

## Rapid Detection of Classical Swine Fever Virus by a Portable Real-Time Reverse Transcriptase PCR Assay

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**A fluorogenic-probe hydrolysis (TaqMan)–reverse transcriptase PCR assay for classical swine fever virus (CSFV) was developed and evaluated in experimentally infected swine. The assay detected CSFV, representing different phylogenetic groupings, but did not amplify viral RNA from related pestiviruses. The assay met or exceeded the sensitivity (1 to 100 50% tissue culture infective doses per ml) of viral cultures of samples from experimentally infected animals. Viral RNA was detected in nasal and tonsil scraping samples 2 to 4 days prior to the onset of clinical disease. The assay can be performed in 2 h or less, thus providing a rapid method for the diagnosis of classical swine fever.**

Classical swine fever (CSF) is a highly contagious and often fatal disease of swine, affecting domestic and wild pig populations. *Classical swine fever virus* (CSFV), the causative agent of CSF, is a member of the genus *Pestivirus*, which belongs to the *Flaviviridae* family (37). Other important animal pathogens within the genus *Pestivirus* are bovine viral diarrhea virus (BVDV) of cattle and border disease virus (BDV) of sheep (26), both of which can naturally infect pigs. Antibodies generated during these infections cross-react with CSFV in serologic assays, making a CSF diagnosis problematic (5, 22, 23, 34).

CSFV is an enveloped virus with a 12.5-kb single-stranded RNA genome of positive polarity (12, 38). The genome comprises a single open reading frame that encodes a 4,000-amino-acid polyprotein that is co- and posttranslationally processed by viral and cellular proteases into 12 polypeptides (19). Both ends of the genome contain untranslated regions (UTR), which are highly conserved among all virus isolates (3, 16, 18, 31, 39).

Clinical signs of CSF can remain undetected, particularly during infections with CSFV strains of low virulence (33). Moreover, gross lesions observed at necropsy are diverse and often not pathognomonic (4, 7, 8, 14, 20, 21, 29, 33, 35, 36). Rapid and precise detection of CSFV is critical for disease containment. Current diagnostic methods, including detection of viral antigens in tonsils by using fluorescent antibodies (28) or antigen capture enzyme-linked immunosorbent assay (6, 32) and detection of genomic RNA by reverse transcription-PCR (3, 11, 13, 15, 39), are relatively rapid diagnostic tests; however, these techniques require centralized laboratory facilities and clinical specimen submissions that might delay disease diagnosis, thus affecting the efficiency of emergency disease management measures.

A rapid, presumptive diagnosis at the site of a suspected disease outbreak would be extremely useful for controlling

CSF. To address this need, a fluorogenic-probe hydrolysis (TaqMan)–reverse transcriptase (RT) PCR assay for CSFV was developed and evaluated in experimentally infected swine.

The assay was designed as a probe hydrolysis (TaqMan)–RT-PCR single-tube assay. Available CSFV nucleotide sequences were aligned by using BioEdit sequence alignment software. Specific oligonucleotide primers and the fluorogenic probe were designed to target a highly conserved region within the 5' UTR of the CSFV genome (GenBank accession number NC\_000294.1). The locations and sequences of the primers and probes were as follows: forward primer, starting at base position 180, 5' CCCTGGGTGGTCTAAG; reverse primer, starting at base 247, 5' CATGCCCTCGTCCAC; and probe, starting at base 139, 5' CCTGAGTACAGGACAGTCGTCAGTATT. The Taqman probe was labeled with a 5' reporter dye, 6-carboxyfluorescein, and a 3' quencher, 6-carboxy-*N,N,N',N'*-tetramethylrhodamine (TAMRA) (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 RT-PCR mixture for a 25- $\mu$ l-volume assay consisted of the 5 $\times$  buffer solution supplied with the kit with the addition of 5 mM Mn<sub>2</sub> 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  $\mu$ l, 0.1  $\mu$ g of bovine serum albumin per  $\mu$ l, and 0.5 M trehalose. The RT-PCR mixture was then dried within the reaction tubes (Fisher Scientific, Atlanta, Ga.). For sample testing, dried reagents were rehydrated with 22.5  $\mu$ l of 1 $\times$  buffer (see above) and 2.5  $\mu$ l of RNA sample material. Cycling consisted of an RT step at 60°C for 10 min, followed by 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 viral RNA and a nontemplate reaction mixture were included with each RT-PCR run.

