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Long-term storage at tropical temperature of dried-blood filter papers for detection and genotyping of RNA and DNA viruses by direct PCR

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Abstract

In tropical countries the diagnosis of viral infections of humans or animals 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. This study describes the use of filter papers for rapid sample collection, and the molecular detection and genotyping of viruses when stored over long periods at elevated temperatures. Infected blood was collected on filter papers, dried and stored at different temperatures (22, 32 and 37 °C) for various periods (up to 9 months). Two animal viruses, African swine fever, a large double-stranded DNA virus and Peste des Petits Ruminants, a negative single-stranded RNA virus, were used to validate the method. Filter papers with dried blood containing virus or control plasmid DNA were cut in small 5 mm^2 pieces and added directly to the PCR tube for conventional PCR. Nucleic acid from both viruses could still be detected after 3 months at 32 °C. Moreover, the DNA virus could be detected at least 9 months after conservation at 37 °C. PCR products obtained from the filter papers were sequenced and phylogenetic analysis carried out. The results were consistent with published sequences, demonstrating that this method can be used for virus genotyping.

Keywords: Filter paper; PCR; Genetic analysis; Phylogeny; African swine fever; Peste des petits ruminants

1. Introduction

Rapid diagnosis of infections is essential for the control of diseases. Not only the detection of the infection but also genotyping of the causative agent is often needed for efficient disease surveillance and control programmes (Zollner, 2004). A reliable procedure is required for sample collection, preservation, transport to laboratories and testing. Generally, samples are preserved using ice packs, dry ice or liquid nitrogen according to the nature of the agent and the expected delay in shipment. However, in some regions, it may be difficult to maintain a cold chain and transport to the laboratory may take more than 3–4 days. The situation is complicated by the international regulations regarding the transport of frozen forms of biohazardous agents (liquid or tissue) surrounded by dry ice or liquid nitrogen. In recent years, several studies have demonstrated the potential interest of filter papers for the collection and storage of biological materials. Using this, the cold chain is not necessary and thus the transport under international regulations is rendered easier. Filter papers have been widely used for blood preservation and antibody detection in the laboratory for human and animal diseases (Behets et al., 1992; De Swart et al., 2001; Hogrefe et al., 2002; Hutet et al., 2003; De la C Herrera et al., 2006). In addition, they have been repeatedly used to detect the genomes of DNA or RNA viruses by PCR (Spagnuolo-Weaver et al., 1998; Hattermann et al., 2002). Filter papers can also be used for the study of genetic variability of viruses (Pitcovski et al., 1999). Different types of filter papers

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have been tested, some have no additives, whereas others are specifically designed for the preservation of genomic material and are impregnated with chemicals that lyse cells and denature proteins (Natarajan et al., 2000). The virus genome can be detected after extraction of the genomic material (Prado et al., 2005; Zhou et al., 2006) or by direct PCR without extraction (Yourno and Conroy, 1992; Pitcovski et al., 1999; Kailash et al., 2002). Filter papers have been shown to be suitable for the conservation of either DNA or RNA viruses for extended periods of time (up to 4–11 years) at moderate or tropical temperatures (Li et al., 2004; Chaisomchit et al., 2005). However, there is no study showing that conventional filter papers can be used for long-term conservation of DNA and RNA viruses at tropical temperature and detection by direct PCR followed by genetic characterisation of the viruses. Two different animal viruses were used as models for the validation of the procedure. Both are responsible of two highly contagious and fatal diseases that are listed by the World Animal Health Organisation (OIE) among the 15 most serious diseases. African swine fever virus (ASFV) is a double-strand DNA enveloped virus that belongs to the family *Asfarviridae*, genus *Asfivirus*. The causative agent of peste des petits ruminants (PPR) is a single-strand negative RNA enveloped virus which is a member of the genus *Morbillivirus* within the family *Paramyxoviridae*. Both viruses were used in this study to determine the threshold for detecting the virus genomes by direct PCR from filter papers. The sensitivity of the method was compared to a conventional diagnostic test and the effect of long-term storage at high temperature was assessed.

2. Material and methods

2.1. Samples and reagents

Pigs free of the specific pathogens listed by the OIE (SPF pigs) were bled and filter papers impregnated with their blood and dried. Filter papers containing dried blood were prepared in the same way from African farmed goats. These goats were confirmed by serology as free of PPR and rinderpest (RP) infections twice. These filter papers served as negative controls in the PCR tests and also as supports for serial dilutions of each virus or plasmid containing the target gene in order to determine the detection limit of the technique. Additionally, 80 pigs and 73 goats from French farms were sampled on filter papers and tested in PCR to assess the specificity of the method. Four pigs, confirmed to be free of ASF infection by antibody detection, were infected experimentally by the intramuscular route with a Spanish strain of ASFV isolated in 1970 (strain E70, Sierra et al., 1990). Two other pigs were challenged with the same strain but by the oronasal route and placed in direct contact with one susceptible pig. Four additional pigs were inoculated by the oronasal route with the Lisbon 60 (L60) ASFV strain isolated in 1960 (Leitao et al., 2001). In this last experiment, four non-infected pigs were kept as negative controls. Blood was collected both in EDTA tubes and on filter papers, on the day of challenge and thereafter at different days post-challenge as indicated in results.

African dwarf goats aged between 2 and 3 years which were shown to be free of PPR and RP antibodies were experimentally infected with four different strains of PPRV corresponding to different lineages (Couacy-Hymann et al., 2005): strains Nigeria 75–1 (Nig/75–1, lineage 1), Côte d'Ivoire 89 (CI89, lineage 2), Ethiopia 94 (Ethio94, lineage 3) and India-Calcutta (Calcutta, lineage 4). Each strain was inoculated subcutaneously in one goat. Blood samples and filter papers were collected at different time post-challenge as indicated in results.

As positive control of PCR and also for detection limit purposes, the target genes of both viruses were cloned in plasmids. The VP72/73 and N gene of ASFV and PPRV, respectively, were selected as target genes as recommended by the Manual of Standards and Diagnostics of the World Animal Health Organisation (OIE). Plasmids were produced in large quantities using the Maxiprep Kit (Qiagen, France) according to the manufacturer's instructions. The DNA concentration was determined by UV absorbance. The number of gene copies in the plasmid preparation was estimated as a relation of the concentration established by UV absorbance and the plasmid size using the following formula 1.0 A_{260} unit dsDNA = 50 μg/ml and 1 μg of 1000 bp DNA = 9.1×10^{11} molecules. For ASFV, 10-fold serial dilutions of the plasmid were prepared with the objective to have copy numbers corresponding to the virus titres. For PPRV, the target N gene of PPRV was cloned under control of the bacteriophage T7 polymerase promoter and transcribed *in vitro* using the T7 RiboMAX Express Large Scale RNA Production System kit (Promega, France) according to the manufacturer's instructions. The number of RNA copies were then estimated as a factor of concentration, as determined by UV absorbance and RNA molecule size by using the formula 1.0 A_{260} unit ssRNA = 40 μg/ml and 1 μg of 1000 b RNA = 18×10^{11} molecules. Both plasmid (ASFV) and RNA (PPRV) serial dilutions were spotted on the filter papers to estimate the detection limit of the method.

A strain of ASFV (BA71V strain isolated in Spain in 1971 and adapted to grow on Vero cells) and the vaccine PPRV strain Nigeria 75/1 (attenuated by serial passages on Vero cells) were amplified and titrated according to the method of Kaerber (1931). Titres were expressed as tissue culture infectious doses 50% ($TCID_{50}$) per ml. Serial dilutions of these viruses were spotted on filter papers and used for the determination of detection limit of the method.

2.2. Filter paper preparation

The Whatman 3MM filter paper (VWR, Fontenay-sous-Bois, France), often used for storage and detection of genetic or protein materials, was primarily selected for this study because of its low cost. However, Whatman FTA cards (Dutcher, Brumath, France), specifically designed for nucleic acid stabilization, were used to compare the detection limit of ASFV.

In order to prepare calibration standards, 5 mm^2 surfaces of filter papers containing dried blood collected from SPF pigs or susceptible goats were impregnated with 2 μl of 1/10 serial dilutions of either plasmids containing the ASFV or PPRV target genes, *in vitro* transcribed RNA from PPRV target gene or

titrated viruses. To compare direct spotting of diluted virus on blood-dried filter papers or spotting of virus diluted first in blood and spotted on filter papers, ASFV and PPRV were serially diluted either in cell culture medium (EMEM, Eurobio, France) or in the blood of one non-infected pig and goat, respectively. Dilutions of the virus in medium were spotted on blood-dried filter papers as previously described. Dilutions in blood were incubated at 37 °C for 30 min and then spotted on filter papers, allowed to dry and tested in the direct PCR.

Bloods from farm pigs and goats were deposited on filter papers, allow to dry and stored at −80 °C until use. Experimentally infected pigs or goats were bled and filter paper strips were immediately impregnated and allow to dry. Once dried, the strips were stored at −80 °C until use. Filter papers found positive by ASFV PCR were stored for 9 months at 22–25 °C or 37 °C. Those found positive by PPRV PCR were stored for 3 months at 32 °C. All filter papers were stored in an environment with 50–70% of humidity. These filter papers were tested once a month.

2.3. PCR

Filter papers containing dried blood from infected pigs were directly processed into the PCR tubes without any nucleic acid extraction. Pieces of 5 mm^2 were placed into 0.2 ml PCR tubes. Reaction mix was added to a final volume of 80 μl to allow proper soaking of filter papers. For DNA extraction from blood collected on EDTA tubes, 100 μl of whole blood were treated with the DNeasy kit (Qiagen) according to the manufacturer's instructions. PCR was then run with 5 μl of extracted DNA in a final volume of 50 μl.

