JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1992, p. 1–8
0095-1137/92/010001-08$02.00/0

Vol. 30, No. 1

Rapid and Biologically Safe Diagnosis of African Swine Fever Virus Infection by Using Polymerase Chain Reaction

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Received 10 June 1991/Accepted 27 September 1991

In order to circumvent the need for infectious virus for the diagnosis of African swine fever (ASF), we established the polymerase chain reaction (PCR) technique for the detection of ASF virus (ASFV) DNA. A 740-bp fragment that originated from the conserved region of the viral genome was partially sequenced. From this sequence, four PCR primers and one oligonucleotide probe were designed and synthesized. A specific 640-bp PCR product was amplified by using oligonucleotides 1 and 5 as primers and extracts of the following samples as templates: organs and plasma obtained from ASFV-infected pigs, ASFV-infected cell cultures, and cloned DNA fragments containing the same conserved genomic region as that in the original 740-bp clone. No specific reaction products were observed in the corresponding controls. The identities of the PCR products were confirmed either by a second amplification with nested primers or by hybridization with a specific, biotinylated oligonucleotide probe. PCR proved to be a quicker and more sensitive method than virus isolation followed by the hemadsorption test when spleen and plasma samples from experimentally ASFV-infected pigs were tested. Furthermore, cloned virus DNA could be used as a positive control in the place of a live virus control. This is advantageous whenever the use of live virus is undesirable.

African swine fever (ASF) is a lethal and economically important disease of domestic pigs. Outside of the natural reservoir in Africa, ASF is enzootic in certain parts of Europe (Portugal, Spain, Sardinia). Occasional outbreaks have been reported recently in Belgium (1985) and The Netherlands (1986), indicating that this disease is a constant threat for ASF-free countries (3). For this reason, the availability of diagnostic tools for rapid detection and identification of the causative agent, ASF virus (ASFV), is essential.

The diagnostic procedures, however, are not simple. Wild-type virus strains do not replicate in conventional cell cultures. Porcine macrophage cultures must be kept ready in order to isolate the virus. An isolate may be identified as ASFV by the hemadsorption test, according to Malmquist and Hay (22). Yet, the existence of nonhemadsorbing virus strains has been reported (30). Although virus isolation is considered the most sensitive test for the diagnosis of ASF, it may be hampered by the fact that, under certain circumstances, the macrophages are not able to support replication of ASFV. The existence of macrophage cultures with reduced susceptibility for ASFV replication has been reported (1). Preliminary data suggest that activated macrophages may be resistant to infection with ASFV (24). For the reasons given above, it is imperative that the susceptibilities of the diagnostic macrophage cultures to infection with control virus be tested. However, many laboratories cannot include this essential control because the use of live ASFV is prohibited by national authorities for reasons of biological safety.

Similar problems are encountered with the identification of ASFV antigens in diagnostic samples by immunological methods (34).

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The organization of the ASFV genome is strikingly similar to that of the *Poxviridae*; i.e., the double-stranded DNA molecule of about 170 kbp contains terminal inverted repetitions and hairpin loop structures that link both strands at the ends (14, 35). A conservative central region of approximately 125 kbp is flanked by variable ends (5).

Tabares (37) and Caballero and Tabares (7) pioneered the potential for biologically safe diagnosis when they described the cloning of a viral DNA fragment from the conserved region of the ASFV genome and its application to the identification of ASFV DNA by hybridization. The genomes of a variety of ASFV strains have been cloned into various vectors by different laboratories (12, 21, 27).

The polymerase chain reaction (PCR) is an in vitro method for the primer-directed enzymatic amplification of specific DNA sequences (32). By the use of PCR, we intended to combine the advantages of the virus isolation method (the most sensitive method) and the hybridization method (the biologically safest method).

In this report, we describe the application of PCR for the diagnosis of ASF. In a first step, a DNA fragment originating from the conserved region of the ASFV genome was partially sequenced. Five oligonucleotides were subsequently synthesized. These oligonucleotides were used as primer pairs for conventional PCR, for PCR with nested primers, and for the identification of the PCR products by hybridization. By these means we were able to amplify cloned ASFV DNA as well as the ASFV DNA present in biological samples (plasma, sera, spleen, lymph nodes) of experimentally infected pigs. PCR proved to be a practical, sensitive, and specific tool for the detection of ASFV DNA in clinical samples from infected pigs; and it allowed diagnosis of the disease within only a few hours. A broad range of ASFV strains was detected. The components used for the tests were synthesized chemically. Cloned DNA fragments served as controls. Thus, the use of live virus was not required at any stage, which makes this method biologically safe.

