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Preclinical Diagnosis of African Swine Fever in Contact-Exposed Swine by a Real-Time PCR Assay

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A fluorogenic probe hydrolysis (TaqMan) PCR assay for African swine fever virus (ASFV) was developed and evaluated in experimentally infected swine. This sensitive and specific one-step single-tube assay, which can be performed in 2 h or less, detected viral DNA in tonsil scraping samples 2 to 4 days prior to onset of clinical disease. Thus, the assay would have application for preclinical diagnosis of African swine fever and surveillance and/or emergency management of a disease outbreak.

African swine fever (ASF) is a highly lethal hemorrhagic disease of domestic swine, with mortality rates approaching 100% (15, 34). ASF occurs in several disease forms, ranging from highly lethal to subclinical infections depending on contributing viral and host factors (7, 9, 14, 15, 20). Hemostatic and hemodynamic changes (hemorrhage, edema, ascites, and shock) resulting from intravascular activation of coagulation are observed in pigs following infection with highly virulent strains of the ASF virus (ASFV) (31–33). ASFV infects cells of the mononuclear-phagocytic system, including fixed tissue macrophages and specific lineages of reticular cells; affected tissues show extensive necrosis following infection with highly virulent viral strains (20, 24). Moderately virulent ASFV strains also appear to infect these cell types, but the degree of tissue involvement and the resulting tissue damage are much less severe.

The causative agent, ASFV, is a unique and genetically complex DNA virus. It is the sole member of the newly named *Asfarviridae* and the only known DNA arbovirus (10). ASFV is a large icosahedral virus which contains a linear double-stranded DNA genome (170 to 190 kbp) encoding approximately 165 viral proteins (8, 29).

In sub-Saharan Africa, cycling of ASFV between soft ticks of the genus *Ornithodoros* and wild pig populations (warthogs, bushpigs, and giant forest hogs) provides a natural reservoir of virus that poses a constant threat to domestic pig populations worldwide (4). ASF has been reported from most African countries south of the Sahara and more recently from Western and North African countries. In 1957 ASF spread to Portugal and eventually to Spain in 1960, where the disease was endemic until its eradication in 1996 (37). Sporadic outbreaks of ASF have also occurred in recent times elsewhere in Europe (France in 1964, 1976, and 1977, Italy in 1967 and 1980, Malta in 1978 to present, Sardinia in 1978, Belgium in 1985, and Holland in 1986), the Caribbean (Dominican Republic in 1978, Haiti in 1979, and Cuba in 1977 to 1980), and South America (Brazil in 1978) (37). These outbreaks were controlled by animal quarantine and slaughter, frequently at a very high cost. Rapid and accurate detection of infected herds would allow for more effective emergency disease management and a reduction in overall economic losses.

Clinical signs of ASF are inapparent at early stages of infection, and at later stages they resemble those of some other swine diseases (20). Rapid and precise detection of ASFV is critical for disease containment. Current diagnostic methods, including detection of infectious virus, viral antigens, and specific antibodies and detection of genomic DNA by PCR, are relatively rapid diagnostic tests (2, 12, 16). However, these techniques require centralized laboratory facilities and clinical specimen submissions that delay disease diagnosis, thus affecting the efficiency of emergency disease management measures.

A rapid, preclinical diagnosis at the site of the suspected disease outbreak would be extremely useful for controlling ASF. To address this need, a fluorogenic probe hydrolysis (TaqMan) PCR assay for ASFV was developed and evaluated in experimentally infected swine. This sensitive and specific one-step, single-tube assay, which can be performed in 2 h or less, detected viral DNA in tonsil scraping samples 2 to 4 days prior to onset of clinical disease. Thus, the assay would have application for preclinical diagnosis of ASF and surveillance and/or emergency management of a disease outbreak.

MATERIALS AND METHODS

Cell culture and viruses. Primary porcine blood macrophage cell cultures were prepared from defibrinated swine blood as previously described (22). Briefly, heparin-treated swine blood was incubated at 37°C for 1 h to allow sedimentation of the erythrocyte fraction. Mononuclear leukocytes were separated by flotation over a Ficoll-Paque (Pharmacia, Piscataway, N.J.) density gradient (specific gravity, 1.079). The monocyte/macrophage cell fraction was cultured in plastic Primaria tissue culture flasks (Falcon; Becton Dickinson Labware, Franklin Lakes, N.J.) containing RPMI 1640 medium with 30% L929 supernatant and 20% fetal bovine serum for 48 h (37°C in 5% CO_2). Adherent cells were detached from the plastic using 10 mM EDTA in phosphate-buffered saline and then reseeded into Primaria 96-well dishes at a density of 5×10^6 cells per ml for use in assays 24 h later.

