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# Development of an optimized protocol for the detection of classical swine fever virus in formalin-fixed, paraffin-embedded tissues by seminested reverse transcription-polymerase chain reaction and comparison with in situ hybridization

S.-K. Ha, C. Choi, C. Chae *

*Department of Veterinary Pathology, College of Veterinary Medicine and School of Agricultural Biotechnology, Seoul National University, San 56-1, Shillim-Dong, Kwanak-Gu 151-742, Seoul, Republic of Korea*

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**Abstract**

An optimized protocol was developed for the detection of classical swine fever virus (CSFV) in formalin-fixed, paraffin-embedded tissues obtained from experimentally and naturally infected pigs by seminested reverse transcription-polymerase chain reaction (RT-PCR). The results for seminested RT-PCR were compared with those determined by in situ hybridization. The results obtained show that the use of deparaffinization with xylene, digestion with proteinase K, extraction with Trizol LS, followed by seminested RT-PCR is a reliable detection method. An increase in sensitivity was observed as amplicon size decreased. The highest sensitivity for RT-PCR on formalin-fixed, paraffin-embedded tissues RNA was obtained with amplicon sizes less than approximately 200 base pairs. An hybridization signal for CSFV was detected in lymph nodes from 12 experimentally and 12 naturally infected pigs. The sensitivity of seminested RT-PCR compared with in situ hybridization was 100% for CSFV. When only formalin-fixed tissues are available, seminested RT-PCR and in situ hybridization would be useful diagnostic methods for the detection of CSFV nucleic acid.


*Keywords:* Classical swine fever virus; Diagnosis; Formalin-fixed tissue; Polymerase chain reaction

## 1. Introduction

Classical swine fever virus (CSFV) is one of the most important viral pathogens in pigs, and its control and prevention are of worldwide concern. CSFV demonstrates an acute course characterized by high fever and multiple hemorrhages, as well as a chronic or clinically inapparent course (van Oirschot, 1999). CSFV is an RNA virus classified within the genus *Pestivirus* of the Flaviviridae family, which also includes bovine viral diarrhea virus (BVDV) and border disease virus (Wengler et al., 1995). The CSFV genome is a single-stranded, positive-sense, non-polyadenylated RNA, about 12.3 kb in length (Meyers et al., 1989; Moormann et al., 1990).

In the field of diagnostic pathology, fresh or frozen tissue is not always available. Formalin fixation is the standard method for tissue preservation in veterinary pathology and this material forms the major source of tissues for many studies. Formalin-fixed, paraffin-embedded tissues have been used for both reverse transcription-polymerase chain reaction (RT-PCR) and in situ hybridization detection of viral RNA (Choi and Chae, 2002; Chung et al., 2002; Choi and Chae, 2003a,b,c). In situ hybridization is, however, of greater technical complexity and expense when compared with RT-PCR (Kim and Chae, 2001, 2002). Therefore, application of the RT-PCR assay for the detection of viral RNA in formalin-fixed, paraffin-embedded tissue is desirable. The development of such techniques is also important as formalin fixation of tissues allows veterinary practitioners to readily ship tissue samples for CSFV identification in a well-preserved, non-infectious state to

* Corresponding author. Tel.: +82-2-880-1277; fax: +82-2-871-5821.
*E-mail address:* swine@plaza.snu.ac.kr (C. Chae).

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avoid accidental transmission of this deadly contagious virus during transportation. The objective of this study, therefore, was to develop an optimized protocol for the detection of CSFV directly from formalin-fixed, paraffin-embedded tissue by seminested RT-PCR and compare this method with in situ hybridization.

## 2. Materials and methods

### 2.1. Classical swine fever virus

CSFV strain SNUVR2345 used in this study was isolated from a 58-day-old pig, in a 200-sow herd located in Chungcheung Province in 1997. In this herd, a pig was presented with severe respiratory disease and growth retardation. The pig was diagnosed by chronic CSFV infection on the basis of clinical signs, virus isolation, immunohistochemistry, and in situ hybridization. CSFV strain SNUVR2345 was considered as a low virulent strain.