TABLE 1. ASFV strains used throughout the PCR experiments[a]

Origin of strain	Designation of isolate	Isolated in:	Yr of isolation	Reference
Europe	France 64	France	1964	19
	Badajoz 71	Spain	1971	13
	Spain 70	Spain	1970	7
	Lisbon 60	Portugal	1960	25
	Malta 78	Malta	1978	40
	Sardinia 82	Italy	1982	9
Africa	Malawi 83 (Lil 30/20)	Malawi	1983	17
	Malawi 83 (Lil 20/1)	Malawi	1983	17
	Uganda (virulent)	Uganda	1960	10
	Kirawira (KWH/12)	Tanzania	1967	15
	Rhodesia (RHO 61/1)	Kenya	1954	11

[a] With the exceptions of strains France 64 and Spain 70, the viral isolates were kindly supplied by P. J. Wilkinson.

MATERIALS AND METHODS

Viral strains, cosmids, and plasmids. The ASFV strains used throughout this study have been characterized previously (Table 1). The genomic locations of the cloned ASFV DNA fragments are indicated in Fig. 1a.

The plasmids pRB, pRC, and pRG represent part of a genomic library obtained from ASFV strain Badajoz 71 (13) and were a generous gift from Eladio Viñuela, Madrid, Spain. The construction and characterization of these plasmids have been described by Ley et al. (21) and Almendral et al. (2).

The plasmid pRPEL2 containing a fragment of the conserved region of ASFV strain Spain 70 was kindly supplied by Enrique Tabares (7).

Cosmid CM15 (cosCM15) was cloned from genomic DNA of ASFV strain France 64 (26, 27).

A 740-bp *Hin*dIII fragment of cosCM15 was subcloned into the Bluescript SKII(+) vector by standard procedures (23). This plasmid was designated pYS.

Cell cultures and virus propagation. (i) Propagation of wild-type ASFV (France 64) in macrophage cultures and hemadsorption test. Propagation of wild-type ASFV (France 64) in macrophage cultures and the hemadsorption test were performed as described previously (22). Briefly, porcine monocytes were isolated from peripheral blood buffy coats by centrifugation through Ficoll-Paque (Pharmacia, Uppsala, Sweden) followed by adsorption onto Falcon tissue culture flasks (25 cm^2). The cells were incubated for 3 days in medium 199 containing 10% (vol/vol) fetal bovine serum at 37°C before the medium was replaced and the cultures were infected with 0.5 ml of ASFV (10^8 50% tissue culture infective doses per ml). After a further 3 days of incubation at 37°C, a freshly prepared suspension of porcine erythrocytes was added in order to test for hemadsorption.

(ii) Propagation of CV-1 cell-adapted ASFV (Lisbon 60). CV-1 cells were propagated in 25-cm^2 Falcon tissue culture flasks by standard protocols (13).

Confluent monolayers were infected with 5×10^6 50% tissue culture infective doses of ASFV per flask. After 24 h, a 90 to 100% cytopathic effect could be observed.

Sequencing and sequence analysis. Cloned DNA was sequenced by the dideoxy sequencing method (23) by using [α-^{35}S]dATP (Amersham, Buckinghamshire, United Kingdom) and the Pharmacia T7 DNA sequencing kit according to the protocol of the supplier (Pharmacia). Oligonucleotide primers KS and SK designed for sequencing of DNA cloned in the pBluescript plasmid (pBluescript Exo/Mung DNA sequencing system) were obtained from Stratagene (Stratagene, La Jolla, Calif.).

Electrophoresis was carried out in 6% (wt/vol) polyacrylamide gels containing 7 M urea at 1,800 V in a Sequi-Gen nucleic acid sequencing cell (Bio-Rad, Richmond, Calif.).

The sequencing data were compared with published data deposited at the EMBL data base (release 25; European Molecular Biology Laboratory, Heidelberg, Germany) by using the FastA search program of W. Pearson (29a).

Oligonucleotide synthesis and biotinylation. Oligonucleotides synthesized at a 0.2-mmol range in a model 381A DNA synthesizer (Applied Biosystems Inc., Foster City, Calif.) were obtained from the Zoological Institute of the University of Zürich, Zürich, Switzerland.

The 3'-end biotin labeling of synthetic oligonucleotides was achieved by using biotin-16-UTP (Boehringer, Mannheim, Germany) and terminal deoxynucleotidyl transferase supplied by Pharmacia, as described by Guitteny et al. (16).

PCR. For PCR, the GeneAmp DNA amplification kit containing the cloned *Thermus aquaticus* polymerase (AmpliTaq; Perkin-Elmer Cetus, Norwalk, Conn.) was used according to the protocol of the supplier. Amplifications were carried out in 10 mM Tris HCl (pH 8.3)–50 mM KCl–1.5 mM $MgCl_2$–0.01% (wt/vol) gelatin–200 μM (each) deoxynucleoside triphosphate–0.05 μM (each) primer–2.5 U of AmpliTaq. The final reaction volume was 100 μl, including 5 to 20 μl (1.2 μg to 1.2 fg) of target (DNA template) (31).

As a standard procedure, 35 consecutive cycles of thermal denaturation at 94°C (30 s), primer annealing at 57°C (30 s), and primer extension at 74°C (60 s) were carried out in a Thermocycler 60 (Biomed, Theres, Germany).