Pathogenic ASFV strain Pretoriuskop/96/4 (Pr4) was isolated from *Ornithodoros porcinus porcinus* ticks collected from the Republic of South Africa in 1996 (17). Pathogenic and cell culture-adapted ASFV isolates, swinepox virus strain Nebraska 99, sheeppox virus strain TU, pseudorabies virus strain Becker, and

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TABLE 1. ASFV isolates used in testing specificity of the ASFV real-time PCR

p72 sequence source and isolate	Source	Yr of isolation	Species of origin	GenBank accession no.
GenBank				
Uganda'65	Uganda	1965	Pig	L27499
E-70	Spain	1970	Pig	S89966
BA-71V	Spain	1971	Pig	U18466
DR-1 (nh)[a]	Dominican Rep.	1979	Pig	L27498
DR-2 (nh)	Dominican Rep.	1979	Pig	L76727
Malawi Lil-20/1	Malawi	1983	Tick	L00966
This study				
TEN	Tengani, Malawi	1962	Pig	AY578704
KER	Kerita, Kenya	1964	Pig	AY578697
KN	Kenya	1966	Pig	AY578698
ZA	Zaire	1967	Pig	AY578708
CAM	Cameroon	1982	Pig	AY578689
VIC	Victoria Falls, Zimbabwe	1983	Pig	AY578705
E-70	Spain	1970	Pig	AY578692
E-75	Spain	1975	Pig	AY578693
HT (nh)	Haiti	1979	Pig	AY578695
WART	Namibia	1980	Warthog	AY578706
MK	Mkuzi, RSA[b]	1978	Tick	AY578700
WB	Warmbaths, RSA	1987	Tick	AY578707
PR-4	Pretoriuskop, RSA	1996	Tick	AY578702
PR-5	Pretoriuskop, RSA	1996	Tick	AY578703
M1	Wildebeeslagte, RSA	1996	Tick	AY578699
K1	Fairfield, RSA	1996	Tick	AY578696
O1	Noord Biabant, RSA	1996	Tick	AY578701
F6	Nooitverwacht, RSA	1996	Tick	AY578694
CRO-1.2	Crocodile, RSA	1996	Tick	AY578690
CRO-3.5	Crocodile, RSA	1996	Tick	AY578691
No sequence available				
SPENCER	RSA	1951	Pig	
KILLEAN-III	RSA	1964	Pig	
DAKAR	Dakar, Senegal	1959	Pig	
LA GRANJA	Spain	1963	Pig	
KITALI	Kenya	1964	Pig	
KIMAKIA	Kenya	1964	Pig	
L-60	Lisbon, Portugal	1960	Pig	
SALA	Salamanca, Spain	1963	Pig	
MAD	Madrid, Spain	1980	Pig	
SOUCHE	France	1964	Pig	
CUBA	Cuba	1979	Pig	
BRAZIL	Brazil	1978	Pig	
HINDE-II	Kenya	1954	Warthog	
UGANDA'61	West Province, Uganda	1961	Warthog	
RSA-W1	RSA	1999	Warthog	
LEE	Kenya	1955	Bushpig	
GR-1	RSA	1981	Tick	
GR-4	RSA	1979	Tick	
ZIM	Zimbabwe	1983	Tick	
CHI	Chiredzi, Kenya	1983	Tick	
MM1	Masa Mari, Kenya	1996	Tick	
JACOBSLOOP/B	North Province, RSA	1996	Tick	

[a] Nonhemadsorbing ASFV isolate.
[b] RSA, Republic of South Africa.

classical swine fever virus (CSFV) isolate Haiti-96, used for assessment of analytical assay sensitivity and specificity, were obtained from the Plum Island Animal Disease Center reference collection (1, 13, 18, 26, 30, 35).

DNA extraction. DNA was extracted from 200 μl of tissue culture supernatant, transport media containing nasal swab samples or tonsil scrapings, and heparinized whole blood samples by using a DNeasy kit (QIAGEN, Stanford, Calif.) and eluted in 100 μl of Tris-EDTA buffer according to the manufacturer's instructions. Viral genomic DNAs were isolated from purified virions using proteinase K and sodium dodecyl sulfate (SDS) lysis followed by phenol extraction and ethanol precipitation (36). For evaluation of assay sensitivity, the p72 gene from the ASFV Pr4 isolate was cloned in the pCR 2.1 TA vector (Invitrogen, San Diego, Calif.) and purified essentially as described by Sambrook et al. (27).