### 2.2. Experimental inoculation and protocol

Twenty four 28-day-old pigs were randomly allocated between an infected ($n = 12$) or control group ($n = 12$). Serum samples from all pigs were tested by the virus neutralization assay for antibodies against CSFV and BVDV before experimental infection. Twelve pigs were inoculated intranasally with 3 ml of the CSFV strain SNUVR2345 (2nd passage) at a concentration of $10^{5.5}$ tissue culture infective doses ($TCID_{50}$) per ml. Twelve control pigs were inoculated with 3 ml of the supernatant of non-infected PK-15 cells. The pigs were housed individually in isolation facilities. Infected and control pigs were humanely euthanized at 7 days post-inoculation (dpi). Tissues were collected from each pig at necropsy. Virus isolation was performed on the superficial inguinal lymph node. Negative tissue controls were also collected from each of 2 calves that had been naturally infected with bovine viral diarrhea virus (BVDV).

### 2.3. Fixation time

To examine the effect of fixation time on the ability to detect CSFV by seminested RT-PCR, tissues from pigs inoculated with CSFV were fixed for various times before embedding. At intervals (1, 30, 45, 60, 90, 120, 150, and 180 days), a representative sample from each tissue was removed, processed, and embedded in paraffin by standard histologic procedures.

### 2.4. Virus isolation

Virus isolation was performed in lymph node from CSFV-infected and negative control pigs as previously described (Choi and Chae, 2003c).

### 2.5. Archived tissues

Twelve formalin-fixed, paraffin-embedded lymph node tissues from each of 12 pigs naturally infected with CSFV were used in the present study. CSFV was isolated from lymph node from all 12 pigs. Four formalin-fixed, paraffin-embedded tissues were 3 years old, 4 were 2 years old, and 4 were 1 year old.

### 2.6. RNA extraction

For each pig, a 10 μm-wide section of lymph node was prepared from a paraffin embedded block, excess paraffin removed and placed into 1.5 ml sterile tubes. The microtome blade, tweezers, and other equipment were carefully cleaned before processing successive tissue block to minimize the risk of cross-contamination.

Tissue sections were deparaffinized with xylene for 10 min and washed twice with ethanol to remove the solvent. The ethanol was then allowed to evaporate under vacuum for 10 min. Three different digestion buffers were used to isolate the genomic CSFV RNA from these deparaffinized tissues. Digestion buffer 1 consisted of 50 mM Tris–HCl (pH 8.0) and 50 mM ethylenediaminetetraacetic acid (EDTA) containing 500 μg/ml of proteinase K (Boehringer Mannheim, Indianapolis, IN, USA). Digestion buffer 2 consisted of 500 mM Tris–HCl (pH 7.6), 10 mM NaCl, 20 mM EDTA, plus 1% SDS containing 500 μg/ml of proteinase K and 10 mg/μl of aurintricaboxylic acid. Digestion buffer 3 consisted of 1 M guanidinium thiocyanate, 25 mM 2-mercaptoethanol, 0.5% Sarcosyl 20 (*N*-lauroylsarcosine), and 20 mM Tris–HCl (pH 7.5) containing 6 mg/ml of proteinase K. To isolate genomic RNA, 500 μl of digestion buffer was added to the extracted dried tissue samples. The resuspended tissues were incubated overnight at 50 °C and then at 95 °C for 8 min to inactivate the proteinase K.

Two different methods were used to purify the RNA from digested samples. The digested samples were extracted three times using either standard phenol–chloroform–isoamyl (PCI) alcohol (25:24:1) or Trizol LS reagent (Gibco BRL, Grand Island, NY, USA) and then precipitated in ethanol to collect the RNA. The final ethanol-washed RNA pellet was air dried and then dissolved in 30 μl of diethyl-pyrocarbonate-treated water.

### 2.7. