An RT-PCR assay for the specific detection of classical swine fever virus in clinical samples

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Original article

An RT-PCR assay for the specific detection of classical swine fever virus in clinical samples

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Abstract – A simple reverse transcriptase-polymerase chain reaction (RT-PCR) assay has been developed for the specific amplification of DNA after reverse transcription of RNA from the classical swine fever virus (CSFV). A pair of oligonucleotides was selected from an area of high homology in the genome of CSFV strains, but which differed from the corresponding sequences in the genome of bovine viral diarrhea virus (BVDV) strains. Using these primers (CSFV1-CSFV2), a CSFV specific DNA band of 174 bp was amplified from the CSFV RNA extracted from four reference strains and 14 field isolates, as well as from 25 organ extracts and eight buffy coats and serum samples of experimentally infected animals. No amplification was observed with the RNA from four BVDV reference and vaccine strains and seven field isolates. This RT-PCR assay made it possible, in a one-step reaction, to detect CSFV rapidly, sensitively and specifically in cell culture supernatants and in clinical specimens. © Inra/Elsevier, Paris

classical swine fever / differential detection / RT-PCR

Résumé – Détection spécifique du virus de la peste porcine classique par amplification en chaîne par polymérase, dans des échantillons cliniques. Un test simple d'amplification en chaîne par polymérase (RT-PCR) a été développé pour l'amplification spécifique de l'ADN après rétrotranscription de l'ARN viral de la peste porcine classique (VPPC). Une paire d'oligonucléotides a été sélectionnée à partir d'une zone à forte homologie du génome de plusieurs souches du VPPC, mais qui différaient des séquences correspondantes du génome du virus de la diarrhée bovine virale. Grâce à l'utilisation de ces amorces (VPPC1-VPPC2), une bande d'ADN spécifique de 174 paires de bases a été amplifiée à partir de l'ARN de VPPC extrait de quatre souches de référence et de 14 isolats du terrain, mais également à partir de 25 extraits d'organes, de huit couches leucocytaires et échantillons de sérum d'animaux infectés expérimentalement. Aucune

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amplification n'a été observée avec l'ARN de virus de la diarrhée bovine virale provenant de quatre souches de référence et vaccins et de sept isolats du terrain. Ce test RT-PCR a donc permis une détection rapide, sensible et spécifique du VPPC dans les surnageants de cultures cellulaires et dans les échantillons cliniques. © Inra/Elsevier, Paris

peste porcine classique / détection différentielle / amplification en chaîne par polymérase après rétrotranscription

1. INTRODUCTION

Classical swine fever virus (CSFV; also known as hog cholera virus) is the causative agent of classical swine fever, a highly contagious disease of swine that causes world wide, significant economical losses in the pig industry [16].

This virus along with viruses of bovine viral diarrhea (BVDV) and border disease (BDV) make up the Pestivirus genus within the *Flaviviridae family* [2, 8]. The pestiviruses are closely related both antigenically and structurally [1]. They also share natural hosts [9] and for this reason it is very important to be able to differentiate between CSFV and BVDV infections in pig herds.

The current standard procedure for the detection of CSFV infection is based on virus isolation, which is then confirmed by immunofluorescence (IF) [23]. Differentiation of CSFV from BVDV and other ruminant pestiviruses is carried out by a further characterization of the viral isolates with genus-specific monoclonal antibodies [18]. However, this assay is laborious, time-consuming and requires the use of containment facilities.

RT-PCR has become a widely used technique for virus detection and characterization as it is a rapid, sensitive and specific diagnostic assay that can complement classical serological procedures (reviewed in Ehrlich et al. [7]). Consequently, over the last few years there have been several reports on the development of RT-PCR assays to detect the different pestiviruses [10, 13, 22], including CSFV [11, 12, 26].

In this report, we described the use of CSFV genotype-specific primer oligonucleotides to set up a simple and sensitive RT-PCR assay for the specific detection of CSFV in the supernatants of infected cell lines and clinical samples from naturally and experimentally infected pigs.

2. MATERIALS AND METHODS

2.1. Viruses

The CSFV reference strains Alfort, Ames, Margarita and PAV-250, and the BVDV strains NADL, Oregon and Singer were used in this study. A collection of eleven CSFV and seven BVDV field strains isolated in Cuba from 1993 to 1997 and three CSFV isolates from the 1997 Spanish outbreak were also analysed. CSFV and BVDV isolates were amplified in PK-15 and MDBK cells, respectively. In addition, tissue samples obtained from experimentally infected rabbits were used as source of lapinized CSFV strain 'C', and a freeze-dried, commercial modified BDV live vaccine (Mucosiffa, Rhône-Mérieux, Lyon, France), resuspended in PBS, was used as the starting material for RNA amplification.

2.2. Clinical samples

The materials analysed included spleen, lymph node or tonsil samples from 25 naturally infected, domestic pigs that were positive to CSFV in an indirect immunofluorescence (IF) assay [23], and from seven non-infected pigs.

For sample extraction, about 1 g of tissue was homogenized in 10 mL of PBS. Suspensions were centrifuged at 3 000 *g* for 20 min, and the supernatants were kept at –70 °C. In addition, sera and buffy coat samples collected from eight pigs before and after their experimental infection with a single standard dose of a commercial vaccine obtained from the lapinized CSFV strain 'C' (Labiofam, S.A. la Habana, Cuba), were also included in the analyses.

2.3. Direct immunofluorescence (IF) assay

The assay was performed as described by Terpstra [23]. Direct IF in tissues was performed from cryostat sections (no more than 4 µm thick) that were mounted on microscope slides. Alternatively, tissue homogenates were inoculated onto confluent monolayers of PK-15 cells that were grown on coverslips within Leighton tubes, and incubated for 48 h. In both cases, the coverslips were washed twice with culture medium, fixed for 10 min at –20 °C in acetone and washed in phosphate buffered saline (PBS). Cells were stained for 1 h at 37 °C in a humid closed chamber with an anti-CSFV swine hyperimmune serum conjugated with fluorescein isothiocyanate (FITC) diluted 1:20 in PBS. The stained cells were washed three times with PBS, mounted on microscope slides with buffered glycerine, and examined by fluorescence microscopy.

2.4. RNA processing

For RNA extraction, 500 µL of the infected cell supernatant was phenol and chloroform extracted, ethanol precipitated and resuspended in 10 µL of distilled water. Alternatively, 500 µL of a ten-fold dilution in PBS of tissue samples, sera or buffy coat samples were incubated with proteinase K (0.1 mg/mL) in the presence of SDS (0.2% wt-vol) for 25 min at 56 °C, and the RNA was extracted as described above.

2.5. Selection of RT-PCR primers

The sequences of CSFV strains Alfort [15], Alfort 187 [21], Alfort A19 (Smondack et al., EMBL Data Bank accession number U90951) and Brescia [17] and BVDV strains Osloss [19], NADL [2] and SD1 [3] were aligned by using the GCG package programs [5]. Gene regions that were both highly conserved between CSFV strains and highly divergent among CSFV strains when compared with BVDV strains, were identified and used to design primer pairs corresponding to CSFV genotype-specific sequences, by using the Hint PCR program [6]. The primer pair selected (CSFV1-CSFV2) defined a fragment of 174 bp within the sequence region corresponding to the non-structural viral protein NS5B *(table I)*. Primers Pest1 (sense) (CCTGATAGGGTGCTGCAGAG) and Pest2 (antisense) (TCAACTCCATGTGCCATGTAC), previously described by Wirz et al. [26], were used to amplify BVDV RNA. Oligonucleotides were purchased from Isogen Bioscience (Maarssen, The Netherlands).

2.6. RT-PCR

A sample of 2 µL of extracted RNA from virus-infected cell culture supernatants, tissue extracts, buffy coat samples or sera from infected pigs was used as starting material for RT-PCR amplifications. Briefly, RNA was incubated in 10 mM Tris (pH 8.3), 50 mM HCl, 1.5 mM $MgCl_2$, 0.01 % gelatine, 100 µM of each deoxynucleoside triphosphate, 40 U of human placental ribonuclease inhibitor, 200 ng of the corresponding antisense primer and 10 U of avian myeloblastosis virus reverse transcriptase (Seikagaku America, Inc., Ijamsville, USA), in a final volume of 100 µL. After 40 min at 42 °C, 200 ng of sense primer and 2.5 U of AmpliTaq polymerase (Perkin-Elmer, Roche, New Jersey, USA) were added to the reaction. Samples were amplified using a program that included first an incubation step at 94 °C for 2.5 min, followed by 35 cycles (denaturation at 94 °C for 15 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s) and a final incubation at 72 °C for 10 min. PCR products were analysed by electrophoresis in 2 % agarose gel containing 0.5 µg/mL of ethidium bromide.

2.7. Detection limit

To assess the sensitivity of the RT-PCR reaction, ten-fold serial dilutions in PBS of a CSFV Alfort strain stock of a known titer were used directly for phenol-chlorophorm RNA extraction, as described above. The RNA obtained was RT-PCR amplified using oliginucleotides CSFV1-CSFV2 and the $TCID_{50}$ corresponding to the last dilution that yielded a DNA fragment of the expected size was considered as the detection limit for the reaction. Likewise, the sensitivity of CSFV amplification from clinical samples, was determined from spleen extracts of negative control animals that were used to prepare serial (ten-fold) dilutions of a titrated CSFV Alfort strain. Upon incubation with proteinase K (0.1 mg/mL) and SDS (0.2%), the RNA was extracted and used to estimate the detection limit in the RT-PCR reaction, as described above.

2.8. cDNA sequencing

cDNAs were purified directly from the reaction mixture using the Wizard PCR Preps (Promega). A 50 ng sample of the recovered DNA, 10 μCi of ^{35}S-dATP and 20 ng of either primer CSFV1 or CSFV2, were used for the sequencing reactions, which were performed using the fmol DNA cycle sequencing system (Promega, Madison, USA).

3. RESULTS

3.1. Amplification of CSFV RNA in supernatants from virus-infected cell cultures

The application of the Hint PCR program to the primer search on the conserved regions of the CSFV genome identified two oligonucleotides (CSFV1 and CSFV2) that were predicted to amplify a 174 bp DNA fragment within the gene corresponding to the non-structural protein NS5B (see *table I*, and Materials and methods for details). When these primers were used in the RT-PCR reaction, they amplified a DNA fragment whose size, estimated in agarose gels stained with ethidium bromide, was consistent with that expected.

Figure 1 shows the 174 bp fragment obtained after RT-PCR amplification, using primers CSFV1-CSFV2, of RNA extracted from cell culture supernatants from three CSFV reference strains: Alfort, Ames and PAV-250. To confirm the specificity of the assay, the nucleotide sequence of the fragment resulting from the amplification of CSFV Alfort and Ames RNAs was determined. In both cases, the sequences obtained corresponded to those expected from the

Table I. Genotype-specific primers used for RT-PCR amplification of CSFV RNA.

Primer	Sequence (5'-3')	Genome position[a]
CSFV1 Sense	CCTGAGGACCAAACACATGTTG	10.230–10.251
CSFV2 Antisense	TGGTGGAAGTTGGTTGTGTCTG	10.403–10.382

[a] Nucleotide numbering corresponds to the genome sequence of CSFV Alfort strain [15].

amplified fragment (data not shown). In addition, no DNA product was observed when RNA extracted from supernatants of BVDV strains NADL and Oregon, or from non-infected cells was used as the starting material for the amplifications.

The specificity of the amplification was further analysed by using CSFV RNA extracted from eleven field isolates from Cuba and three viruses isolated in Spain *(table II)*. Positive amplification of a DNA band of the expected size was achieved with all the CSFV field isolates analysed. In addition, no amplification was observed with the RNA from four BVDV reference and vaccine strains and seven field isolates *(table II)*. In all cases, the presence of RNA in the BVDV samples was confirmed as they were efficiently amplified using primers Pest1/Pest2 *(table II)*. An example of the amplification obtained with primers CSFV1-CSFV2 and RNA extracted from supernatants of four CSFV field isolates, as well as with primers Pest1/Pest2 and RNA from the BVDV reference strains NADL, Oregon, Singer and the Cuban field isolate 1/96 is shown in *figure 2*. These results confirmed the specificity of the RT-PCR amplification based on the use of primers CSFV1-CSFV2.

3.2. Amplification of CSFV RNA in clinical samples of naturally and experimentally infected animals

The potential of the procedure to detect CSFV in field samples was first analysed using RNA extracted from different organs of naturally infected pigs. Three different samples from the lymph nodes, spleen and tonsils, were assayed. The amplifications obtained as well as the corresponding results of CSFV detection by IF are compared in *table III*. Positive RT-PCR amplification was achieved in all but one of the 25 samples from the infected animals analysed, while direct detection of CSFV in the tissue by IF was positive in only 22 of the samples tested. The presence of virus in the two samples that were positive to RT-PCR amplification (32/97 and 33/97) was con-

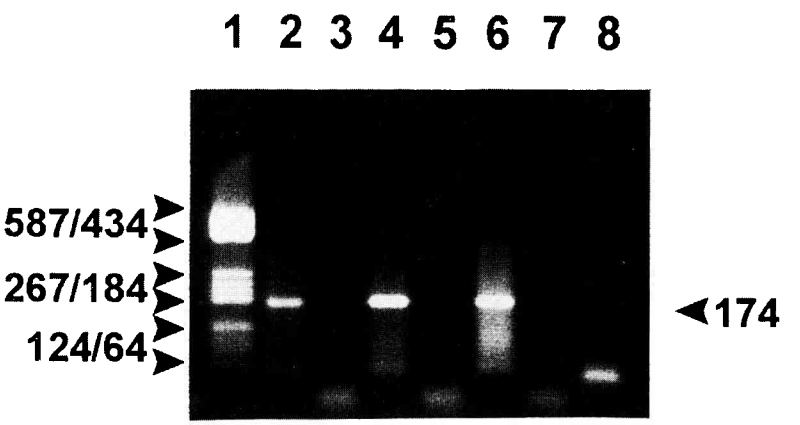

Figure 1. RT-PCR amplification with primers CSFV1-CSFV2 of RNA extracted from supernatants of cell cultures infected with CSFV or BVDV. Lane 1: molecular markers, fragment sizes (in base pairs) are indicated on the left; lane 2: CSFV Alfort; lane 3: BVDV NADL; lane 4: CSFV PAV-250; lane 5: BVDV Oregon, Lane 6: CSFV Ames; lane 7: BVDV Singer; lane 8: negative control.

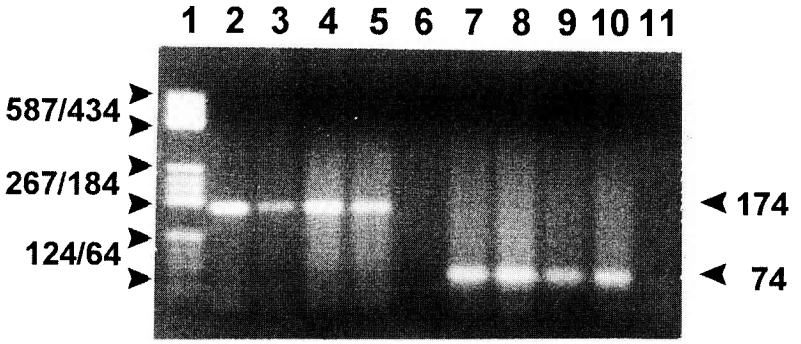

Figure 2. RT-PCR amplification with primers CSFV1-CSFV2 (lanes 2–5) or Pest1-Pest2 (lanes 7–11) of RNA extracted from supernatants of cell cultures infected with the following viruses: lanes 2–5, CSFV Cuban field isolates 157/93, 198/93, 34/96, 38/96, respectively; lane 7, BVDV NADL, lane 8, BVDV Oregon; lane 9, BVDV Singer; lane 10, BVDV Cuban field isolate 1/96. Lane 1: molecular markers, fragment sizes (in base pairs) are indicated on the left.

firmed as positive IF was observed in the supernatants of PK-15 cells infected with the corresponding tissue extracts. In addition, and confirming the specificity of the assay, no amplification was noticed in samples from non-infected animals.

Similar results were obtained in the analysis of blood samples taken from eight animals experimentally infected with a single dose of the CSFV cuban commercial vaccine *(table IV)*. Sera and buffy coat samples obtained from eight pigs before and 7 days after CSFV inoculation were tested by RT-PCR. All samples from CSFV inoculated pigs yielded the expected DNA segment, while no RT-PCR amplification products were detected in any of the clinical specimens obtained from these animals before infection.

3.3. Detection limit

Positive RT-PCR amplification was achieved when CSFV dilutions corresponding to 0.4 $TCID_{50}$ or higher were used as the starting material for RNA extraction (data not shown). To estimate the sensitivity of the assay from tissue extracts, we explored the ability of the procedure to amplify viral RNA extracted from serial dilutions of CSFV in spleen extracts of a negative control animal extraction (see Materials and methods for details). The lowest dilution resulting in positive amplification of viral RNA corresponded to 2 $TCID_{50}$ (data not shown).

4. DISCUSSION

Identification of genotype-specific primers is one alternative for the differential RT-PCR amplification and diagnosis of viral RNAs [20, 26]. In this report we describe a highly sensitive and specific RT-PCR procedure for the differential detection of CSFV RNA. The strategy is based on the use of a primer pair complementary to highly conserved sequences within the region of non-structural gene products, that allowed amplification of a

Table II. RT-PCR amplification from virus infected cell culture supernatants of CSFV or BVDV RNA, using primers CSFV1-CSFV2 and Pest1-Pest2, respectively.

Strains or isolates[a]	Amplification[b]	
	CSFV1–CSFV2	Pest1–Pest2
CSFV		
ALFORT	+	+
PAV-250	+	+
AMES	+	+
MARGARITA	+	+
C	+	+
157/93	+	+
198/93	+	+
34/96	+	+
38/96	+	+
41/96	+	+
95/96	+	+
146/96	+	+
167/96	+	+
253/96	+	+
14/97	+	+
15/97	+	+
Sp1/97[c]	+	+
Sp2/97[c]	+	+
Sp3/97[c]	+	+
BVDV		
NADL	–	+
OREGON	–	+
SINGER	–	+
MUCOSIFFA	–	+
1/96	–	+
2/96	–	+
3/96	–	+
4/96	–	+
5/98	–	+
6/97	–	+
7/97	–	+

[a] The different viruses analysed are indicated as follows: reference and vaccine strains, capital letters; field isolates, low case letters.

[b] The detection of a DNA fragment of 174 bp, upon the analysis of the RT-PCR reaction products on an agarose gel, was considered as positive CSFV amplification with primers CSFV1-CSFV2. Likewise, the detection of a DNA fragment of 74 bp was considered as positive pestivirus amplification with primers Pest1-Pest2.

[c] Virus isolated in Spain in 1997.

DNA fragment of 174 bp. Primers were identified with the aid of a computer program that allowed selection of sequences highly conserved among CSFV isolates but that were also divergent enough when compared to other pestiviruses. The specificity of the reaction was confirmed by nucelotide sequencing of the DNA amplified product and further supported by the positive results obtained with RNAs extracted from supernatants of cells infected with five CSFV reference and vaccine strains, eleven Cuban field isolates and three viruses isolated during the 1997 Spanish outbreak. Preliminary results based on the analysis of the E2 gene sequences indicated that the CSFV Cuban isolates used in this report were related to that of PAV-250 strain and defined an independent cluster within phylogenetic group I, (Diaz et al., unpublished results). Thus RNA from CSFV isolates that belonged to the two main phylogenetic groups previously defined [14, 24], group I (PAV-250, Margarita and 'C' strains, and the Cuban isolates) and group II (Alfort, and the Spanish isolates) were amplified with the primers used in this study. The specificity of this RT-PCR procedure was further confirmed by the lack of amplification observed when RNA from four reference and vaccine strains and seven BVDV Cuban field isolates were used in the assay. Experiments are in progress to evaluate the specificity of this assay with BDV isolates, a related pestivirus that can be transmitted from sheep to the pig. Thus, even while further work is required to ensure the specificity of this assay, the results available support the potential of the procedure to detect CSFV.

A high sensitivity, up to 0.4 $TCID_{50}$, was obtained from the amplification of RNA serial dilutions of CSFV. The procedure also made the amplification of RNA possible directly from samples of infected animals, for which the limit of detection was estimated as two $TCID_{50}$.

Table III. RT-PCR amplification of CSFV RNA in field samples from naturally infected pigs.

Pigs	Organ	RT-PCR amplification	IF[a]
Infected			
253/96	Spleen	+	+
169/96	Lymph node	+	+
121/95	Spleen	+	+
168/96	Lymph node	+	+
164/96	Tonsil	+	+
165/96	Spleen	+	+
192/96	Spleen	+	+
163/96	Spleen	+	+
110/96	Spleen	+	+
141/96	Spleen	+	+
185/96	Spleen	+	+
199/96	Tonsil	+	+
125/95	Tonsil	+	+
275/96	Spleen	+	+
281/96	Spleen	+	+
14/97	Spleen	+	+
85	Spleen	+	+
88	Spleen	+	+
1-67	Spleen	+	+
2-62	Spleen	+	+
25-2/97	Spleen	+	+
25-M/97	Spleen	+	+
32/97	Spleen	+	+[b]
33/97	Spleen	+	+[b]
34/97	Spleen	–	–
Non-infected			
68/96	Spleen	–	–
77/96	Spleen	–	–
78/96	Tonsil	–	–
26/96	Tonsil	–	–
106/96	Spleen	–	–
35/96	Spleen	–	–
34/97	Spleen	–	–

[a] Immunofluorescence assay [23]. [b] These samples were IF negative when the tissue was directly assayed, but positive IF was observed after one passage in PK-15 cells.

Table IV. RT-PCR amplification of CSFV RNA in serum and buffy coat samples from pigs before and 7 days post experimental infection.

Sample	RT-PCR	
	Before	After infection
Serum 11	–	+
Serum 39	–	+
Serum 92	–	+
Serum 97	–	+
Buffy coat 65	–	+
Buffy coat 69	–	+
Buffy coat 93	–	+
Buffy coat 94	–	+

Consistently, the ability of the RT-PCR procedure to detect CSFV in organ samples from slaughtered animals, without previous viral isolation in cell culture, was higher than that of direct IF. Positive RT-PCR amplification was achieved from 24 out of 25 samples analysed, while only 22 of these samples were positive to direct IF. The two RT-PCR positive samples that were negative to IF detection in tissue tested positive upon viral isolation in cell culture, providing support for the high sensitivity of the RT-PCR amplification.

Conventional virological methods do not provide a tool for the reliable detection of the virus in the case of viral persistence. Thus, RT-PCR is particularly attractive for the detection and study of persistently infected animals, which may play an important role in the spreading of the virus [4, 25]. As a first step to study the potential of this RT-PCR assay as a diagnostic tool in live animals, we analysed serum and buffy coat samples collected from animals experimentally infected with the Cuban lapinized commercial vaccine. Positive amplification was detected in the eight samples from the infected animals analysed, whereas negative results were obtained from samples collected before infection, as expected. Further analyses of sera and buffy coat samples are in progress to draw definitive conclusions concerning the sensitivity and the specificity of this RT-PCR procedure. Even while additional experiments are required to assess the potential of this procedure for amplifying CSFV from persistently infected animals, the possibility of performing RT-PCR amplifications from serum samples offers a simple procedure for large-scale screenings aimed at CSFV detection.

The efficiency of the amplification obtained with primers CSFV1-CSFV2 was, in all cases, equivalent to that obtained when primers pest1-pest2 were used in our reaction conditions. These primers were previously described by Wirz et al. [26], and they hybridize with sequences highly conserved among pestiviruses in the 5' non-coding RNA region. Therefore, the combined use of primers CSFV1-CSFV2 and Pest1-Pest2 may be used to further differentiate CSFV from other pestivirus.

In summary, the RT-PCR assay developed offers a rapid and sensitive diagnostic tool for the specific detection of CSFV in cell culture viral supernatants, organ extracts, buffy coat and serum samples from infected domestic pigs.

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