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Highly Sensitive PCR Assay for Routine Diagnosis of African Swine Fever Virus in Clinical Samples

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This work provides a novel, highly sensitive, hot start PCR method for rapid and specific detection of African swine fever virus (ASFV) that can be used as a routine diagnostic test for ASFV in surveillance, control, and eradication programs. A confirmatory test of the specificity of this method based on restriction endonuclease analysis was also developed.

African swine fever (ASF) is a highly contagious disease of swine caused by a complex DNA virus that has been classified as an *Asfivirus* (10). It produces great economic losses in the affected countries, due to the high mortality rates of the acute and peracute forms and its potential for extensive and rapid geographical spread. Domestic pigs and European wild boars are very susceptible, showing a wide range of clinical forms (1, 4, 15). African wild boars, bushpigs, and warthogs present unapparent clinical infections, acting as reservoir hosts in Africa (1, 7). Soft ticks, especially *Ornithodoros erraticus* and *Ornithodoros moubata*, are reservoir and transmission vectors of ASF virus (ASFV) (2, 13, 14).

At this time, ASF is present in sub-Saharan countries of Africa, where the disease has acquired great importance since 1997, because of the increasing number of outbreaks that have affected many countries (3). Outside Africa, ASF is currently endemic in Sardinia (Italy).

Epidemiological studies have demonstrated that the entrance of ASFV in ASFV-free areas is primarily related to feeding pigs with contaminated garbage from international airports and seaports (15). This fact, together with the extensive commercial trade, keeps ASFV-free countries at constant risk of having the disease introduced in their territory. Since there is no vaccine available at present, control and eradication strategies are mainly based on rapid and accurate laboratory diagnosis of ASFV-positive and carrier animals and on the enforcement of strict sanitary measures. Furthermore, the great similarity of ASF clinical symptoms and lesions with those of other hemorrhagic pig diseases, particularly classical swine fever, makes differential laboratory diagnosis compulsory in order to distinguish ASF from other pig diseases with compatible clinical presentations (15). Therefore, diagnostic laboratories must have rapid and accurate procedures for specific ASFV detection, such as PCR, available.

Only a few PCR-based ASF diagnostic assays have been previously described for detection and identification of ASFV (8, 16, 17). In this paper, a novel, fast, highly sensitive, and specific gel-based hot start PCR test has been specially designed for routine laboratory diagnosis of ASFV.

Different specific primer pairs for ASFV (in the VP73 coding region of the genome) were selected with the aid of Primer Express (Applied Biosystems) computer program. The nucleotide sequences of the VP73 genes of seven different ASFV strains, available in GenBank (5, 9, 11, 12, 18, 19) were aligned using Clustal W software, to assess conservation of the primer sequences in all strains. The primer set PPA1-PPA2 (PPA-1/2) (PPA1, 5′-AGTTATGGGAAACCCGACCC-3′; PPA2, 5′-CCCTGAATCGGAGCATCCT-3′), which delimits an amplicon of 257 bp, was finally selected as providing the best results. The primer set recommended by the Office International des Epizooties (OIE) (Paris, France), primer 1 (5′-ATGGATACCGAGGGAATAGC-3′) and primer 2 (5′-CTTACCGATGAAAATGATAC-3′) (17), were used for comparison.

Different DNA extraction systems were evaluated, and the method that was the most efficient was selected. Briefly, total DNA was purified from 200 μl of a sample (cell culture samples, serum samples, blood samples treated with EDTA, or tissue homogenate samples in 10% phosphate-buffered saline) by using the High Pure PCR template preparation kit (Roche Molecular Biochemicals), following the manufacturer's instructions. The final elution volume was 50 μl.

Total RNA was extracted from 100 μl of a sample with a commercial reagent (Tripure isolation reagent; Roche Molecular Biochemicals) under the conditions recommended by the manufacturer. RNA was resuspended in 10 μl of MilliQ water.

Optimal conditions for the PCR assay were established as follows: 2 μl of sample DNA, 1× PCR buffer II (50 mM KCl, 10 mM Tris-HCl), 2 mM $MgCl_2$, 0.2 mM concentrations of the four deoxynucleoside triphosphates (Roche Molecular Biochemicals), 0.2 μM concentrations of both primers (PPA-1/2 or the OIE *Manual of Standards for Diagnostic Test and Vaccine* primer pair [primers 1 and 2] described previously [17]) and 0.625 U of *Taq* Gold polymerase (Applied Biosystems), in a total volume of 25 μl. When the PPA-1/2 primer set was used, the reaction mixture was treated as follows: (i) incubated for 10 min at 95°C; (ii) subjected to 40 cycles of PCR, with 1 cycle consisting of 15 s at 95°C, 30 s at 62°C, and 30 s at 72°C; and (iii) incubated for 7 min at 72°C. When the OIE primer set was used, the reaction mixture was treated as follows: (i) incubated