One-step real-time PCR assay for ASFV. The one-step real-time PCR assay was designed as a single-tube PCR probe hydrolysis (TaqMan) assay to target a gene encoding a highly conserved ASFV structural protein, p72 (5, 38). Twenty-six available full-length p72 sequences (Table 1) were aligned using BioEdit sequence alignment software. Specific oligonucleotide primers and the fluorogenic probe were designed to target a highly conserved region within the p72 open reading frame. The location and sequence of the primers and probe were the following: forward primer, starting at base position 1466, 5′ CCTCGGCGA

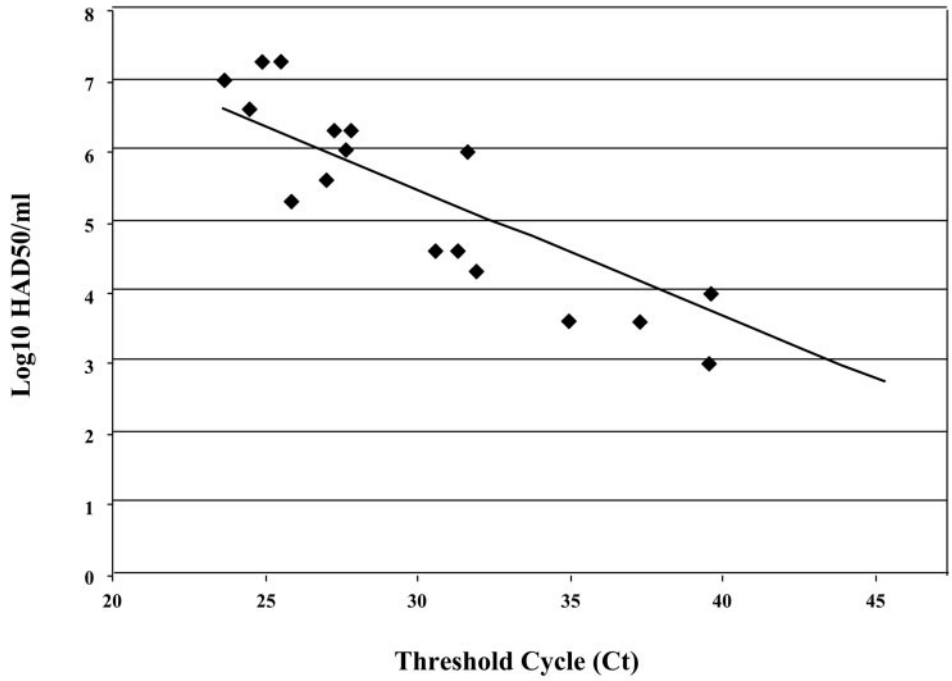

FIG. 1. Sensitivity of the ASFV real-time PCR assay was calculated by using ASFV samples obtained from infected cell culture supernatants or blood samples from infected pigs. An inverse linear relationship ($r = 0.9237$) exists between cycle threshold and the logarithm of HAD_{50} per milliliter present in the sample.

GCGCTTTATCAC 3′; reverse primer, starting at base position 1528, 5′ GGA AACTCATTCACCAAATCCTT 3′; probe, starting at base position 1486, 5′ CGATGCAAGCTTTAT 3′. The TaqMan probe was labeled with a 5′ reporter dye, 6-carboxyfluorescein, and a 3′ minor grove binder nonfluorescent quencher (PE Biosystems, Foster City, Calif.).

Reagents from Perkin-Elmer (EZ-RT-PCR; PE Biosystems) were used to prepare master-mixture recipes according to the guidelines of the manufacturer for individual component concentrations. The final PCR mixture for a 25-μl-volume assay consisted of the 5× buffer solution supplied with the kit with the addition of 5 mM Mn_2-acetate, a 0.3 M concentration of each primer, 0.1 mM dATP, dCTP, or dGTP, 0.2 mM dUTP, 0.1 U of recombinant *Tth* DNA polymerase per μl, 0.1 μg of bovine serum albumin per μl, and 0.5 M trehalose. The PCR mixture was then dried within the reaction tubes (Fisher Scientific, Atlanta, Ga.). For sample testing, dried reagents, which can be stored at room temperature for at least 1 year (J. D. Callahan, unpublished data), were rehydrated with 22.5 μl of 1× buffer (see above) and 2.5 μl of DNA sample material. Cycling consisted of 45 amplification cycles (95°C for 2 s and 60°C for 30 s). The assay was optimized for use on a SmartCycler (Cepheid, Inc., Sunnyvale, Calif.), a 22-lb portable instrument that is operated by a laptop computer. Positive and negative controls consisting of ASFV DNA and a nontemplate reaction mixture were included with each PCR run.