Usually, 5 to 20 μl of the reaction product was analyzed electrophoretically (60 V, 1 h, TAE buffer [1× TAE is 0.04 M Tris acetate, 0.002 M EDTA; pH 8.5]) in a 1.8% (wt/vol) agarose gel. The bands were visualized after staining with ethidium bromide.

Primers for the specific amplification of pseudorabies virus DNA were synthesized and used as described by Bélak et al. (4).

Methods of sample preparation. Precautions were taken in order to prevent the well-known problems of contamination (20). The precautions included the preparation of samples and reaction mixtures in completely separate laboratories. Furthermore, samples for PCR were prepared with as few steps as possible, as described by Bélak et al. (4). Serum, plasma, as well as various spleen, lymph node, and erythrocyte samples were collected in previous experiments (and stored at −20°C) from experimentally infected (ASFV strain France 64) and noninfected pigs (19). Organs obtained from pigs infected with a variety of European and African strains of ASFV (Table 1) as well as matched organ controls were kindly supplied by P. J. Wilkinson.

(i) Serum and plasma samples. Serum or plasma (100 μl) was diluted in 1 ml of phosphate-buffered saline (PBS). The samples were vortexed, boiled for 15 min, and centrifuged in an Eppendorf centrifuge at 14,000 rpm for 10 min. A total of 10 to 20 μl of the supernatant was used for PCR.

(ii) Organs. Pieces of organs (20 mg) were homogenized and diluted in PBS (0.2% [wt/vol]). The samples were boiled and centrifuged as described above. A total of 5 μl of the supernatant was used for the PCR (4).

(iii) Erythrocyte samples. A total of 500 μl of lysis buffer (0.32 M sucrose, 10 mM Tris HCl [pH 7.5], 5 mM $MgCl_2$, 1% [vol/vol] Triton X-100) and 500 μl of whole blood was mixed and centrifuged for 20 s, at 14,000 rpm in an Eppendorf centrifuge. The supernatant was discarded, and the pellet

was resuspended in 1 ml of lysis buffer and recentrifuged. After repeating this step twice, the pellet was resuspended in 200 μl of lysis buffer supplemented with 2 μl of proteinase K (20 mg/ml). After an incubation period of 1 h at 60°C, the proteinase K was inactivated (10 min at 100°C) and the samples were centrifuged for 5 min at 14,000 rpm. A total of 5 μl of the supernatant was taken for PCR.

(iv) Cell cultures. ASFV-infected and mock-infected macrophage cultures were disintegrated by repeated freezing and thawing. The resulting suspensions were transferred to separate Falcon tubes and centrifuged at 700 × *g* for 10 min. The pellets were resuspended in 1 ml of PBS, transferred to Eppendorf tubes, boiled, and centrifuged as described above. Amounts of 10 to 20 μl of supernatant were used for PCR (41).

ASFV-infected and mock-infected CV-1 cells were harvested by scraping the tubes with a disposable rubber policeman. The suspensions were transferred to separate Falcon tubes and treated exactly as described above for infected and uninfected macrophage cultures.

Identification of PCR products. The PCR products were identified by at least one of the following three methods: (i) determination of the apparent molecular mass of the PCR product by agarose gel electrophoresis; (ii) amplification of the primary PCR product by using a second set of primers (nested primers); or (iii) hybridization of the PCR product with specific probes.

Determination of the molecular mass of the PCR product. A total of 10 μl of PCR product was separated electrophoretically in a TAE-buffered 1.8% (wt/vol) agarose gel (60 V, 1 h; Bio-Rad gel apparatus) before being stained with ethidium bromide, according to standard procedures (23). *Hin*fI-cleaved pBR328 (Boehringer) was used as a molecular mass standard (in base pairs), as follows: 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 298, 234, 220, 154, 154.

Nested primers. After a first round of PCR, a sample of the PCR product (0.1 to 1 μl) was removed and amplified in the presence of a second pair of primers (28). The reaction conditions were the same as described above for the first round of amplification.

The reaction products (5 to 20 μl) were separated by agarose gel electrophoresis and visualized by ethidium bromide staining as described above.

Hybridization of the PCR products. The electrophoretically separated DNA samples were transferred to Hybond N^+ nylon membranes according to the instructions of the manufacturer (Amersham).

Prehybridization of the membranes was carried out for at least 1 h at 65°C with 5× SSPE (1× SSPE consisted of 0.18 M NaCl, 0.01 M sodium phosphate, 0.002 M EDTA [pH 7.7]), 5× Denhardt solution (23), and 0.625% (wt/vol) sodium dodecyl sulfate (SDS) in the presence of 10 μl (50 mg/ml) of heparin (Sigma Co., St. Louis, Mo.) per 10 ml of prehybridization solution.

The membranes were hybridized overnight at 65°C in prehybridization buffer supplemented with 40 μl (10 mg/ml) of sonicated and denatured herring sperm DNA (Boehringer) per 10 ml of hybridization solution and the [α-^{32}P]dATP-labeled, 440-bp PCR product. The DNA labeling was carried out by using the Multiprime Labelling Kit (Amersham).