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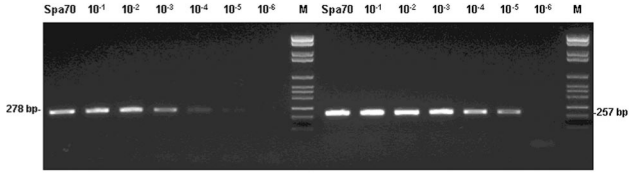

FIG. 1. Sensitivity of the ASFV PCR assay using the OIE primer pair or novel PPA-1/2 primer set. DNAs extracted from serial dilutions, in serum, of a suspension of ASFV strain Spain 70 (Spa70) with a titer of 1.6×10^6 $UHAD_{50}$/ml were employed as templates in the PCR assays under reaction conditions described in the text using OIE primers (left) or novel PPA-1/2 primers (right). M, molecular weight marker VI (Roche Molecular Biochemicals).

for 10 min at 95°C; (ii) subjected to 35 cycles, with 1 cycle consisting of 30 s at 94°C, 30 s at 53°C, and 30 s at 72°C; and (iii) incubated for 7 min at 72°C. Amplification products were analyzed by electrophoresis on a 2% agarose gel containing 0.5 μg of ethidium bromide per ml.

In order to determine the detection limits of the test, PCR assays were performed on serial 10-fold dilutions of a viral suspension of ASFV Spain 70 with a titer of 1.6×10^6 50% hemadsorption units ($HADU_{50}$)/ml. The sensitivity was consistently observed to be 0.12 $HADU_{50}$ per PCR mixture (Fig. 1). The amplification efficiency and detection limit of the assay were further examined in a comparative PCR study using PPA-1/2 and the recommended OIE *Manual of Standards for Diag-*

TABLE 1. Viruses used to test the specificity of the ASFV PCR assay

Virus	Virus
ASFV	Disparate strains
West Indies and South American strains[a,b]	Kanagawa 74
Brazil 78	
Haiti 78	Bovine viral diarrhea virus strains[a,c,d]
Dominican Republic 79	Type 1
European strains[a,b]	Sanders
Spain 70	NADL
Spain 75	Oregon
Spain 94	Type II
Italy Ca78/1	61/138, Low Saxony, Germany
Italy Nu 84WB	61/120, Low Saxony, Germany
Italy Nu 90/1	
Italy Nu 95/2	
Italy Nu 98	Border disease virus strains[c,e,f]
Lisbon 57	X818
Lisbon 60	Moredun
Malta 78	Fritjers
African strains[a]	137/4
Uganda 64	
Mozambique 64	Foot-and-mouth disease virus strains[a]
Katanga 67	Serotype A
Angola 72	A_{22} Iraq (IRQ24/64)
Cape Verde Islands 97	Serotype O
Cape Verde Islands 98	O_1-Manisa/Turkey 69
Ivory Coast 99	Serotype C
Nigeria 2001	C_1-Noville Switzerland/65
	Reference strains of the serotypes:
Classical swine fever virus strains[a,c]	Sat 1
Subgroup 1.1	Sat 2
Alfort 187	Sat 3
Eystrup	Asia 1
Subgroup 1.2	
Brescia	Vesicular stomatitis virus serotypes[a]
Subgroup 2.1	Indiana
Spain 97	New Jersey
V1240/97 Germany	
Subgroup 2.2	Swine vesicular disease virus[a]
Parma 98	Reference strain UK-72
Subgroup 2.3	
2699/Osterode 82	Porcine respiratory and reproductive syndrome strains[a]
Switzerland IV/93	European
1189/1 Belgium	5710
VI3837/38 Germany 99	American
Spain 2001	VR2332
Barcelona 2001	
Visbek/Han 95	Aujeszky disease virus[a]
	Phylaxia reference strain

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[c] Classical swine fever virus European reference laboratory, Institute of Virology, Hannover, Germany.
[d] Veterinary Laboratories Agency, Weybridge, United Kingdom.
[e] Elizabeth Macarthur Agricultural Institute, Camden, Australia.
[f] Institute for Animal Science and Health-Lelystad, Lelystad, The Netherlands.

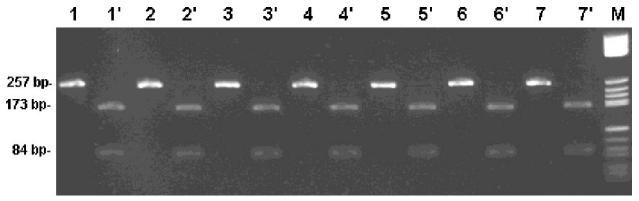

FIG. 2. *Bsm*AI restriction endonuclease analysis of amplification products of different ASFV strains. Lanes 1 and 1′, Brazil 78; 2 and 2′, Haiti 78; 3 and 3′, Spain 70; 4 and 4′, Lisbon 60; 5 and 5′, Cape Verde Islands 97; 6 and 6′, Ivory Coast 99; 7 and 7′, Nigeria 2001. Lanes 1, 2, 3, 4, 5, 6, and 7 are amplification products. Lanes 1′, 2′, 3′, 4′, 5′, 6′, and 7′ are amplification products after digestion with *Bsm*A. Reaction conditions are described in the text. M, molecular weight marker V (Roche Molecular Biochemicals).

nostic Test and Vaccine primer set (17) (Fig. 1). The sensitivity of the assay was increased 10-fold when the PPA-1/2 primer set was used.

The specificity of the PCR was tested using DNA from 22 different ASFV isolates from different geographical origins and isolation years and DNA or RNA from related viruses (Table 1). In addition, DNA from tissue homogenates (spleen, kidney, and tonsils), blood samples treated with EDTA, and serum samples from healthy pigs, noninfected tissue homogenates from *O. erraticus*, and several noninfected cell lines (PK15, MDBK, and BHK-21) was employed in the PCR specificity test. PCR gave positive results for all 22 ASFV isolates, but PCR did not give any positive results when other related viruses, cell lines, or noninfected tissue samples were assayed (data not shown).

Sequence analysis of the genome region delimited by the PPA-1/2 primer set enabled us to determine the location of a *Bsm*AI restriction endonuclease site, splitting the amplicon into two fragments of 173 and 84 bp in length. The *Bsm*AI site was conserved in the sequences of the seven isolates available in GenBank.

*Bsm*AI digestion of the amplification products were performed in 20-μl reaction mixtures, using 5 μl of the PCR amplification product and 5 U of the enzyme. The reaction mixtures were incubated for at least 2 h at 55°C, and restriction fragments were analyzed by electrophoresis on a 3% agarose gel. *Bsm*AI digestion of the corresponding amplicon from the 22 ASFV isolates resulted in the two expected specific restriction fragments in all cases. The results obtained after analysis of seven representative isolates of the 22 ASFV isolates are shown in Fig. 2.

In order to assess the validity of the method in clinical specimens, samples from pigs experimentally inoculated with two different ASFV isolates were tested. Serum and blood samples obtained at 0, 1, 2, 3, 4, and 7 days postinoculation (dpi) from two pigs (1-year-old) intramuscularly inoculated with 10^4 $HADU_{50}$ of the Spain 75 strain, which has low virulence, were analyzed by PCR using the novel PPA-1/2 primer set and the OIE primer set. The results are shown in Fig. 3. The ASFV genome was detected 2 dpi in blood samples treated with EDTA when the PPA-1/2 primers were employed, 2 days before the first clinical signs (fever) were observed, which indicates the power of this procedure for early diagnosis. When the OIE primers were used, positive results were detected by PCR at 3 dpi. Similarly, blood, kidney, liver, lung, lymph node, spleen, and tonsil samples obtained at 5 dpi from a 3-month-old pig experimentally inoculated with 10^7 $UHAD_{50}$ of Lisbon 60 strain were analyzed by PCR using the PPA-1/2 primer set (Fig. 4) or the OIE primer set (data not shown). All the samples analyzed gave positive results by both tests.

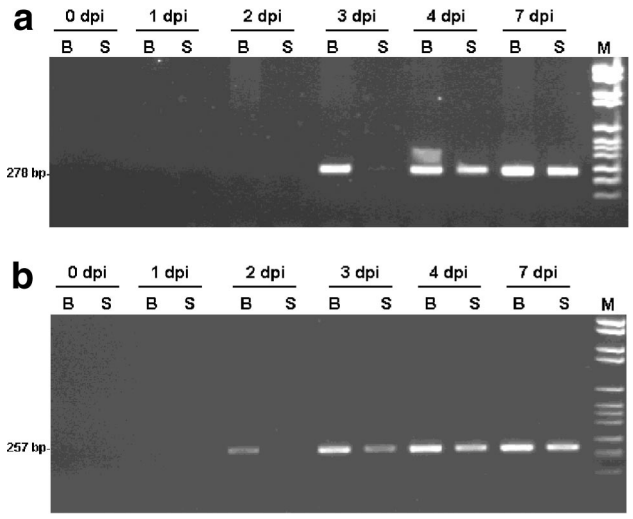

FIG. 3. ASFV detection by PCR in blood (B) or serum (S) samples from experimentally infected animals using OIE (a) or PPA-1/2 (b) primer pairs. Samples of pigs were obtained at different dpi, as described in the text. M, molecular weight marker VI (Roche Molecular Biochemicals).

To determine the sensitivity of the PCR method on badly preserved samples, pieces of kidney tissue obtained from a pig experimentally infected with ASFV strain Lisbon 60 were stored at room temperature for 0, 14, or 28 days, homogenized, and analyzed by PCR using PPA-1/2 and OIE primer sets. The viral genome was detected when both primer sets were used, even after 28 days of tissue sample storage at room temperature when the samples were putrefied (Fig. 5). However, a stronger signal was obtained when the PPA-1/2 primer set was employed.

Finally, a retrospective study was also performed by analysis of a collection of 70 frozen samples, including 18 serum samples, 4 blood samples treated with EDTA, and 48 tissue samples recovered from ASF outbreaks that occurred in Spain

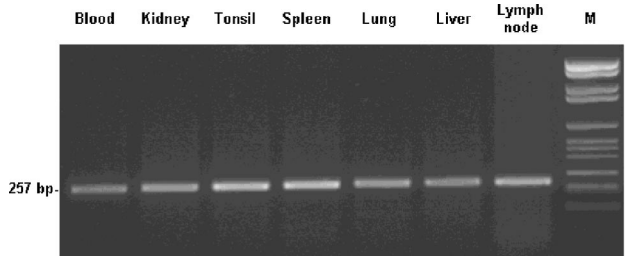

FIG. 4. ASFV detection by PCR in blood and pig tissue samples. Samples of blood (treated with EDTA), kidney, liver, lung, lymph nodes, spleen, and tonsils were obtained from a pig experimentally inoculated with Lisbon 60 at 5 dpi and analyzed by PCR using PPA-1/2 primers. M, molecular weight marker VI (Roche Molecular Biochemicals).

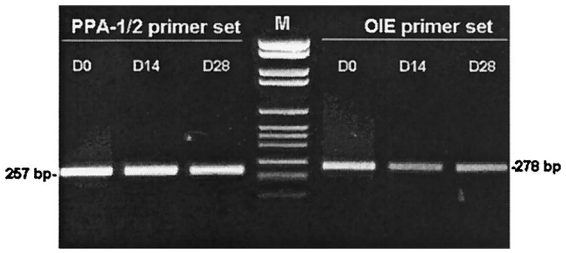

FIG. 5. PCR detection of ASFV on badly preserved samples. Pieces of kidney from a pig infected with ASFV isolate Lisbon 60 were homogenized and analyzed by PCR using either the PPA-1/2 or OIE primer set after 0, 14, or 28 days of storage at room temperature. M, molecular weight marker VI (Roche Molecular Biochemicals).

from 1960 to 1994 and, more recently, in Cape Verde Islands (1997 and 1998), Ivory Coast (1999), and Nigeria (2001). These samples were previously confirmed to be ASFV positive by virus isolation or hemadsorption or by FAT reference tests (and for the samples obtained more recently, by the OIE PCR protocol [17]). All samples gave positive results by PCR when the PPA-1/2 primer pair was used (data not shown).

In summary, the novel ASFV PCR assay possesses a higher sensitivity (0.12 $HADU_{50}$) than that recommended by the OIE *Manual of Standards for Diagnostic Test and Vaccine* (17) and allows the detection of ASFV strains worldwide, as it was established after the analysis of 22 ASFV strains from different years and geographical locations. This method is both rapid (the results can be obtained in less than 5 h) and specific (no false-positive reactions were observed when samples from healthy pigs, noninfected ticks, uninfected cell lines, and related porcine viruses were assayed). Other advantages of this method is that it has been standardized so that it can be used on tissue samples, blood samples treated with EDTA, and serum samples, and it can be performed even on poorly preserved or putrefied tissues, while previous PCR methods did not have such wide applications (8, 16, 17). The ability to check the specificity of the amplicons by simple restriction endonuclease digestion, instead of sequencing, broadens the range of laboratories in which this diagnostic technique can be used with confidence.

In addition, the results of this study show that the DNA extraction method used efficiently removes potential inhibitors that are present in the clinical samples, since all the ASFV-positive tissue, serum, and blood samples analyzed were confirmed by PCR. A simpler DNA preparation procedure has been described previously (16, 17) which sometimes gives false-negative results, as recently demonstrated by Gonzague et al. (6), probably due to the presence of inhibitors which are not removed during sample preparation.

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