samples. The virus was detected at 2–3 days post-challenge and throughout the clinical phase.

Performance of ASFV detection methods on 3-MM filter papers containing dried blood collected from animals in the field

A similar approach was used on field samples collected in Madagascar, an endemically infected country, and in Côte d'Ivoire, which has been free of the disease for more than 10 years. The results are summarized in Table 3. Again, the real-time UPL and conventional PCRs performed better. Virus isolation was not performed on field samples because not enough material was available.

UPL PCR robustness

In the previous sections, the UPL PCR was shown to have the best performances with 3-MM filter papers containing dried blood. We then checked the robustness of this assay. The robustness test consisted of repeated tests on the same 3-MM filter papers spotted with serial dilutions of the virus, sampled at different times and tested randomly on two real-time PCR machines. All ASFV-positive 3-MM

Table 1. Detection limit of ASFV on 1.25-mm^2 3-MM filter papers containing dried blood by direct (no nucleic acid extraction) conventional PCR, direct real-time UPL PCR and virus isolation compared to standard procedures. The virus was not isolated from blood

		Detection limit				
		3-MM filter papers containing dried blood			Blood	
Estimated virus titres (TCID50)	Dilution	Conventional PCR	UPL PCR (C_t value)	Virus isolation	Conventional PCR	UPL PCR (C_t value)
$3 \times 10^{4.8}$	1/3	+	29.8	+	+	27.51
$1 \times 10^{4.8}$	1/9	+	32.7	+	+	29.55
$3 \times 10^{3.8}$	1/27	+	34.9	+	+	31.48
$1 \times 10^{3.8}$	1/81	+	39.4	+	+	34.89
$3 \times 10^{2.8}$	1/243	+	39.9	+	+	37.28
$1 \times 10^{2.8}$	1/729	+	41.4	+	+	38.90
$3 \times 10^{1.8}$	1/2187	−	No C_t	−	+	40.56

+ = detected.

− = non detected.

Table 2. Comparison of test performances for ASFV detection on filter papers containing dried blood collected from experimentally challenged pigs. The positive/negative status of the pig was defined by considering a combination of the experimental treatment administered to the animal and the result of the standard detection procedure on EDTA blood (see bottom of the table for definitions). The results of the different methods used on 3-MM filter papers are shown as a function of this status

Test on filter paper	Conventional PCR		King real-time PCR		UPL real-time PCR		Virus isolation	
Pig status	Detected	Undetected	Detected	Undetected	Detected	Undetected	Detected	Undetected
Positive[a]	54	15	45	24	66	3	66	3
Uninfected (negative)[b]	0	168	0	168	0	168	0	88
Sensitivity	78.3% [95% CI, 68.4–88.2]		65.2% [95% CI, 53.7–76.7]		95.7% [95% CI, 90.8–100]		95.7% [95% CI, 90.2–100]	
Specificity	100% [95% CI, 99.–100]		100% [95% CI, 99.7–100]		100% [95% CI, 99.7–100]		100% [95% CI, 99.4–100]	
Positive predictive value	100% [95% CI, 99.1–100]		100% [95% CI, 98.9–100]		100% [95% CI, 99.2–100]		100% [95% CI, 99.2–100]	
Negative predictive value	91.8% [95% CI, 87.7–95.9]		87.5% [95% CI, 82.7–92.3]		98.2% [95% CI, 96.1–100]		96.7% [95% CI, 92.9–100]	
K value (Kappa test)	0.90		0.72		0.96		0.96	

[a]Infected pigs with at least one positive result in standard testing of EDTA-blood samples.
[b]Infected pigs but PCR negative by standard detection procedure and control animals not infected considered as true negative.

Table 3. ASFV detection performance using field samples, 3-MM filter papers compared to standard detection procedures. The pig status is characterized both by the origin of the pigs (infected versus infection free regions, i.e. Madagascar versus Côte d'Ivoire) and the result of conventional and real-time PCRs obtained on the corresponding tissue samples. The results for the evaluation of the different methods used for 3-MM filter papers are shown as a function of this status