Animal infections. Commercial crossbred Yorkshire pigs (30 to 35 kg) were used. Two pigs were intramuscularly inoculated in the left rear leg with 10^4 50% hemadsorption doses (HAD_{50}) of the pathogenic ASFV isolate, Pr4. Twenty-four hours postinoculation, the infected animals were placed in contact with six noninfected swine. Heparinized whole blood, nasal swabs, and tonsil scrapings were obtained daily from all animals until death. Nasal and tonsil samples were collected with a cotton swab and placed in 1 ml of transport medium (RPMI 1640 containing antibiotics). Clinical signs of ASF (fever [a rectal temperature greater than or equal to 104°F], anorexia, lethargy, shivering, cyanosis, and recumbency) were monitored daily. Animal experiments were conducted under federal guidelines and Plum Island Animal Disease Center policies on animal care and use.

Virus titration. ASFV titers in tissue culture supernatants, blood samples, nasal swabs, and tonsil scrapings were determined in swine macrophage cell cultures as previously described (23). Virus titers were calculated by the method of Spearman-Karber and expressed as HAD_{50} per milliliter (11).

Statistical analysis. Performances of the ASFV real-time PCR assay were compared with virus isolation and/or ASFV DNA detection in tonsil scrapings, nasal swabs, and blood samples collected from contact-exposed pigs. Sensitivity, specificity, predictive values, test accuracy, and 95% confidence intervals were estimated using disease-test relation calculators found at the University of Oklahoma Health Sciences Center website (http://www.fammed.ouhsc.edu/robhamm/cdmcalc.htm).

RESULTS AND DISCUSSION

Assay analytical sensitivity and specificity. To assess assay sensitivity, viral DNA was extracted from ASFV-infected mac-

TABLE 2. Swine survival, fever response, and viremia following infection with ASFV Pr4[a]

Group (*n*)	No. of animals that survived/total no. inoculated	Fever				Viremia		
		Days to death	Days to onset	Duration (days)	Mean temp (°F)	Days to onset	Duration (days)	Max titer (log_{10} HAD_{50}/ml)
A (2)	0/2	9.0 (0.0)[b]	4.0 (0.0)	4.5 (0.5)	106.0 (0.5)	3.0 (0.0)	6.0 (0.0)	7.3 (0.3)
B (6)	0/6	16.3 (1.3)	11.0 (1.0)	5.3 (0.9)	105.7 (0.6)	9.8 (0.9)	6.5 (0.6)	7.0 (0.2)

[a] Two pigs (group A) were inoculated intramuscularly with 10^4 HAD_{50} of ASFV isolate Pr4. Six pigs (group B) were contact exposed to the two experimentally inoculated animal.

[b] Standard errors of means are shown in parentheses.