Subsequently, the membranes were washed twice at room temperature for 10 min each time with 2× SSPE–0.1% (wt/vol) SDS, once for 30 min at 65°C with 1× SSPE–0.1% (wt/vol) SDS, and once for 30 min at 65°C with 0.1× SSPE–0.1% (wt/vol) SDS. After washing, the wet membranes were sealed in hybridization bags and exposed for autoradiography with Hyperfilm-MP (Amersham) at −70°C.

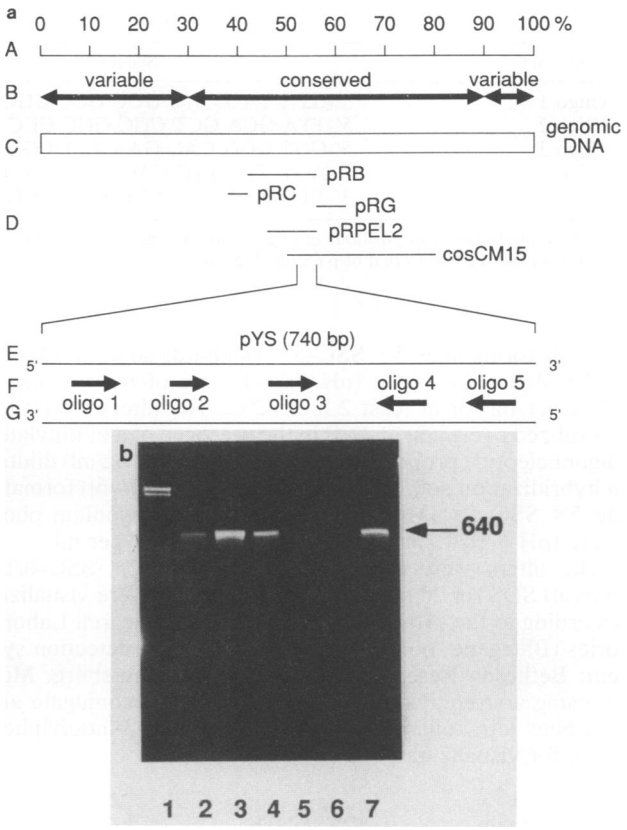

FIG. 1. (a) Relative localization of the cloned ASFV DNA and the synthetic oligonucleotides used in the PCR experiments. (A) Map units of the ASFV genome. (B) Schematic representation of the variable and conserved regions within the viral genome. (C) Schematic representation of the ASFV genome with covalently linked ends. (D) Localization of the cloned ASFV DNA fragments relative to each other and to the viral genome. (E) Blowup and orientation of the positive strand of pYS. (F) Positions and orientations of oligos 1 through 3 on the positive DNA strand and of oligos 4 and 5 on the negative DNA strand. (G) Blowup of the negative strand of pYS. (b) The PCR primers oligo 1 and oligo 5 were used to amplify cloned ASFV DNA originating from different parts of the viral genome. The products were separated electrophoretically (1.8% agarose gel, stained with ethidium bromide). Molecular mass markers are given in lane 1. The specific PCR product of 640 bp could be observed when cosCM15 (lane 2) or the plasmids pYS (lane 3), pRPEL2 (lane 4), and pRB (lane 7) were used as templates. No positive test signal could be obtained from clones pRG and pRC (lanes 5 and 6). The arrow points to the 640-bp PCR product.

Alternatively, dot blotted PCR products were identified by hybridization to a biotinylated oligonucleotide probe. PCR product (10 μl) was diluted in 100 μl of 20× SSC (1× SSC consisted of 0.15 M NaCl plus 0.015 M trisodium citrate [pH 7.0]) and was denatured by boiling for 10 min. The probes were then transferred to enzyme-linked immunosorbent assay plates and diluted in 10× SSC to 1:10,000 of the original concentration. By using a Bio-Rad dot blot apparatus, 100 μl of each dilution was transferred to HYBOND-C EXTRA nitrocellulose filters (Amersham), which had been presoaked in 10× SSC. The filters were baked for 2 h at 80°C in a vacuum oven and subsequently rehydrated in 2× SSC.

Prehybridization of the membranes was carried out in 50%

TABLE 2. Description of the synthetic oligonucleotides

Designation[a]	Sequence	Length (bp)	Strand orientation[b]
Oligo 1	5′-GTA TAG GAG GGC GCC GGC TT-3′	20	Positive
Oligo 2	5′-TTA GCA GCT CGC GGC GCC T-3′	19	Positive
Oligo 3	5′-GGT GGG CAC GAA TTT CGG ACA GT-3′	23	Positive
Oligo 4	3′-AGT TTG TTC CAC TGC TCC GAA C-5′	22	Negative
Oligo 5	3′-TTG AAA CCG GTG GTC ACG GA-5′	20	Negative

[a] The approximate map positions of oligos 1 to 5 are given in Fig. 1a.
[b] Orientation relative to that of pYS (cf. Fig. 1a).