Different primer pairs were initially designed and tested on filter papers and the best pair was selected for further testing (data not shown). The proof reading polymerase (Taq pol Pfu, Stratagene, Amsterdam) was used to allow direct sequencing of the PCR products. For ASFV detection, the reaction mix consisted of 0.4 μM of each primer [forward: 5′-TCggAgATgTTCCAggTAgg-3′, reverse: 5′-CgCAAAAggATTTggTgAAT-3′], 250 μM dNTP, 2.5 units of Pfu polymerase. After amplification (5 min at 95 °C, then 35 cycles, 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C, and finally 7 min at 72 °C), a DNA fragment of 346 base pairs was visualized on agarose gels. However, to ensure that the most sensitive PCR methodology was used, two other polymerases and PCR protocols for ASFV detection were also evaluated. One uses the master mix of Eppendorf (Dutcher) which has the advantage that is ready to use. The other is the hotstart immolase DNA polymerase (Bioline, Abcys, France) and was used by Basto et al. (2006) to detect ASFV in ticks. In all PCR runs, a negative control consisting of a dried-blood filter paper from a SPF pig was included. DNA from ASFV-infected cell culture was the positive control. This DNA was extracted with the DNeasy kit (Qiagen).

A one step RT-PCR (Qiagen) was performed on a single punched disc from infected goats, without any extraction of the viral RNA. The primer set NP3/NP4 (Couacy-Hymann et al., 2002), used in the study was designed from the nucleoprotein gene sequences to amplify specifically PPRV. Pieces of filter paper of 5 mm^2 were placed into 0.2 ml PCR tubes and 33 μl of RNase free water were added. The tubes were heated at 95 °C for 10 min and then immediately placed on ice. Seventeen microlitre of reaction mix consisting of 10× PCR reaction buffer, 0.6 μM of each primer [forward: 5′-gTCTCggAAATCgCCTCACAgACT-3, reverse: 5′-CCTCCTCCTggTCCT CCAgAATCT-3′], 400 μM of each dNTP and 2 μl of the Qiagen OneStep RT-PCR Enzyme Mix (Qiagen). After reverse transcription and amplification (30 min at 50 °C, 15 min at 95 °C then 35 cycles, 30 s at 95 °C, 30 s at 53 °C and 30 s at 72 °C, and finally 10 min at 72 °C), DNA bands of the expected 352 bp size were obtained. In the PCR reactions, a negative control was included, which consisted of RNA extracted from non-infected cell cultures with the "Nucleospin RNA virus" kit (Macherey Nagel, France).

2.4. Sequencing and phylogenetic analysis

Phylogenetic analysis was carried out on the sequences obtained from PCR products amplified from filter papers collected from a pig infected with the Lisbon 60 (L60) ASFV strain and from 2 goats, one infected with the PPRV Côte d'Ivoire 1989 (CI89) and the other with the Ethiopian strain (Ethio94). PCR products amplified from filter papers were purified from the gels using the "Qiaquick PCR purification" kit (Qiagen) and directly sequenced by GATC (Germany) using the same PCR primers. Analysis of sequences was performed using Vector NTI-9 package (Invitrogen, USA). Multiple alignments of sequences were done with the Clustal Wallis application included in the Vector NTI package. Sequences were retrieved from Genbank (Table 1) or generated in this study (Michaud et al., unpublished). The alignments were exported in msf file format for conversion in a Phylip 3.2 format using Bioedit software to allow phylogenetic analysis (Hall, 1999). Phylogenetic analysis was carried out using the neighbor-joining method based on the principle of parsimony (Saitou and Nei, 1987), included in the Darwin5 software (Perrier et al., 2003). Dissimilarities and distances between the sequences were first determined by Darwin5 and trees were generated with the TreeCon MATRIXW program (Van de Peer and De Wachter, 1993) included in Darwin5. Tree construction was based on the unweighted neighbor-joining method proposed by Gascuel (1997). Bootstraps were determined on 1000 replicates.

3. Results

3.1. Determination of the detection limit of PCR on filter papers containing dried blood

The limits for detection of virus nucleic acid by PCR amplification from filter papers containing dried blood were determined using serial dilutions of either ASFV or PPRV viruses or corresponding plasmids containing the target gene. The use of FTA cards instead of Whatman 3MM with Taq pfu did not improve the analytical sensitivity of the method (data not shown). The Taq polymerase Bioline, when use for the detection of ASFV

Table 1
List of sequences retrieved from Genbank and used for the phylogenetic analysis