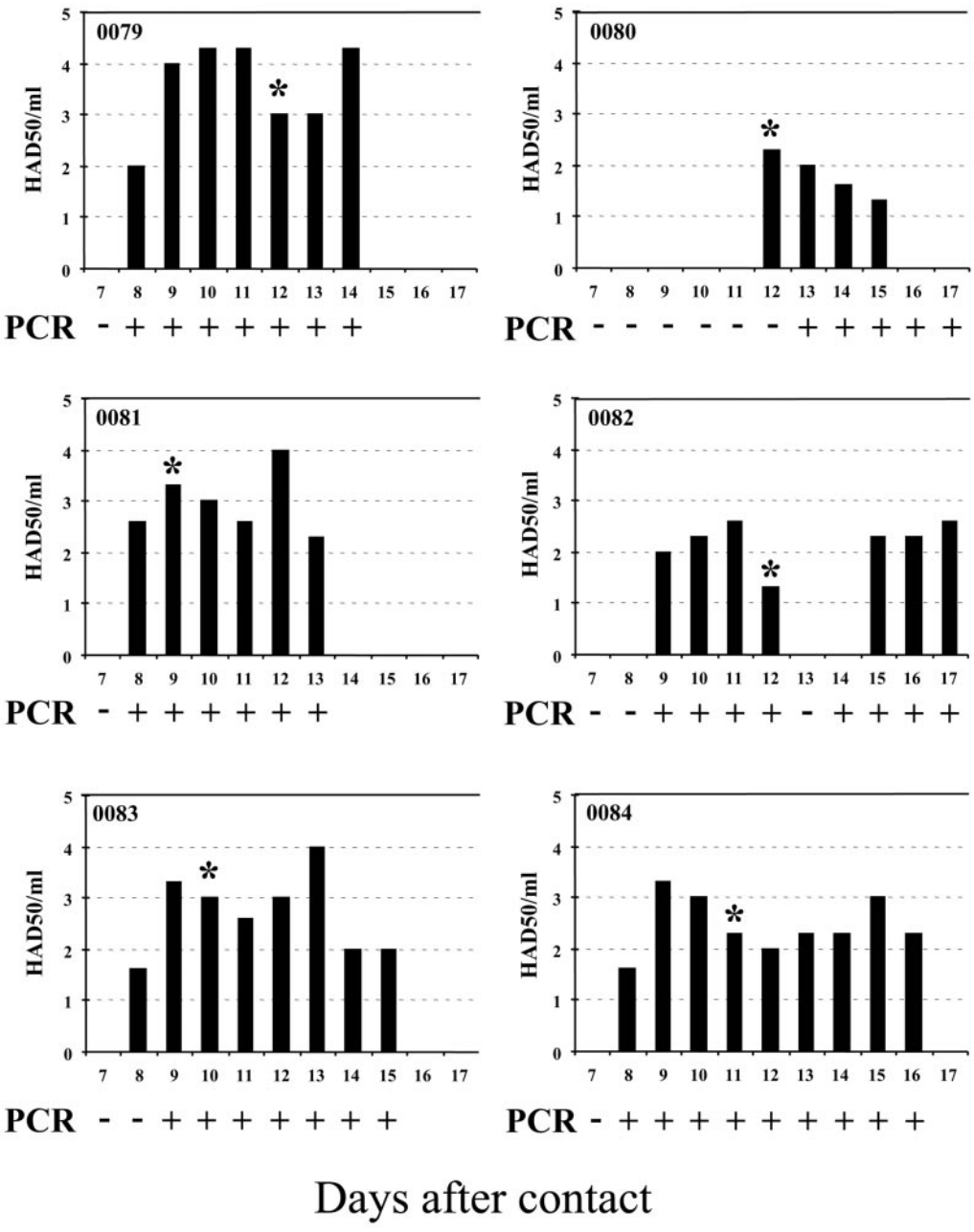

FIG. 2. Detection of ASFV in tonsil scraping samples. Six pigs (animals 0079, 0080, 0081, 0082, 0083, and 0084) were contact exposed to experimentally infected pigs. The bars represent virus titers expressed as HAD_{50} per milliliter. PCR results are expressed as positive (+) or negative (−). Asterisks indicate the time of appearance of ASF clinical symptoms.

rophage cell culture supernatants and blood samples from pigs infected with the ASFV Pr4 isolate. The samples were diluted in log 10 steps in RPMI 1640 medium and/or whole swine blood, and DNA was extracted and tested to determine the end point dilution at which a positive amplification signal could be obtained. Prior to DNA extraction the same samples were titrated in porcine macrophage cell cultures and the titers were expressed as the HAD_{50} per milliliter. The results demonstrated that, regardless of sample origin, the sensitivity of the PCR assay ranged between 1,000 and 10,000 HAD_{50} per ml of the sample. In six independent determinations, the assay range of linearity was at least 4 log dilutions of extracted ASFV DNA (Fig. 1). After the sample volume used was adjusted for the DNA extraction volume (200 μl), elution volume (100 μl), and the volume tested (2.5 μl), assay sensitivity was estimated to be between 5 and 50 HAD_{50}.

To further assess sensitivity, purified ASFV genomic DNA and plasmid constructs containing the cloned p72 gene were used to determine genomic equivalents necessary to yield a positive fluorogenic signal. One microgram of DNA was serially diluted in log 10 steps, and real-time PCR amplifications were performed to determine the end point dilution at which