For all experiments, RNA was extracted from 150  $\mu$ l of tissue culture supernatant, nasal swab samples, tonsil scrap-

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TABLE 1. Pestiviruses used in testing specificity of CSF real-time RT-PCR

Pestivirus isolate (original source, yr)	Source <sup>a</sup>
<b>CSFV</b>	
Haiti-96 (Haiti) .....	PIADC
DR-1 (Dominican Republic).....	PIADC
Bavaro (Dominican Republic) .....	PIADC
CS vaccine strain (Russia).....	Ivanovsky Institute
Shimen (China).....	Ivanovsky Institute
Brescia (Italy).....	Ivanovsky Institute
CSF 0901 (Alfort).....	EU Reference Lab
CSF 0906 UD (Bergen).....	EU Reference Lab
CSF 0216 V1081/94.....	EU Reference Lab for CSFV
CSF 0410 (congenital tremor).....	EU Reference Lab for CSFV
CSF 0309 (Kanagawa).....	EU Reference Lab for CSFV
CSF 123 (Germany, 1995).....	EU Reference Lab for CSFV
CSF 277 (Germany, 1997).....	EU Reference Lab for CSFV
CSF 573 (Italy, 1998).....	EU Reference Lab for CSFV
CSF 634 (Germany, 1999).....	EU Reference Lab for CSFV
<b>BVDV</b>	
NADL.....	NADC
Singer.....	NADC
NY-1.....	NADC
890.....	NADC
SD-1.....	NADC
1373.....	NADC
<b>BDV</b>	
Idaho 207.....	NADC
CB02.....	NADC
CB05.....	NADC

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ings, or blood samples with an RNeasy kit or an RNA blood extraction kit (Qiagen, Stanford, Calif.) and resuspended in 40 µl of RNase-free water.

Viral RNAs extracted from CSFV-infected cell supernatants with approximate viral titers of 10<sup>6</sup> 50% tissue culture infectious doses (TCID<sub>50</sub>) per ml were tested with the real-time RT-PCR assay. All CSFVs were detected with cycle threshold (C<sub>t</sub>) values ranging from 25 to 30 (data not shown). Positive fluorogenic signals were observed when testing RNA obtained from tissue culture supernatant of SK6 cells infected with CSFV isolates Haiti-96, DR-1, Brescia, CS, and Shimen, CSF 901 (Alfort), CSF 906 UD (Bergen), CSF 216 V1081/94, CSF 0309 Kanagawa, CSF 0410, CSF 123, CSF 277, CSF 573, and CSF 634 (Table 1). Importantly, the assay detected geographically and temporally distinct viruses belonging to different phylogenetic groups (17) (i.e., Brescia, 1945, Italy; Haiti-96, 1996, Haiti; and recent isolates from Germany, from 1995, 1997, and 1999).

No fluorogenic signal was observed in the CSFV assay with samples containing viral RNA, detected by the classic RT-PCR that is described elsewhere (30), of BVDV strains NADL, Singer, NY-1, 890, SD-1, and 1373 and BDV strains Idaho 207, CB02, and CB05 (Fig. 1), which indicated 100% specificity for

the selected panel. Additionally, no false-positive results were observed for nasal swab samples taken from 100 clinically healthy pigs from a farm in the United States (data not shown).

To assess assay sensitivity, viral RNA was extracted from tissue culture supernatants of CSFV-infected SK6 cells and from clinical samples (nasal swabs and tonsil scrapings) obtained from experimentally infected pigs. The samples were diluted in log-10 steps in Dulbecco's minimal essential medium, and RNA was extracted and tested to determine the end-point dilution at which a positive amplification signal could be obtained. The results demonstrated that, regardless of sample origin, the sensitivity of the assay ranged between 1 and 100 TCID<sub>50</sub> per ml of the sample. In eight independent determinations, the assay range of linearity was at least 5 log dilutions of extracted CSFV RNA (Fig. 2). After the sample volume used was adjusted for the RNA extraction (140 µl), the yield of purified RNA (40 µl), and the volume tested (2.5 µl), assay sensitivity could be estimated to be between 0.01 and 1 TCID<sub>50</sub>. It must be emphasized that this is an estimate based on RNA extraction, reverse transcription, and PCR efficiencies that are less than 100%.