Test on filter papers	Conventional PCR		King real-time PCR		UPL real-time PCR	
Pig status	Detected	Undetected	Detected	Undetected	Detected	Undetected
Infected[a]	14	2	12	4	15	1
Uninfected[b]	0	75	0	75	0	421
Sensitivity	87.5% [95% CI, 70.9–100]		75% [95% CI, 53.3–96.7]		93.8% [95% CI, 81.7–100]	
Specificity	100% [95% CI, 99.1–100.0]		100% [95% CI, 95.1–100]		100% [95% CI, 99.2–100.0]	
Positive predictive value	100% [95% CI, 78.5–100]		100% [95% CI, 80.6–100]		100% [95% CI, 80.6–100]	
Negative predictive value	97.4% [95% CI, 93.8–100]		94.9% [95% CI, 87.6–98]		99.8% [95% CI, 99.4–100]	
Kappa test	0.92		0.83		0.96	

[a]Pigs with positive PCR on tissue samples, samples considered as true positive.
[b]Pigs from an infected region but PCR negative or pigs from an infection free region.

filter papers were repeatedly detected by the UPL PCR assay in the two independent runs performed, while negative 3-MM filter papers remained undetected (data not shown).

Antibody detection

A limited comparative study of the capacity of 3-MM filter papers to preserve antibodies for subsequent detection by ELISA was carried out. Paired samples of sera/3-MM filter papers containing dried blood collected from experimentally challenged pigs were used, as described above. Although only small numbers of samples were tested, the performances of the ELISA on 3-MM filter papers are encouraging compared to results generated on corresponding sera. The sensitivity, specificity, PPV and NPV for antibody detection on 3-MM filter papers compared to sera are summarized in Table 4. Except for one positive serum, all 3-MM filter papers gave similar results. The Kappa test showed very good agreement between the two methods.

Thermal stability of 3-MM filter papers

The virus suspended in EDTA blood and dried on 3-MM filter papers was still detectable after one and 2 months of storage at 4 or 37°C as shown by the UPL PCR test.

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Table 4. Comparison of filter papers containing dried blood and sera for ASF-specific antibody detection by ELISA

	Sera		
	Negative	Positive	Total
Filter papers			
Negative	30	1	31
Positive	0	15	15
Total	30	16	46
Sensitivity		93.8% [95% CI, 71.7–98.9]	
Specificity		100% [95% CI, 88.6–100]	
Positive predictive value		100% [95% CI, 79.6–100]	
Negative predictive value		96.8% [95% CI, 83.8–99.4]	
Kappa		0.95	

In addition, virus collected on slaughtered pigs in Madagascar and dried on 3-MM filter papers and initially found to be positive remained positive for up to 9 months at 22–25°C when checked by conventional, King and UPL real-time PCRs.

Discussion

In this study, the performance of Whatman 3-MM filter papers for the collection of blood samples and their storage at room temperature (>22°C) for long periods of time was investigated with currently used diagnostic procedures for African swine fever. One originality of this study was the detection of ASFV specific antibodies from blood dried on 3-MM filter papers using a commercial ELISA kit originally designed for serum. Our results suggest that 3-MM filter papers can be used for antibody detection by ELISA as previously shown for other diseases and other species (Hutet et al., 2003; Joseph and Melrose, 2010; Curry et al., 2011). The agreement between the sera and the 3-MM filter papers in the ELISA test was very good although a slight reduction in sensitivity was observed. The only conflicting result (positive serum and negative 3-MM filter paper) concerned the paired samples collected 23 days after the experimental challenge, suggesting that low antibody concentrations may not be detected in 3-MM filter papers compared to sera. This reduction in sensitivity needs to be assessed more precisely on a larger number of field samples in the future. However, assuming the reduction in sensitivity is confirmed, one possible solution would be to increase the targeted number of samples collected in sero-surveillance programs to find a balance between the need for more tests but a simpler procedure to collect and store the samples in the field. As the procedure for collecting blood on 3-MM filter paper is easy (blood droplets obtained by scarifying the skin of the pig's ear), considering the advantage of 3-MM filter paper for long-term storage of biological material at ambient temperature, this should not be a major disadvantage. Using 3-MM filter papers instead of sera for serological testing in the laboratory is less convenient. Indeed as indicated in the Material and Methods section, 3-MM filter papers stored at room temperature have to be eluted for several minutes or hours by stirring in the appropriate buffer to allow the recovery of antibodies.