Name of virus isolate	Country of origin	Date of isolation	Reference or year of submission to GenBank	GenBank accession nos
African swine fever virus				
DRC/67	DRC	1967	Zsak et al. submitted 2004	AY578708
Ba71v	Spain	1971	Lopez-Otin et al., 1990	M34142
DR-2	Dominican Republic	?	Yu et al., 1996	L76727
L60	Portugal	1960	Bastos et al., 2003	AF301539
CAM/82	Cameroon	1982	Bastos et al., 2003	AF301544
KEN/64	Kenya	1964	Zsak et al. submitted 2004	AY578697
Haiti/79	Haiti	1979	Zsak et al. submitted 2004	AY578695
E75	Spain	1975	Zsak et al. submitted 2004	AY578693
Mkuzi/79	RSA	1979	Kutish and Rock submitted 2003	AY261362
ZIM/83	Zimbabwe	1983	Zsak et al. submitted 2004	AY578705
Toliara/98	Madagascar	1998	Michaud et al, unpublished	DQ875934
Moronda/02	Madagascar	2002	Michaud et al, unpublished	DQ875935
RSA/96-5	RSA (Noord Biabant)	1996	Zsak et al. submitted 2004	AY578701
RSA/96-4	RSA (Wildebeeslagte)	1996	Zsak et al. submitted 2004	AY578699
RSA/96-3	RSA (Fairfield)	1996	Zsak et al. submitted 2004	AY578696
RSA/96-2	RSA (Nooitverwacht)	1996	Zsak et al. submitted 2004	AY578694
NAM-Warth	Namibia	?	Kutish and Rock submitted 2003	AY261366.
Tengani/62	Malawi	1960	Bastos et al., 2003	AF301541
RSA-Warm	RSA	?	Kutish and Rock submitted 2003	AY261365
RSA/96-1	RSA (Crocodile)	1996	Zsak et al. submitted 2004	AY578691
RSA/96-6	RSA (Pretorisuskop)	1996	Kutish and Rock submitted 2003	AY261363
KEN/50	Kenya	1950	Kutish and Rock submitted 2003	AY261360
UGA/65	Uganda	1965	Yu et al., 1996	L27499
Lil20/1	Malawi	?	Yozawa et al., 1994	U03762
Peste des petits ruminants virus				
Nig75/1	Nigeria	1975	Kwiatek et al., 2007	DQ840160
Nig76/1	Nigeria	1976	Kwiatek et al., 2007	DQ840164
Ghana78	Ghana	1978	Kwiatek et al., 2007	DQ840166
Nig75/3	Nigeria	1975	Kwiatek et al., 2007	DQ840162
Nig75/2	Nigeria	1975	Kwiatek et al., 2007	DQ840161
Mali1	Mali	1999	Kwiatek et al., 2007	DQ840192
Iran98	Iran	1998	Kwiatek et al., 2007	DQ840185
Saoudi/7	Saudi Arabia	1999	Kwiatek et al., 2007	DQ840195
Saoudi/8	Saudi Arabia	1999	Kwiatek et al., 2007	DQ840197
Turkey96	Turkey	1996	Kwiatek et al., 2007	DQ840184
Israel95/3	Israel	1995	Kwiatek et al., 2007	DQ840181
Israel/2	Israel	1998	Kwiatek et al., 2007	DQ840178
Israel	Israel	1993	Kwiatek et al., 2007	DQ840173
Iran/3	Iran	1994	Kwiatek et al., 2007	DQ840186
India94	India	1994	Kwiatek et al., 2007	DQ840176
Calcutta	India	1995	Kwiatek et al., 2007	DQ840177
Oman83/2	Oman	1983	Kwiatek et al., 2007	DQ840168
UAE86	United Arab Emirats	1986	Kwiatek et al., 2007	DQ840169
Sudan72	Sudan	1972	Kwiatek et al., 2007	DQ840158
Ethio96	Ethiopia	1996	Kwiatek et al., 2007	DQ840183
Ethio94	Ethiopia	1994	Kwiatek et al., 2007	DQ840175
Guinea88	Guinea	1988	Kwiatek et al., 2007	DQ840170
CI89	Ivory coast	1989	Kwiatek et al., 2007	DQ840199
Burki88	Burkina Faso	1988	Kwiatek et al., 2007	DQ840172
Bissau89	Guinea-Bissau	1989	Kwiatek et al., 2007	DQ840171
Seneg94	Senegal	1994	Kwiatek et al., 2007	DQ840174
Seneg68	Senegal	1968	Kwiatek et al., 2007	DQ840165

in ticks was completely inefficient on dried-blood filter papers either collected on Whatman 3MM or FTA cards. In contrast, the Eppendorf master mix compared to Taq pfu, gave a lower sensitivity on Whatman 3MM but a higher sensitivity on FTA cards. Since FTA cards were more expensive and required extra-steps of washings before running the PCR and since a secondary aim of this project was also to sequence the amplified product, it was therefore decided to use Whatman 3MM and Taq pfu to avoid errors during amplification.