(wt/vol) formamide–5× SSC–5× Denhardt solution (23)–25 mM sodium phosphate (pH 6.5)–0.5 mg of herring sperm DNA per ml for at least 2 h at 42°C. The filters were then hybridized overnight at 42°C in the presence of a biotinylated oligonucleotide probe (final concentration, 250 ng/ml) diluted in hybridization solution consisting of 45% (wt/vol) formamide–5× SSC–1× Denhardt solution– 20 mM sodium phosphate (pH 6.5)–0.2 mg of herring sperm DNA per ml.

The filters were then washed twice in 2× SSC–0.1% (wt/vol) SDS for 20 min at 42°C. The signals were visualized according to the protocol of the Bethesda Research Laboratories (Bluegene, nonradioactive nucleic acid detection system; Bethesda Research Laboratories, Gaithersburg, Md.) by using a streptavidin-alkaline phosphatase conjugate and nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate for visualization.

RESULTS

Primer designation. In order to select primer pairs suitable for the amplification of ASFV DNA by PCR, a 740-bp DNA fragment, pYS, which was subcloned from the conserved central region of ASFV, was partially sequenced. Although a computer search indicated that the resulting ASFV sequences were relatively unique among animal viruses, a certain homology to vaccinia virus DNA was observed. A sequence of maximal homology (78.9%) within 52 bp was found, as were several regions with less homology.

Five regions (termed oligonucleotides [oligo] 1 through 5) consisting of 19 to 23 nucleotides were selected for oligonucleotide synthesis, in order to be used either as primers for the PCR or as tools for the identification of the PCR product. Repetitive and GC-rich regions, as well as sequences homologous to each other, were not included as much as possible. The genomic map positions of these oligonucleotides are shown in Fig. 1a, while the sequences are shown in Table 2.

The regions of oligos 1 and 5 were also sequenced with DNA obtained from ASFV strain Spain 70. Homology of 100% was observed (data not shown).

With the exception of oligo 5, none of the synthetic nucleotides showed significant homology to published viral sequences. Interestingly, oligo 5 was apparently partially homologous to sequences found within the glycoprotein C gene of herpes simplex virus (86.6% identity in a 15-bp overlap), within the genome of Epstein-Barr virus (86.7% identity in a 15-bp overlap), and the glycoprotein I gene of bovine herpesvirus type 1 (100% identity in an 11-bp overlap). Furthermore, stretches of identity were found within the genomes of encephalomyocarditis virus, mouse hepatitis coronavirus, and equine influenza virus. It must be mentioned, however, that the homologous regions always included the 5′ but not the 3′ ends of this oligonucleotide. For this reason, it could be expected that this oligo 5 would probably bind to non-ASFV DNAs without functioning as an initiator of chain elongation (36).

Amplification of cloned ASFV DNA by PCR. The two outmost oligonucleotides, termed oligos 1 and 5, were used to amplify cloned ASFV DNA by PCR. The map positions of the templates are shown in Fig. 1a. pRB, pRPEL2, and cosCM15 originated from the same genomic region as pYS. They represented different viral strains, however. As expected, a PCR product of approximately 640 bp was generated with these primers and templates. Cloned fragments originating from adjacent genomic regions (pRC and pRG) were used as controls. With these templates, no PCR product was generated. These results are shown in Fig. 1b.

It could be concluded from these experiments that the primers oligo 1 and oligo 5 were appropriate for further applications.

Specificities of primers oligo 1 and oligo 5. In order to test the specificity of the reaction by using primers oligo 1 and oligo 5, various samples originating from different biological sources were used as templates. A number of controls were included.

Vaccinia virus DNA was not amplified. This control was included because vaccinia virus is, in terms of its genome structure and organization, closely related to ASFV (6, 39).

Primers used for the amplification of pseudorabies virus

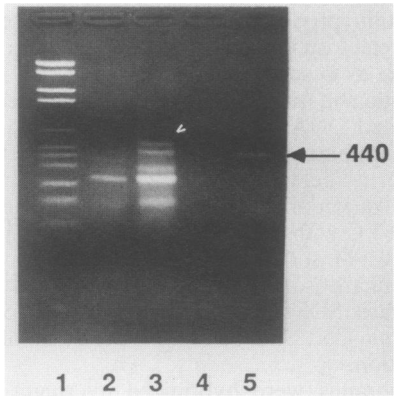

FIG. 2. Application of PCR primers and nested primers on erythrocyte samples obtained from a healthy and an experimentally infected pig. Lane 1, molecular mass marker. ASFV-negative (lane 2) and -positive (lane 3) erythrocyte samples were used after amplification with oligos 1 and 5 and after a second amplification with the nested primers oligo 2 and oligo 4 (lane 5). The specific product (440 bp) was absent in the negative control (lane 4). A total of 5 μl of each reaction was loaded onto a 1.8% agarose gel and stained with ethidium bromide. The arrow points to the 440-bp PCR product. The arrowhead indicates the approximate position of the 640-bp PCR product.