The ability of the assay to detect CSFV during acute infection was assessed in experimentally infected pigs. Two 40- to 50-lb pigs were inoculated intramuscularly in the neck with 10<sup>6</sup> TCID<sub>50</sub> of the moderately virulent CSFV isolate Haiti-96 and placed in contact with four naïve animals 24 h postinfection. Nasal swabs, tonsil scrapings, and blood samples were obtained daily from all animals after contact and examined by RT-PCR and virus isolation. CSFV titers in clinical samples were determined on 96-well plates (Costar, Cambridge, Mass.) with SK6 cells. After 4 days of culture, virus infection was determined by an immunoperoxidase assay (1) using CSFV monoclonal antibody 303 (10) and a Vectastain ABC kit (Vector Laboratories, Burlingame, Calif.). Titers were calculated by the method of Reed and Muench and expressed as TCID<sub>50</sub> per milliliter (27).

The contact pigs developed fever (body temperature above 104°F) and anorexia 11 to 13 days postexposure. Nasal swabs and tonsil scraping samples were positive by RT-PCR for all contact-exposed pigs at 7 to 8 days postcontact, and the samples were positive for virus isolation, beginning at 11 to 13 days postcontact (Fig. 3).

Thus, the CSF RT-PCR assay outperformed virus isolation, which is considered the diagnostic standard for CSF (8, 24, 28). CSFV Haiti-96 was detected in the nasal swabs and tonsil scrapings of contact-infected pigs (n = 4) 3 to 5 days before detection by virus isolation (Fig. 3). This enhanced sensitivity suggests the suitability of the assay for early detection of CSF. During contact infection, Haiti-96, a moderately virulent CSFV strain, apparently replicated at very low levels in the tonsils and nasal cavities of contact-infected pigs (Fig. 3) at early stages of infection and was undetectable by virus isolation with SK6 cells for 3 to 5 days. The failure to isolate the virus early in infection could be due to poor replication of moderately virulent CSFV strains in SK6 cells. Given the normal growth kinetics of Haiti-96 in SK6 cells, this does not appear to be the case (G. Risatti, unpublished data). Alternatively, it may be the case that little to no infectious virus is present during the earliest stages of oronasal infection (2). Haiti-96 virus titers observed in oronasal samples tend to be low, indicating that early replication events might be impaired, resulting in less