Results for the selected protocol using Taq pfu to amplify target genes spotted as either plasmid, RNA transcript or virus on Whatman 3MM filter papers are shown in Fig. 1. For ASFV, PCR from filter papers detected less than two copies of the VP72 gene and less than one $TCID_{50}$. The detection limit for PPRV was

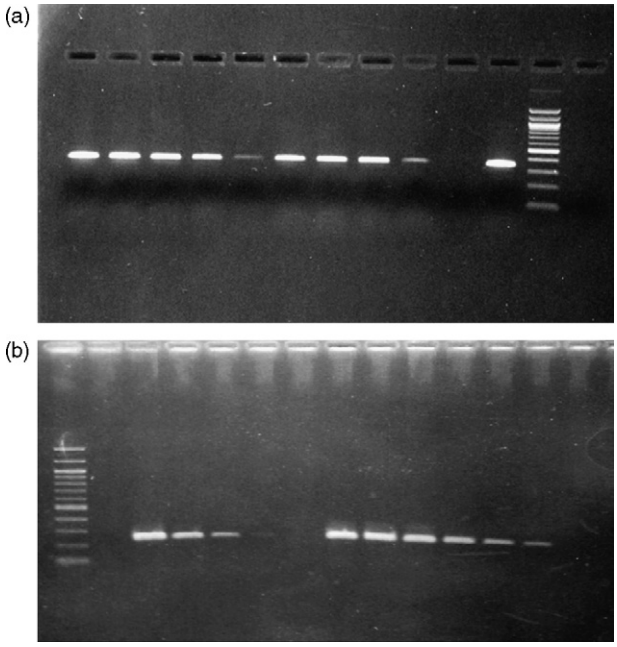

Fig. 1. Determination of the detection limit of direct PCR on blood-dried filter papers. This figure show one of two repetitions of the assay (see results section). (a) Detection of ASFV DNA virus spotted on FP as serial dilutions of either $TCID_{50}$ or plasmid DNA copies containing the VP72 virus gene. Lanes 1 to 5 are 10-fold serial dilutions of ASFV, from 1260 to 0.126 $TCID_{50}$. Lanes 6 to 10 are 10-fold serial dilutions of VP72 plasmid, from 1260 to 0.1260 gene copies. Lanes 11, 12 and 13 are control + (ASFV extracted from infected cell cultures), ladder and control—(DNA extracted from non-infected cell cultures), respectively. (b) Detection of PPRV RNA virus as dilutions of either $TCID_{50}$ or RNA transcript copies from the N gene. Lane 1 is the ladder and lanes 2 and 7 are negative controls (RNA extracted from non-infected cell cultures). Lanes 3–6 are 10-fold serial dilutions of PPRV, from 398 to 0.398 $TCID_{50}$. Lanes 8 to 14 are 10-fold serial dilutions of PPRV N transcripts, from 2×10^9 to 2×10^3.

2×10^4 copies of N-RNA transcript and less than one $TCID_{50}$ (Fig. 1). Nonetheless, the PCR from filter papers was able to detect at least one copy of the N gene cloned into a plasmid (data not shown). The determination of the detection limit for the two viruses was repeated and the method could detect at least two $TCID_{50}$ of each virus spotted on blood-dried filter papers. When the viruses were first diluted in the blood and incubated for 30 min at +37 °C before spotting on the filter papers, there was no loss of sensitivity as compared with the first procedure.

3.2. Application of PCR on filter papers collected from farms or experimentally infected pigs and goats

The direct PCR carried out on blood-dried filter papers collected on 80 farm pigs and 73 farm goats gave a negative result as expected which gives an estimated specificity higher than 96% on the two separate populations and higher than 98% on the whole population. ASFV DNA in blood of infected pigs was detected by PCR in parallel from blood collected in EDTA tubes and dried on filter papers. The results are shown in Table 2. All samples were found negative on the day of the challenge. Three days post-challenge in trial 1, all filter papers were positive by PCR and a good agreement was observed with the results from the samples from EDTA tubes. In trial 2, the results were very similar except that fewer samples were found positive on day 3. All non-infected pigs in trial 2 were found negative both for filter papers and EDTA tubes all over the experimental period. The percentage agreement between PCR from samples collected in EDTA tubes and filter papers (Kappa coefficient) was 89.5%, which is satisfactory (Jakobsson and Westergren, 2005). For both ASFV and PPRV infected animals, the kinetics of virus genome detection in peripheral blood was established (Table 3). Virus was detected from the two infected pigs with the same kinetics, starting from day 8 and lasting for at least 17 days post-challenge. The third pig, placed in direct contact with the two others, developed a DNAemia almost at the same time, suggesting that this animal had been infected at the same time as the other, presumably by inoculum discharges. However, the virus dose received by this contact pig was probably reduced since the DNAemia did not last after day 12 post-challenge. RNAemia in the infected goats was variable according to the virulence of the strain. Infected goats 2 and 3 were infected by highly virulent strains and died at day nine post-challenge. They had PPRV RNA in their blood as soon as day 4 or 5 after the oronasal challenge. In contrast, the two other goats survived the infection and PPRV RNA was detected in their blood, 6 or 9 days after challenge and thereafter.