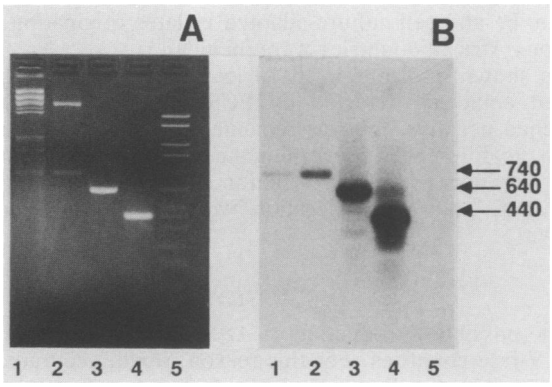

FIG. 3. In order to show the homology of the second PCR product to the cloned ASFV DNAs as well as to the first PCR product, two experiments were done. (A) The samples (see lane descriptions below) were separated electrophoretically on a 1.8% agarose gel and stained with ethidium bromide. (B) Subsequently, the gel was transferred to a nylon membrane, hybridized with the [α-^{32}P]dATP-labeled, 440-bp nested primer DNA fragment, and autoradiographed. Lane 1, *Hin*dIII-cleaved cosCM15; lane 2, *Hin*dIII-cleaved pYS; lane 3, PCR product obtained after the first amplification; lane 4, DNA fragment obtained with the nested primers; lane 5, molecular mass marker. The arrows point to the DNA fragments, with molecular masses (in base pairs) indicated on the right.

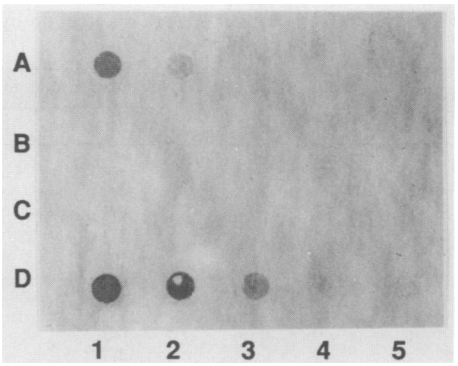

FIG. 4. Application of a biotin-labeled oligonucleotide for identification of the ASFV PCR product. Tenfold dilutions of cloned DNA fragments and PCR products were spotted onto the membrane. The denatured samples were hybridized with the biotinylated oligo 3 probe before visualization with alkaline phosphatase, as described in the text. Row A, 35-ng (A1) through 3.5-pg (A5) samples of *Hin*dIII-cleaved pYS; row B, 1-μg (B1) through 0.1-ng (B5) samples of *Hin*dIII-cleaved Bluescript SKII(+) vector; row C, dilutions of PCR product obtained from a spleen sample of a healthy pig; row D, dilutions of PCR product obtained from the spleen of an experimentally ASFV-infected pig.

DNA did not react with ASFV DNA or, vice versa, with ASFV-specific primers.

Additional negative controls included suspected contaminants of diagnostic samples. DNAs extracted from normal pig spleen, normal pig macrophages, *Escherichia coli*, and *Erysipelothrix rhusiopathiae* were not amplified.

Finally, DNA extracted from uninfected CV-1 cells, which were used for the propagation of cell culture-adapted strains of ASFV, proved to be nonreactive with the ASFV primers.

In contrast, the samples containing genomic ASFV DNA were specifically amplified by these primers. In some instances, however, nonspecific bands appeared following PCR with this first set of primers. This was a particular problem with samples which contained relatively large amounts of erythrocytes (Fig. 2, lanes 2 and 3).

Identification of PCR products. In addition to the determination of the molecular masses, the PCR products were identified either by the application of a set of nested primers or by hybridization to a synthetic oligonucleotide homologous to a central region situated between the first set of primers.

(i) Amplification of PCR product by nested primers. In cases in which nonspecific products were obtained with the first pair of primers (Fig. 2, lanes 2 and 3), a second round of amplification with the nested primers made the discrimination of positive from negative samples possible (Fig. 2, lanes 4 and 5). For this purpose, oligos 2 and 4 (Fig. 1a and Table 2) were used as a set of nested primers. An aliquot of the reaction product was used for a second amplification. Only probes containing the target DNA gave a second PCR product. As expected, the nested primer set produced a DNA fragment of approximately 440 bp (Fig. 2).

In order to show the homology of the second PCR product to the first PCR product, as well as to the cloned ASFV DNA fragments contained in pYS and cosCM15, the second PCR product was radioactively labeled and was used as a probe for hybridization with the DNAs mentioned above. The results of this hybridization experiment are shown in Fig. 3.

(ii) Biotin-labeled oligonucleotide probe. In order to identify the PCR products by an alternative method, without the need for separation in agarose gels, spleen samples which were positive or negative for the presence of ASFV were subjected to PCR with the primers oligo 1 and oligo 5. After amplification, samples of the PCR products were spotted onto a membrane and hybridized with an ASFV-specific biotinylated oligonucleotide probe (oligo 3; Fig. 1a and Table 2). The results, which were obtained following visualization of the reaction, indicated that specific identification of positive PCR samples was possible by this method (Fig. 4). Several dilutions of the positive spleen sample, as well as at least 3.5 ng of target sequence (pYS), were easily detected.

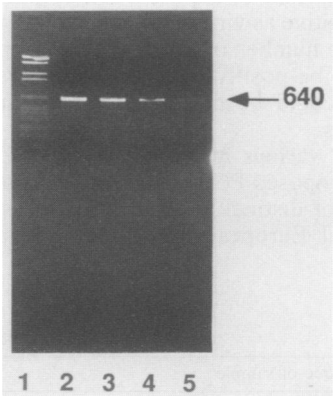

FIG. 5. In order to define the sensitivity of the PCR, *Hin*dIII-cleaved pYS was diluted to a final probe concentration of 1.2 fg/10 μl (representing 1,500 copies of the original template) before amplification. A total of 3 μl of each reaction product was loaded onto the 1.8% agarose gel, electrophoresed, and stained with ethidium bromide. Lane 1, molecular mass marker; lane 2, 1.2 μg of template; lane 3, 120 pg of template; lane 4, 12 fg of template; lane 5, 1.2 fg of template. The arrow points to the 640-bp PCR product.

TABLE 3. Comparison of ASFV detection by hemadsorption and PCR

Sample	Day of collection[a]	No. positive/total no. tested by:	
		Hemadsorption	PCR
Spleen	0	0/4	0/4
	9	1/1	1/1
	11	0/1	1/1
	14	1/1	1/1
	18	1/1	1/1
	107	0/7	2/7
Plasma	0	0/7	0/7
	7	4/7	3/7
	14	0/6	4/6
	25	0/6	3/6

[a] Day collected after experimental infection with ASFV.

Negative samples did not stain, even after prolonged periods of visualization.

Sensitivity of ASFV DNA detection by PCR. (i) Serial dilution of target sequence. In order to quantify the amount of detectable target sequence, pYS was diluted and the DNA concentration was determined. Subsequently, 35 cycles of PCR, using primers oligo 1 and oligo 5, were performed.

Samples representing starting DNA concentrations ranging from 1.2 μg to 1.2 fg were separated by agarose gel electrophoresis. The results are shown in Fig. 5. Even the highest dilution, which represented 1,500 copies of the original template, yielded a faintly visible band on the agarose gel. The corresponding negative controls are not shown.

(ii) Detection of ASFV DNA in tissues of infected pigs. In order to estimate roughly the sensitivity of ASFV DNA detection in diagnostic probes, samples either with high amounts of virus (spleen) or with only trace amounts of virus (plasma) were tested. The classical test of virus detection by hemadsorption was performed for comparison. Plasma samples were taken at intervals after experimental infection of pigs with ASFV. Spleens were obtained from necropsied pigs at various times after infection.

As shown in Table 3, the detection of ASFV by PCR was at least as sensitive as virus isolation and hemadsorption. By PCR a greater number of both spleen and plasma samples were found to be positive. Even at 107 days postinfection, ASFV DNA could be detected by PCR in two of seven spleen samples.

Detection of various ASFV strains. In order to evaluate whether the proposed PCR represented a broadly applicable tool, a range of distinct strains and isolates of ASFV was tested. Several European isolates as well as a variety of wild-type and cell culture-adapted isolates originating from various African countries were included.

As shown in Table 4, all African and European strains tested could be detected by PCR, whereas individually matched negative controls remained nonreactive. Interestingly, no differences concerning the molecular masses of the PCR products and their restriction enzyme patterns following *Hin*fI and *Hha*I digestion were observed (data not shown).

DISCUSSION

The possible introduction of ASF is a constant threat to ASFV-free countries. For this reason, rapid, accurate, and biologically safe diagnostic procedures are of high value. Here we reported that PCR can be applied to the diagnosis of ASF without ever using live ASFV.

Following the partial sequence determination of a DNA fragment originating from the conserved region of the ASFV genome, oligonucleotides consisting of 19 to 23 nucleotides were synthesized. With regard to their future application as primers and tracers, care was taken not to include repetitive and/or GC-rich sequences.

The genome of ASFV is, at least in terms of genome structure and organization, related to that of vaccinia virus. Certain ASFV promoters are recognized by enzymes specified by vaccinia virus (6, 39). For this reason, it was important to check the available data bases for sequence homologies between ASFV and vaccinia virus and to include vaccinia virus as a control in the PCR process. Despite these homologies, the selected oligonucleotides exclusively amplified DNA of ASFV but not that of vaccinia virus.

Analysis of other positive and negative organs and a number of organisms that could possibly contaminate the diagnostic probes demonstrated that the ASFV probes were specific. The specificity of the reaction was further confirmed, as follows.

The 640-bp PCR product could be synthesized when the plasmids pRB (cloned from strain Badajoz 71) and pRPEL2 (cloned from strain Spain 70), as well as cosCM15 and pYS (cloned from strain France 64) were used as templates. Although these clones originated from different virus strains, they originated from the same genomic region. In contrast, plasmids containing DNA fragments originating from other genomic regions of ASFV could not be used to generate this PCR product. When the use of live virus is not permitted for reasons of biological safety, it would be possible to apply these plasmids as positive and negative controls.

A second PCR product of the expected size was obtained by using a pair of nested primers. In certain cases, especially when erythrocytes were used as samples, nonspecific PCR products with molecular weights different from the expected ones appeared after the first round of PCR (Fig. 2). The

TABLE 4. Detection of different ASFV strains in various samples by PCR

Source of sample[a]	Viral strain detected by PCR[b]
HAD test-positive organs of infected pigs	France 64, Malta 78, Rhodesia (RHO 61/1), Sardinia 82, Uganda (virulent)
In vitro-infected porcine macrophages	France 64, Lisbon 60, Malawi 83 (Lil 30/20), Malawi 83 (Lil 20/1), Malta 78, Sardinia 82,
ASFV-infected CV-1 cells	Lisbon 60
Cloned ASFV DNA	Badajoz 71, France 64, Spain 70

[a] Used as the template for PCR.

[b] Identical to viral strain assayed for in a specific sample (note that the availability of samples and viral strains was restricted).

nested amplification overcame this problem and increased the capacity to accurately discriminate between positive and negative samples.

The biotinylated oligonucleotide could be used for the identification of PCR products by hybridization without the need of separating the PCR products by agarose gel electrophoresis. Nevertheless, this method of identification was apparently less sensitive than the other methods. In our experiments, the detection limit was in the nanogram range (3.5 ng), although it is possible that optimization of the reaction protocols could increase the level of sensitivity.

In our experiments, we put more emphasis on the specificity of the reaction than on the sensitivity of the methods. There are both biological and methodological reasons for this.

First, ASFV is present at high titers and for long periods of time in the bloodstreams and lymphatic tissues of infected animals (10, 33). The organs used for diagnostic purposes include spleen and lymph nodes, as well as blood samples.

Second, 90% of infectious ASFV is associated with erythrocytes in vivo. This association explains why plasma and sera represent poor diagnostic samples. Unfortunately, the erythrocytes represent the least suitable ingredient for PCR because hemoglobin apparently interferes with the *Taq* polymerase (18). We often observed nonspecific bands when erythrocytes were used as diagnostic samples. It was therefore of particular interest to know whether ASFV could be demonstrated in such material. In fact, PCR could identify more positive plasma samples (obtained from viremic pigs) than could the virus isolation method. The PCR technique therefore appeared to be at least as sensitive as virus isolation. Pastor and Escribano (29) reported only recently that other in vitro methods, such as immunoblot and hybridization, are between 500 and 2,000 times less sensitive than virus isolation.

Because of the extreme sensitivity of PCR, contaminants and false-positive results are major problems of the diagnostic application of PCR (20). Regarding the consequences which would follow the positive diagnosis of ASF, it is of the utmost importance to avoid errors. Consequently, we suggest that diagnostic samples be prepared at different dilutions, and the nested primer method should be used to amplify the target sequence in a second cycle of PCR. In our hands, this strategy was particularly useful when samples from apparently healthy pigs, which had been infected with ASFV several months before, were tested.

An important goal of this study was to show that the proposed diagnostic PCR represents a broadly applicable tool. Dixon (12) reported that the conserved central regions of the genomes of certain ASFV isolates from Africa are clearly different from the genomes of strains from Europe. Furthermore, deletions and additions to the viral genome during the adaption to cell cultures have been reported (38). It was therefore surprising to notice not only that all of the tested isolates were amplified (Table 4) but also that they gave, according to the molecular masses and the restriction enzyme patterns, indistinguishable PCR products. Differences, however, might still exist at the level of the nucleotide sequence. Yet, the selected genomic region is apparently highly conserved among ASFV strains, even among hemadsorption-negative virus strains. Although hemadsorption-negative virus strains were not available and therefore could not be integrated into this study, two observations support the hypothesis mentioned above.

First, the possibility is unlikely that a variable marker, such as hemadsorption, is located in a genomic region characterized by such a high degree of conservation.

Second, Cistué and Tabares (8) reported that virus protein 73, the major structural antigen, mapped in the genomic region represented by pRPEL2. Virus protein 73, a highly conserved protein, is routinely used as the antigen for serology (34) and has no hemadsorbing activity.

We conclude from the results of our experiments that the proposed methods are highly specific and sensitive. We hope that they may provide a useful tool for the control and prevention of ASF.

ACKNOWLEDGMENTS

This work was supported by the Swiss Federal Veterinary Office and by the Swiss National Science Foundation (grant 31-8832.86).

We thank P. J. Wilkinson, E. Tabares, and E. Viñuela for providing viral strains and cloned viral DNAs. We also thank Martin Schwyzer for advice and discussions; Anne Bridgen, Robert Wyler, and Kenneth McCullough for reviewing the manuscript; and Werner Fraefel, Robi Tschudin, and Marie-Paule Farkas for excellent technical support.

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