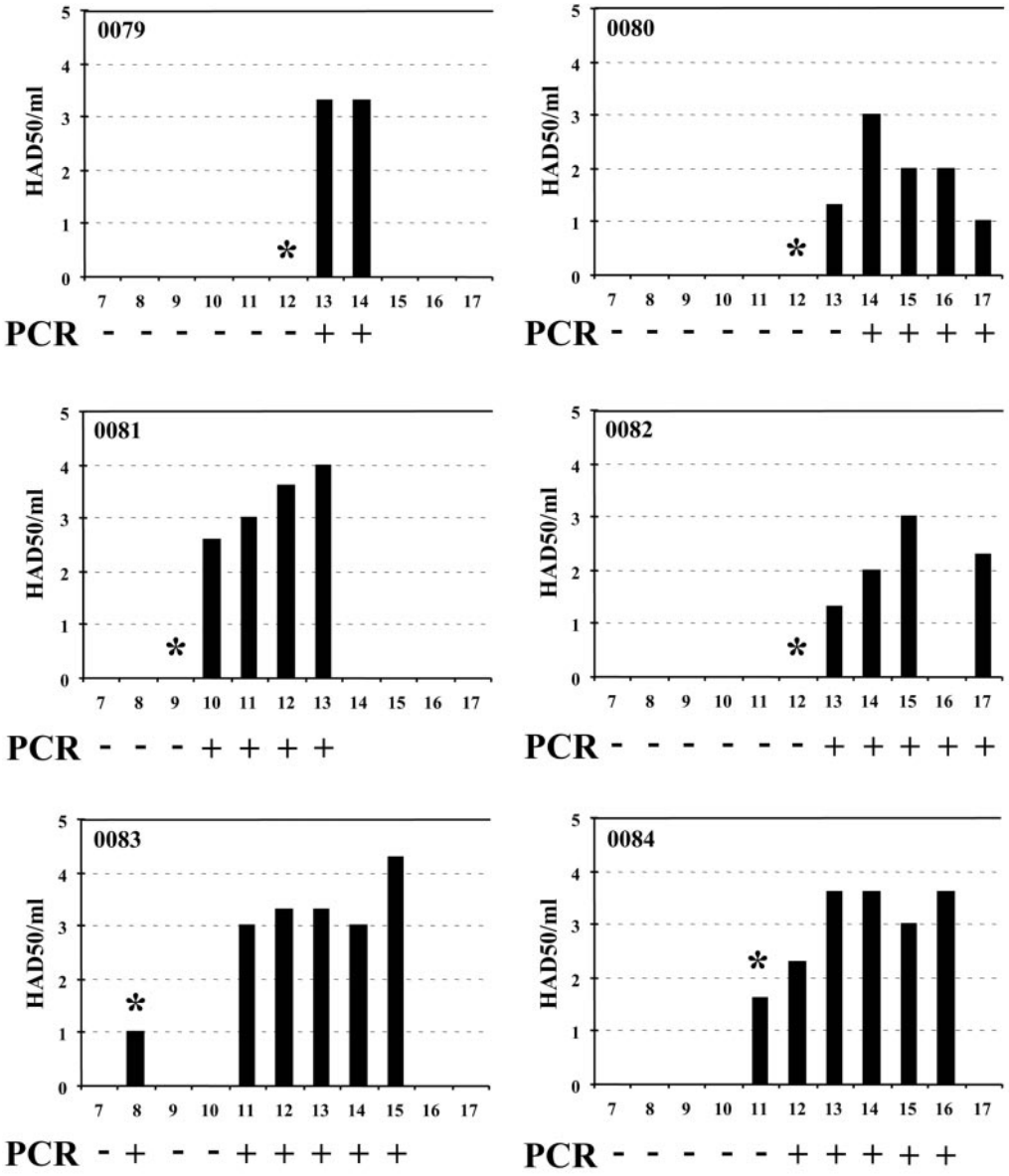

FIG. 3. Detection of ASFV in nasal swab samples of contact-exposed pigs. Six pigs (animals 0079, 0080, 0081, 0082, 0083, and 0084) were contact exposed to experimentally infected pigs. The bars represent virus titers expressed as HAD_{50} per milliliter. PCR results are expressed as positive (+) or negative (−). Asterisks indicate the time of appearance of ASF clinical symptoms.

positive signal could be observed. The assay detected 1.4 to 8.4 viral genomic equivalents and 2.6 to 4.5 cloned p72 DNA molecules (data not shown). It should be emphasized that this is an estimate based on DNA extraction, ratios of the optical densities at 260 and 280 nm, and PCR efficiencies that are less than 100%.

Viral DNAs from 48 ASFV isolates were extracted from infected macrophage cell culture supernatants and tested in the real-time PCR assay. All ASFVs were detected with cycle threshold (*Ct*) values ranging from 15 to 28 (data not shown). Positive fluorogenic signals were observed for all 48 ASFV isolates (26 strains with known p72 sequences and 22 isolates with no available p72 sequence information) (Table 1). Importantly, the assay detected geographically and temporally distinct viruses isolated from different hosts (pig, warthog, bushpig, and tick). No fluorogenic signal was detected in the ASFV assay with samples containing DNAs from swinepox virus, sheeppox virus, and pseudorabies virus, or viral RNA extracted from CSFV-infected cell culture supernatants. Additionally, no false-positive results were observed for DNA samples extracted from pig macrophage cell cultures or lymphoid tissues (spleen, tonsil, or lymph nodes) from uninfected swine (data not shown).