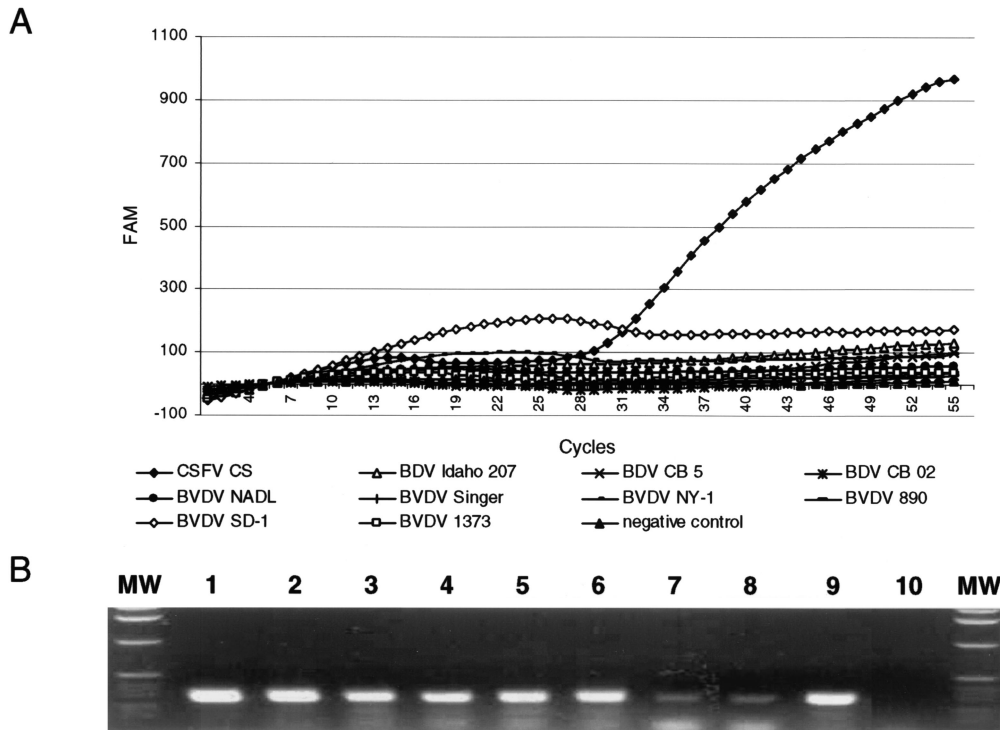


FIG. 1. Specificity of CSFV RT-PCR assay. (A) Genomic RNAs obtained from CSFV strain CS, BDV strains Idaho 207, CB02, and CB05, and BVDV strains NADL, Singer, NY-1, 890, SD-1, and 1373 were assayed by CSFV-specific real-time RT-PCR. (B) RT-PCR amplification of genomic RNA obtained from the pestiviruses listed above by a general pestivirus RT-PCR assay. Amplicons of the 5' UTR of viral genomic templates were resolved in a 1% agarose gel stained with ethidium bromide. Shown are results for BVDV strains NADL, Singer, NY-1, 890, SD-1, and 1373 (lanes 1 to 6); BDV strains CB02 and Idaho 207 (lanes 7 and 8); CSFV strain CS (lane 9); and the nontemplate control (lane 10). Lanes MW contain molecular weight standards. FAM, 6-carboxyfluorescein.

infectious virus. Thus, the amount of infectious virus present in the oronasal cavity early after contact infection is not enough to be detected by virus isolation; however, viral RNA, which is likely to be present in much greater quantities, is detectable by the RT-PCR assay. Although virus isolation is the “gold stan-

dard” for CSFV detection, its use as a screening test, with complete blood samples, in clinically healthy pig herds showed little predictive value, with 4.5% of infected herds detected during the 1997 to 1998 CSF epizootics in The Netherlands (9, 25). In the experiment described here, CSFV Haiti-96 was

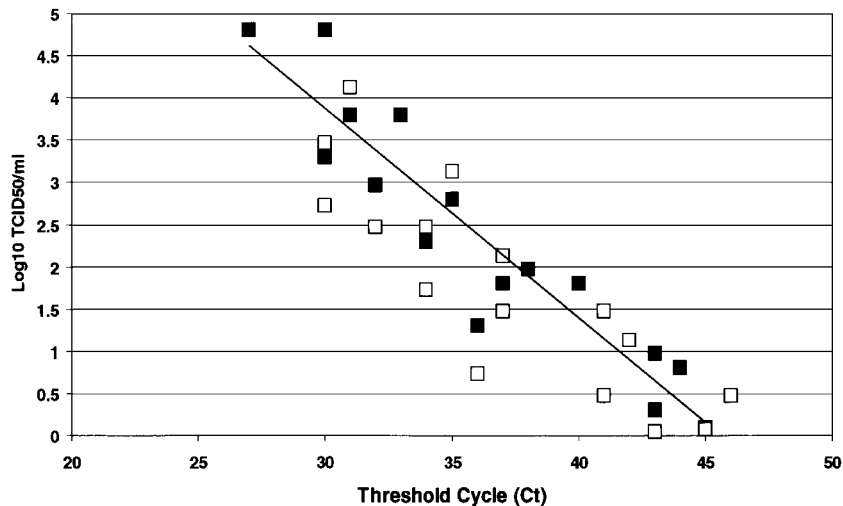
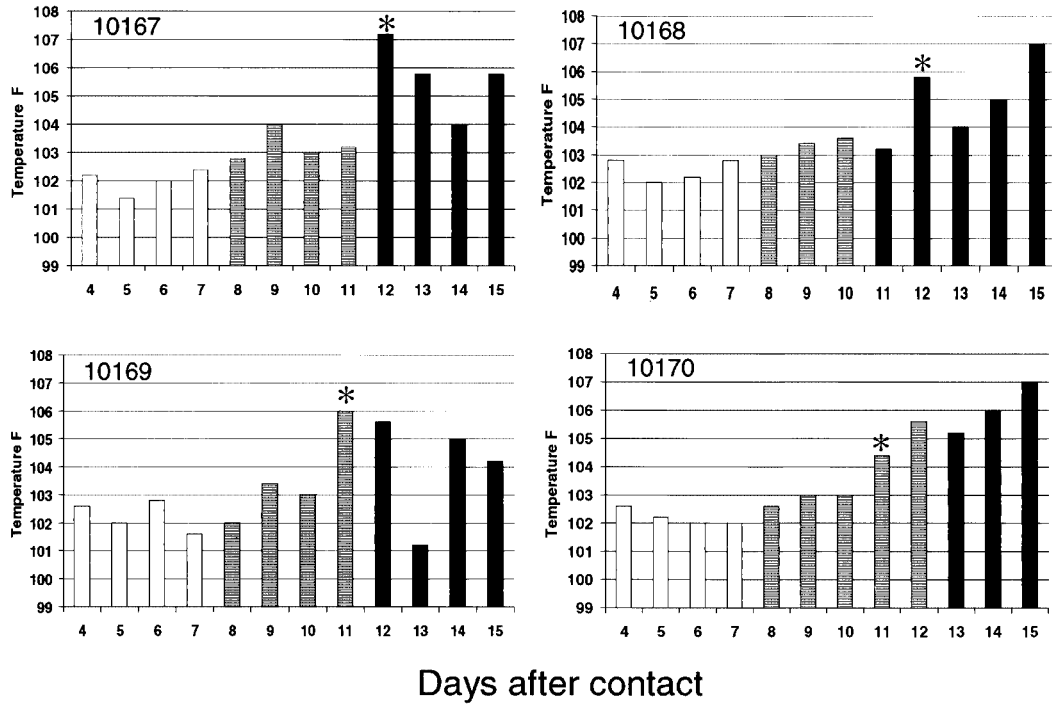