Fortunately, this drawback does not exist for molecular techniques which, after serological testing, are the most popular diagnostic procedures used for rapid identification of animal and human diseases. In this study, it was even shown that 3-MM filter papers have an outstanding advantage compared to conventional biological materials, as extraction of nucleic acids is not required. Thus, to be able to perform direct conventional or real-time PCRs implies a considerable reduction in both the time required for molecular diagnosis and the cost. Also, another potential advantage is the reduction of potential contaminations during sample process. In all circumstances, very high specificity was observed with the different molecular tests evaluated in this study, whereas sensitivity was more variable. Thus, the first real-time PCR (King et al., 2003) run for ASFV detection was less sensitive than the other molecular tests. Indeed, this study and a previous one by Michaud et al. (2007) showed that the likelihood a validated test for conventional biological materials will not give satisfactory results on 3-MM filter papers is even higher. Viral DNA was detected easily using real-time PCR (Braae et al., 2013). It was possible to use Whatman 3-MM filter papers collected as early as 2–3 days after infection in direct conventional or real-time PCRs for early detection of ASFV genome in the three different experiments.

The recent UPL procedure was shown to be more sensitive than the reference test designed by King et al. (2003) (Fernández-Pinero et al., 2012). This difference was not only apparent on 3-MM filter papers but also on tissues or blood (data not shown). The UPL method uses a commercial Universal Probe Library (UPL) probe combined with a specifically designed primer set to amplify an ASFV DNA fragment within the VP72 coding genome region. It is also cheaper than the Taqman technology and, combined with direct detection on filter papers, is probably more suitable for laboratories with a limited budget. Whatman 3-MM filter papers are not only cheap but are also suitable for large-scale surveillance in remote rural and tropical areas. In this study, ASFV continued to be detected for at least 9 months at ambient temperature (22–25°C), and it is likely that even higher temperatures will not interfere with the preservation of the material, as previously shown by other authors (Michaud et al., 2007; Uttenthal et al., 2013). This study showed that samples originally diagnosed as positive were still positive after being stored for 2 months at 37°C. However, a slight reduction in the analytical sensitivity of PCR was observed for 3-MM filter papers compared to standard

procedures on tissue samples. This may be due to the reduced amounts of viral DNA present on filter papers containing dried blood, or alternatively, to the presence of PCR inhibitors. PCR inhibitors in blood have been identified and include natural blood components, mainly heme (Akane et al., 1994) and leucocyte DNA (Morata et al., 1998). To prevent inhibitory effects, we suggest using smaller pieces of filter paper for negative tests. In this study, the size of the 3-MM filter papers had little influence on analytical sensitivity, underlining the robustness of the method. From our results, we suggest a 2-mm^2 piece is the most convenient. To control PCR inhibiting factors, the parallel detection of an internal control, for example, a house-keeping gene from pig blood would be possible. As laboratories located in remote areas may find it difficult to acquire punchers to punch out standard pieces of 3-MM filter paper for the methods used in this study, which are less prone to variations in size, common laboratory consumables such as Petri dishes and sterile scalpel blades could be used to prepare small pieces of 3-MM filter paper thus limiting the risk of cross-contamination between samples.

Interestingly, another new approach we tested in this study was the detection of ASFV through isolation of the virus from the blood of infected animals dried on 3-MM filter papers. The results were comparable with those of direct conventional and real-time PCRs, even on 3-MM filter papers conserved at 37°C for 2 months. Even higher temperatures may be encountered in some tropical countries. However, it is important to note that in our case, filter papers conserved at 37°C for more than 2 months could not be satisfactorily eluted for subsequent virus isolation. Therefore, keeping 3-MM filter papers at temperatures higher than 37°C or for more than 2 months should be avoided or at least, assessed before implementation in the field. This means that in countries where the temperature can exceed 37°C, the use of a closed box to limit overheating should be considered. Direct isolation of virus from filter papers can only be achieved with filter papers such as 3-MM filter papers which, unlike FTA cards for instance, do not contain impregnated matrix for nucleic acid preservation. Whatman 3-MM filter papers do not inactivate the material and are therefore less suitable for biosafety shipment of the material. However, in some circumstances, being able to isolate the virus may be necessary to better characterize the virus and to provide absolute proof that it is the cause of an outbreak.

In summary, Whatman 3-MM filter papers are a cheap, simple and rapid support for blood collection, preservation and ASF disease diagnosis either by ELISA, direct conventional and real-time PCRs, and virus isolation. Other advantages of 3-MM filter paper strips include the smaller volume of blood that is required and the ability to collect a large number of samples (Dubay et al., 2006). Further, work is required to establish the robustness of the assay in large-scale filter paper sampling in an endemic country. The Whatman 3-MM filter papers used in this study are less expensive and although they are not specifically designed for the preservation of nucleic acids, they proved to be efficient in this study and others (Kailash et al., 2002). Whatman 3-MM filter papers can therefore be used as a multivalent support for multipurpose diagnosis under tropical conditions.

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