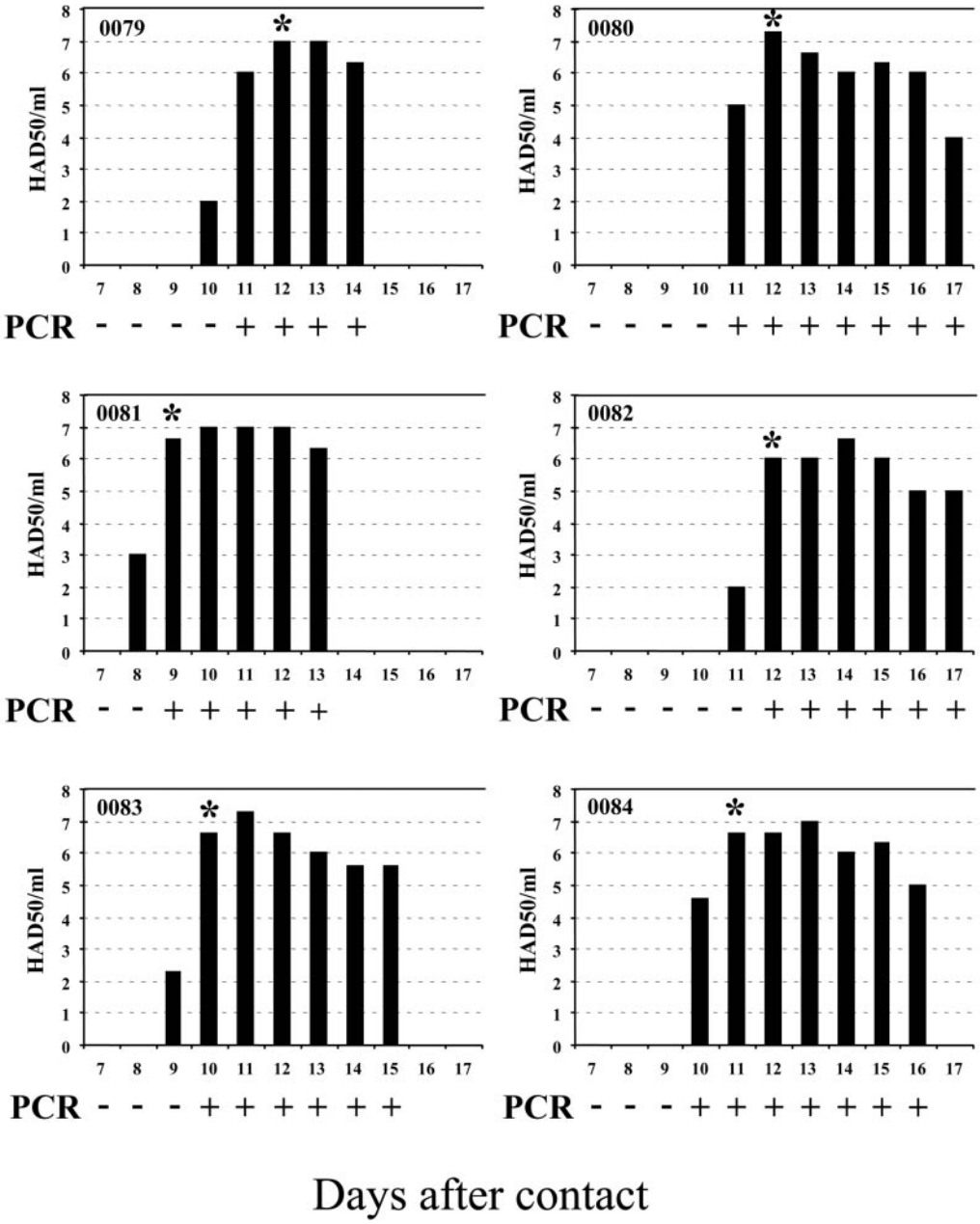

FIG. 4. Detection of ASFV in blood samples of contact-exposed pigs. Six pigs (animals 0079, 0080, 0081, 0082, 0083, and 0084) were contact exposed to experimentally infected pigs. The bars represent virus titers expressed as HAD_{50} per milliliter. PCR results are expressed as positive (+) or negative (−). Asterisks indicate the time of appearance of ASF clinical symptoms.

Assay diagnostic sensitivity and specificity. The ability of the assay to detect ASFV during acute infection was assessed in experimentally infected pigs. Two pigs (30 to 40 kg) were inoculated intramuscularly with 10^4 HAD_{50} of the pathogenic ASFV isolate, Pr4, and placed in contact with six uninfected animals 24 h postinfection. Results of this experiment are shown in Table 2. Infected donor pigs presented with clinical disease (febrile response for 4 to 5 days and high blood viremia titers of 7.3 $\log_{10}$ HAD_{50}/ml) and died within 9 days postinoculation. Contact animals presented with clinical signs of acute ASF 9 to 12 days postexposure and died within 18 days. No significant differences in disease course (number of days of fever, mean temperature, days of viremia, and maximum viremia titers) were noted for the two groups (Table 2).

Nasal swabs, tonsil scrapings, and blood samples were obtained daily from all animals after contact and examined by real-time PCR and virus isolation and titration. Contact pigs developed fever 9 to 12 days postexposure. DNA samples from tonsils were positive by PCR for all but one contact-exposed pig at 8 to 9 days postinoculation (Fig. 2). Virus was detectable both by PCR and virus isolation from nasal swabs only at later time points, generally after the appearance of clinical symp-

TABLE 3. Comparison of performances of ASFV real-time PCR assay with virus isolation and/or ASFV DNA detection in tonsil scrapings, nasal swabs, and blood[a]

Sample type and parameter	% (no. of samples with evaluated result/total no. tested)	95% confidence interval
Tonsil scrapings		
Sensitivity	95.4 (42/44)	89.3–101.6
Specificity	100.0 (12/12)	100.0–100.0
Positive predicted value	100.0 (42/42)	100.0–100.0
Negative predicted value	85.7 (12/14)	67.3–104.0
Test accuracy	96.4 (54/56)	91.5–101.2
Nasal swabs		
Sensitivity	92.8 (42/44)	83.3–102.4
Specificity	100.0 (12/12)	100.0–100.0
Positive predicted value	100.0 (42/42)	100.0–100.0
Negative predicted value	93.3 (12/14)	84.4–102.2
Test accuracy	96.4 (54/56)	91.5–101.2
Blood		
Sensitivity	89.7 (42/44)	80.2–99.2
Specificity	100.0 (12/12)	100.0–100.0
Positive predicted value	100.0 (42/42)	100.0–100.0
Negative predicted value	80.9 (12/14)	64.1–97.7
Test accuracy	92.8 (54/56)	86.1–99.6

[a] ASFV DNA detection involved PCR amplification and sequencing of two additional ASFV genes (8-DR and p22).

toms (Fig. 3). Viremia was first detected at 9 to 11 days postexposure, indicating a 1- to 2-day delay between virus replication in tonsils and generalization of infection (Fig. 4). Interestingly, pig 0080 revealed a high viremia both by PCR and virus isolation prior to appearance of virus in tonsil or nasal swab samples, suggesting that infection likely occurred parenterally by virus-containing blood (20).

The PCR assay performed with 100% specificity and 92.8 to 96.4% accuracy for the 168 clinical samples evaluated during the experiment (Table 3). Four samples from tonsil scrapings and nasal swabs positive by real-time PCR but negative by virus isolation (Fig. 2 and 3) were examined in greater detail by PCR amplification of two additional ASFV genes (a CD2-like gene, 8-DR [6] and a structural protein, p22 [38]), followed by sequencing of the amplified products. All four samples contained ASFV genomic DNA, indicating that these represented true-positive samples (data not shown).

The real-time PCR assay showed somewhat less sensitivity (89.7%) for blood samples compared to other clinical samples (95.4% for tonsil scrapings and 92.8% for nasal swabs), especially when viremia titers were low (2 to 3 $\log_{10}$ HAD_{50}/ml) (Table 3 and Fig. 4). While virus isolation-titration methods detect infectious particles, sensitivity of PCR correlates with the number of viral DNA molecules. Since ASFV viremia is red blood cell associated and the virus does not replicate in these cells, it is reasonable to speculate that the lower sensitivity of the PCR was due to the reduced amounts of viral DNA present in these samples. It is also possible that PCR amplification of ASFV DNA from blood was reduced or blocked by the presence of PCR-inhibitory substances. PCR inhibitors in blood have been identified and include natural blood components, mainly heme (3) and leukocyte DNA (21), or added anticoagulants, such as heparin (28).

Although the hemadsorption test is a highly sensitive and routinely used method to detect ASFV in cell culture, there are ASFV isolates that do not hemadsorb (19, 20). Nonhemadsorbing isolates are identified by cytopathic effect and then confirmed by detecting the presence of ASFV antigen by indirect immunofluorescence test or peroxidase-based immunoassays using monospecific anti-ASFV antibodies (25). Depending on viral titers, detection of nonhemadsorbing ASFV can take several days (2 to 7 days), and the sensitivity of detection is approximately 10-fold lower compared with the HAD_{50} method (25). Since the real-time PCR assay for ASFV targets a highly conserved structural protein, p72, it would perform consistently with both hemadsorbing and nonhemadsorbing viruses, making the assay extremely useful for detecting ASFV in samples of unknown origin.

Notably, ASFV was detected in tonsil scrapings 2 to 4 days before the appearance of clinical symptoms (Fig. 2). Tonsil scrapings are also the sample of choice during the early stages of CSFV infection (26). Thus, a tonsil sample would not only be useful for preclinical diagnosis of ASF but for differential diagnosis of these two highly significant swine diseases.

In summary, the assay described here, a fluorogenic probe hydrolysis (TaqMan) PCR assay for rapid detection of ASFV, is performed in a single tube that contains all PCR reagents dried, and test results are obtained by using a portable detection instrument in real time, thus simplifying all pre- and post-PCR operating procedures. The simplicity, speed, and portability of the assay allow for its use as a pen-side assay for rapid preclinical detection of ASFV. This should facilitate surveillance and/or emergency management of a disease outbreak.

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