FIG. 2. Sensitivity of CSFV real-time RT-PCR assay was calculated by using CSFV samples obtained from tissue culture (filled squares) or clinical samples (open squares). An inverse linear relationship ( $r = 0.89$ ) exists between cycle threshold and the logarithm of TCID<sub>50</sub> per milliliter originally present in the sample.

**A** Tonsil



**B** Nasal swab

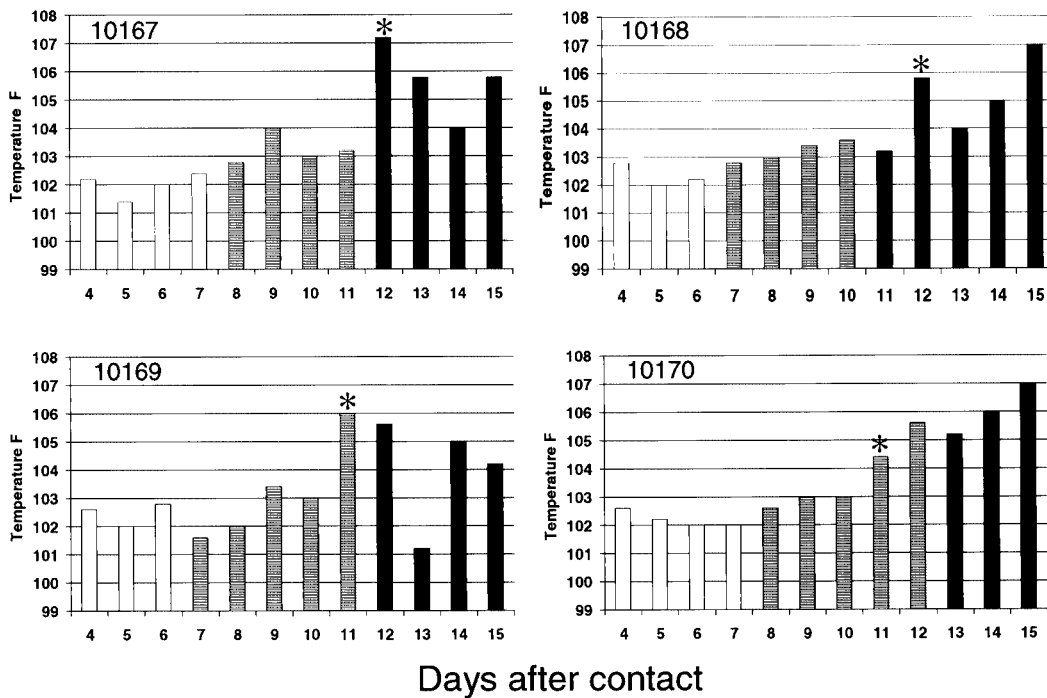


FIG. 3. Preclinical detection of CSFV in tonsil scraping (A) and nasal swab (B) samples. Four pigs (animals 10167, 10168, 10169, and 10170) were contact-exposed to experimentally infected pigs. The bars represent body temperature after contact exposure. Empty bars represent samples negative by both real-time RT-PCR and virus isolation, hatched bars represent samples positive by PCR and negative by virus isolation, and filled bars represent nasal swabs samples positive by PCR and virus isolation. Asterisks indicate the time of appearance of CSF clinical symptoms.

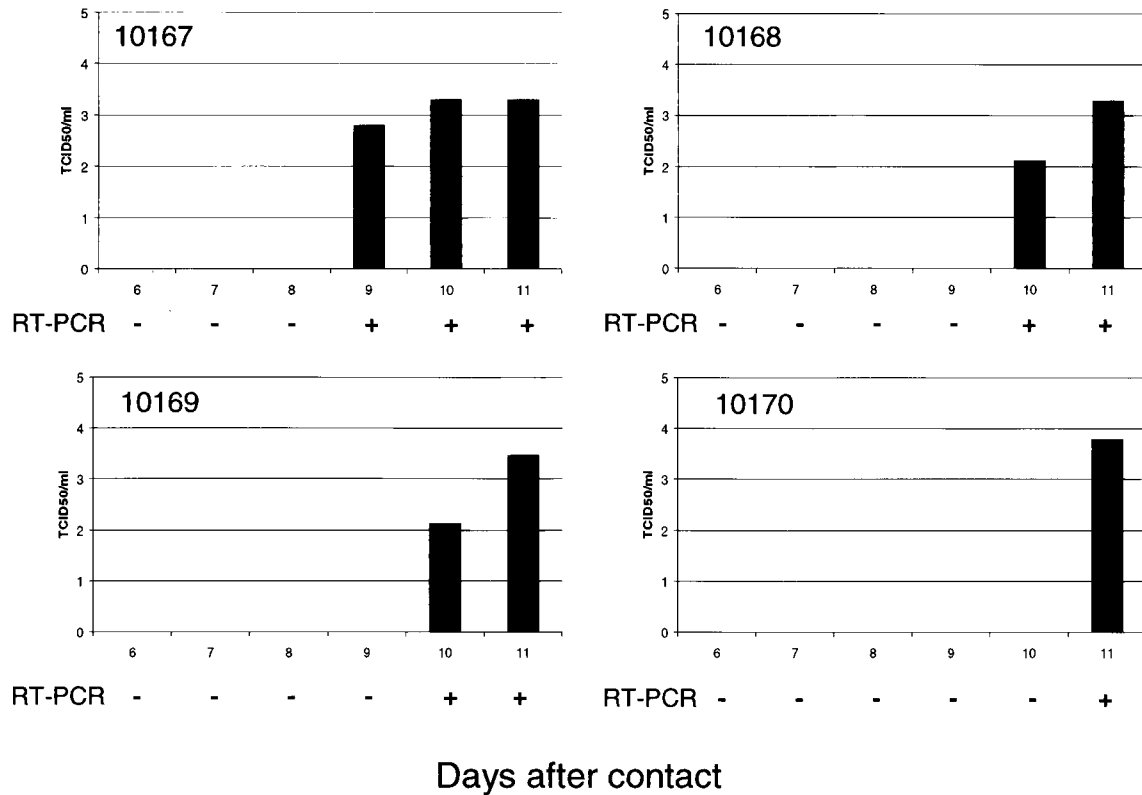


FIG. 4. Detection of CSFV in blood samples from contact-exposed pigs (animals 10167, 10168, 10169, and 10170) by virus isolation and real time-RT-PCR. The bars represent viremia titers expressed as TCID<sub>50</sub> per milliliter. RT-PCR results are expressed as positive (+) or negative (-).

isolated from blood samples at the same time (9 to 10 days postcontact) that it was detected by PCR (Fig. 4). These data clearly indicate that nasal swabs and tonsil scrapings are the samples of choice during the early stages of infection, with blood becoming the sample of choice at later times. Nasal and tonsil samples may have a higher predictive value of detecting CSFV infection during disease surveillance screening.

In summary, the assay described here, a fluorogenic-probe hydrolysis (TaqMan)-RT-PCR single-tube assay for rapid detection of CSFV, is performed in a single tube that contains all RT-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 and portability of the assay allow for its use as a pen-side assay for rapid on-site detection of CSFV.

Importantly, the assay detected the presence of the virus before the appearance of the disease. Virus was detected in the oronasal area 3 to 5 days before the appearance of clinical symptoms and in blood samples 0 to 3 days before the appearance of clinical disease (Fig. 3 and 4). The early detection of CSFV suggests two potential uses in disease control for the assay: as a surveillance tool in areas free of the disease and as a screening assay for monitoring a disease outbreak in real time.

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