Table 2
Results of ASFV DNA detection in blood of pigs infected by the intramuscular route with the strain E70 and by the oronasal route with strain L60

Days post-challenge	0		3		5–6		7	
	Filter papers	EDTA blood	Filter papers	EDTA blood	Filter papers	EDTA blood	Filter papers	EDTA blood
Trial 1 (strain E70 intramuscular)								
Pig 1	–	–	+	+	ns	–	ns	ns
Pig 2	–	–	+	ns	+	ns	ns	ns
Pig 3	–	–	+	+	+	ns	ns	ns
Pig 4	–	–	+	+	–	ns	ns	ns
Trial 2 (strain L60 oronasal)								
Pig 5	–	–	–	–	+	+	+	–
Pig 6	–	–	–	+	+	+	dead	dead
Pig7	–	–	–	ns	+	+	dead	dead
Pig 8	–	–	+	+	+	+	dead	dead

Pigs were bled in parallel on EDTA tubes and on filter papers.
ns: not sampled.

Table 3
Results of the detection of ASFV and PPRV genome in blood collected on filter papers from pigs or goats infected experimentally by the oronasal route

Days post-infection	1	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Pig challenge trial																
Infected pig 1	–	–	ns	ns	–	–	+	+	+	+	ns	ns	+	+	+	+
Infected pig 2	–	–	ns	ns	–	–	+	+	+	+	ns	ns	+	+	+	+
Contact pig	–	–	ns	ns	–	–	–	+	+	+	ns	ns	–	–	–	–
Goat challenge trial																
Infected goat 1	–	–	–	–	–	–	–	+	+	+	+	–	–	–	ns	ns
Infected goat 2	–	–	+	+	+	+	–	dead								
Infected goat 3	–	–	–	+	+	+	+	dead								
Infected goat 4	–	–	–	–	+	+	–	–	–	–	–	–	–	–	ns	ns

ns = not sampled.
ASFV strain was the E70. Goats 1 to 4 were infected by PPRV strains Nigeria 75–1 (Nig/75-1, lineage 1, low virulent), Ethiopia 94 (Ethio94, lineage 3, high virulent), Côte d'Ivoire 89 (CI89, lineage 2, high virulent) and India-Calcutta (Calcutta, lineage 4, low virulent), respectively.

3.3. Long-term storage of blood-dried filter papers at high temperatures

Filter papers initially scored positive for ASFV by PCR were still detected as positive after storage for 9 months at 22–25 °C or 37 °C. Filter papers positive for PPRV by PCR were also still positive after 3 months at 32 °C. No loss of sensitivity was observed after this period. Negative filter papers remained negative.

3.4. Genotyping of the strains collected on filter papers

After PCR, the amplicons were purified and sent for sequencing. The ASFV sequence obtained from filter papers was identical to the Lisbon 60 sequence deposited on Genbank (accession no AF449480). The sequence of PPRV strain Ethio94 was identical to the one previously established (Roeder et al., 1994). The other sequence obtained for strain CI89 differed by only one nucleotide. It is believed that the initial sequence determined previously was incorrect at this position (Diallo, personal communication) since a pyrimidine-to-purine transversion (U → G) was observed whereas the other 27 strains sequenced in this study have a G at this position. The sequences generated from filter papers were aligned with other established sequences and phylogenetic trees were generated as described in material and methods. Fig. 2 shows the result for one strain of ASFV and two strains of PPRV belonging to two different lineages. As expected, the ASFV sequence clustered within the group I consisting of European, West African and South American strains. The maximum number of variable positions observed in this 346 bp region was 29 (11.9%). The resulting phylogenetic tree was almost identical to the one produced by Bastos et al. (2003) and the positions of the groups in Fig. 2a was directly derived from their work. Interestingly, the partial sequence of the VP72 gene used in this phylogenetic analysis was not in the same region than the one used by Bastos et al. (2003), thus illustrating that different regions of the VP72 gene may be used for the phylogenetic analysis of ASFV strains. Similarly, CI89 and Ethio94 strains sequenced from PCR products containing the nucleoprotein gene amplified from the filter papers were clustered into lineages 2 and 3 as already established by Dhar et al. (2002) on partial sequence of the fusion protein gene and latter on confirmed by our group on partial sequence of the N gene (Kwiatek et al., 2007). The maximum nucleotide divergence observed in the 255 bp region was 52 (20%).

4. Discussion

Filter papers containing dried blood are interesting sampling systems for the conservation of biological materials when the use of cold chain is impracticable, for instance under extreme climatic conditions encountered in Africa. They can serve for many diagnostic purposes, including the detection of serum proteins like antibodies, genetic material like somatic DNA for the diagnosis of genetic diseases and virus or parasite genomes. A relatively high number of viruses have already been detected on filter papers by PCR among which, the Human immunodeficiency virus (Yourno and Conroy, 1992; Beck et al., 2001), Measles virus (De Swart et al., 2001; Katz et al., 2002; Mosquera et al., 2004), Hepatitis C virus (Abe and Konomi, 1998), dengue virus (Prado et al., 2005), Human papillomavirus (Kailash et al., 2002) and various animal DNA viruses (Hattermann et al., 2002; Wang et al., 2002; Guy-Gonzague et al., 2003) and RNA viruses (Spagnuolo-Weaver et al., 1998; Moscoso et al., 2005; Dubay et al., 2006). This illustrates that sample collection and storage on filter papers can be adapted to a wide range of RNA and DNA viruses. Generally, the use of filter papers requires the pre-treatment of the material in order to extract the biomolecules to be detected and/or to be sequenced. In this study, filter papers were used after long-term storage at high temperatures in a direct PCR test without any previous extraction of nucleic acids. Whatman 3MM filter papers used in this study are cheap and although they are not specifically designed for nucleic acids preservation, they proved to be efficient in this study and others (Kailash et al., 2002). In other publications, FTA Whatman cards were used, which allow cell lysis and the binding of nucleic acids (Beck et al., 2001; Moscoso et al., 2005). These FTA cards have been shown to preserve genetic materials for extended periods of time: 4 years at 22–24 °C (Li et al., 2004) and up to 11 years at ambient tropical conditions (Chaisomchit et al., 2005). However, FTA cards and elution buffer are expensive compared to Whatman 3MM, which can be a handicap for large-scale epi-

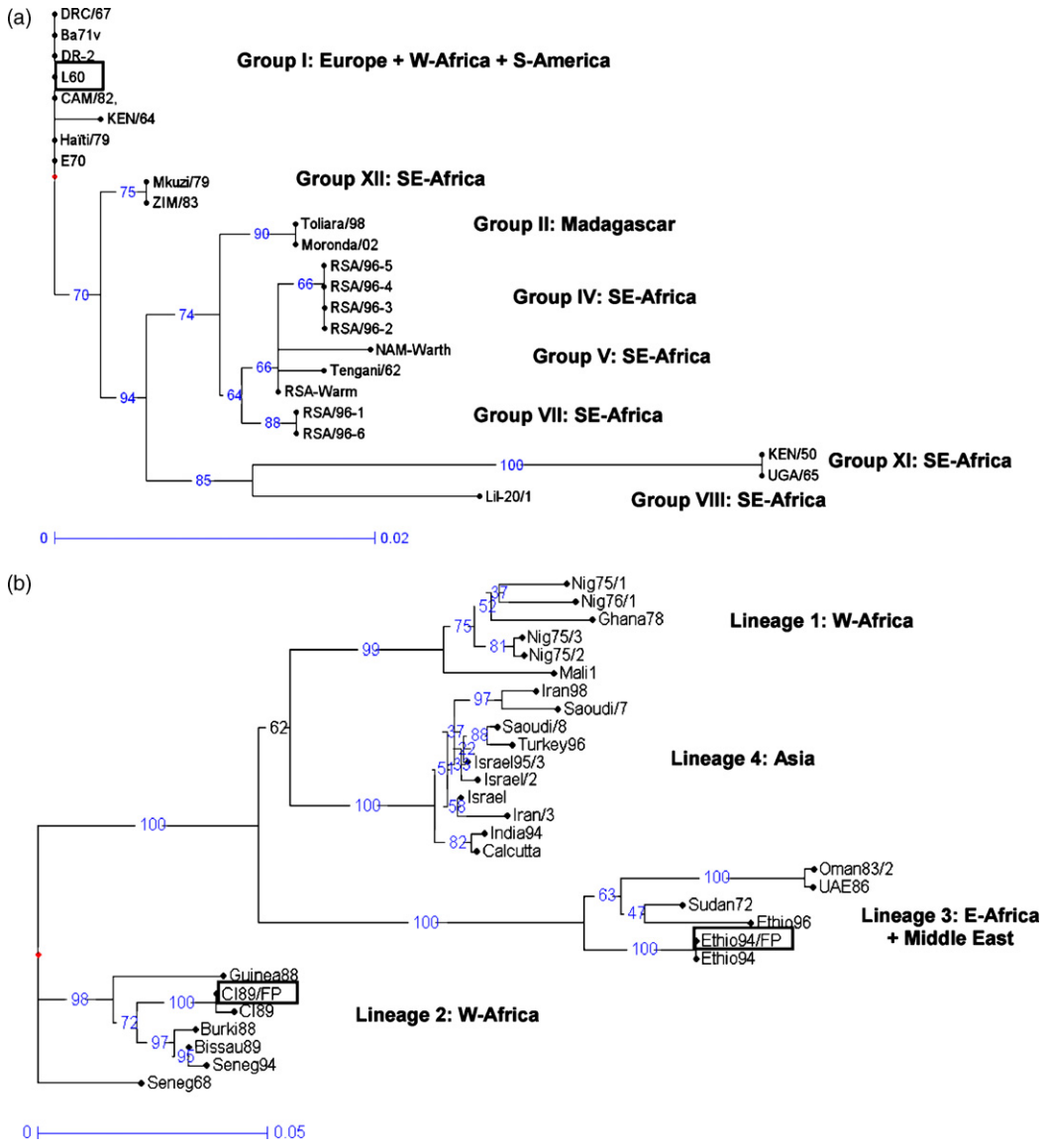

Fig. 2. Phylogenetic analysis of ASFV (a) and PPRV (b). Sequences derived from blood-dried filter papers collected on infected pigs or goats are shown in boxes. Sequencing and analysis of sequences were done as indicated in Material and methods. Consensus trees were generated on 1000 replicates. Only bootstraps higher than 60 are shown. The strains L60 (a), CI89 and Ethio94 (b) amplified and sequenced from filter papers were found in Group I Lineages 2 and 3, respectively as expected according to the work of Bastos et al. (2003), Dhar et al. (2002) and Kwiatek et al. (2007).

demiological surveys. Also, the elution step before PCR adds 1 h to the protocol. Although FTA cards may improve the sensitivity of detection, several factors may make them inconvenient and expensive for routine large-scale surveys. Our data show that Whatman 3MM papers are an effective cheaper alternative. Subsequently, a satisfactory analytical sensitivity was obtained with these filter papers (around 1–2 $TCID_{50}$), which is considered as sensitive enough to detect ASFV infected pigs as illustrated in the *in vivo* trials. In this study, this approach was validated for a DNA virus known to be highly resistant in the environment and a RNA virus which is considered to be very labile outside the host. For the two viruses used in this study, the defined protocol gave an excellent sensitivity (around 1–2 $TCID_{50}$ detected). The discrepancy between the number of DNA copies and tissue culture infectious doses 50 detected (1–10 for ASFV and PPRV) can be ascribed to the fact that viruses which are non-viable but still contain undamaged genome or free genomes are probably released during *in vitro* replication as already reported *in vitro* but also *in vivo* in other virus infection systems (MacLachlan et al., 1994; Tedder et al., 1998; Spagnuolo-Weaver et al., 1998). With PPRV, an important difference in the number of DNA and RNA copies detected on filter papers was noticed: 1 DNA copy versus 2.10^4 RNA copies. This difference is probably more resulting from a reduced yield of cDNA during the reverse transcription of RNA than from RNA denaturation on filter papers. Indeed, Katz et al. (2002) also found a sensitivity of 10^4 copies of measles virus RNA in a single-step RT-PCR done on soluble RNA. Previous studies have shown that filter papers enabled storage of double or single stranded RNA viruses for 1 month at 37 °C (Pitcovski et al., 1999; De Swart et al., 2001) and at least 3 months at 30 °C for ASFV (Guy-Gonzague et al., 2003). In this study, filter papers were allowed to dry shortly after soaking, once dried,

they were stored for extended periods of time. It is shown that the stability can be longer than 9 months at 37 °C for ASFV, thus reinforcing the excellent capacity of filter papers containing dried blood to store genetic material. With the single stranded RNA virus used as a model in this study, filter papers containing dried blood could maintain nucleic acids more than 3 months at 32 °C. PPRV belongs to the same genus *Morbillivirus* as measles virus. In a previous study, measles virus RNA could be stored for 1 month at 37 °C and the sensitivity of the detection was 100 $TCID_{50}$ for a single PCR and 3 $TCID_{50}$ for a nested PCR (Katz et al., 2002). This work shows that with another *morbillivirus* an analytical sensitivity of 1–2 $TCID_{50}$ can be achieved with a single PCR. Although, a relatively limited number of paired samples (blood from EDTA tubes and from filter papers) was used, a satisfactory agreement was found between the two collection materials. Discrepant results were seen for two couples of filter paper/EDTA blood out of 19. These couples pertaining to the trial 2 and collected at days 3 and 7 post-challenge provided inverse results −/+ and +/−, thus giving no clear advantage to one of these collection materials. The use of filter papers is also compatible with genetic characterization of the strains as shown in this study and others (Nerurka et al., 1993; Pitcovski et al., 1999; De Swart et al., 2001). Direct sequencing from filter papers allowed to rapidly group the strains into phylogeographic dendogrammes. In that case, molecular sequencing of the strains is particularly useful to trace the geographic origin of the infection (Mosquera et al., 2004; Verbeeck et al., 2006).

5. Conclusion

The new protocol proposed in this study is rapid, does not need previous extraction of nucleic acids, limits the risk of cross-contamination between samples, allows long term-storage of blood at relatively high temperatures and simplifies shipment to the laboratory without the need for cold chain. The unique constraint is the necessity of using a set of forceps and scissors for individual preparation of filter papers in the direct PCR. However, these instruments can be easily decontaminated and used again. The method described here could be adapted to any DNA and RNA viruses using peripheral blood for circulation within the host. Development perspectives for this method are the use of the same filter paper samples for the combined direct detection of nucleic acids, antibodies and antigens as already done for measles virus (De Swart et al., 2001). In addition, the use of filter papers for quantitative detection of virus genomes will be shortly evaluated as already done before for the duck hepatitis B virus (Wang et al., 2002).

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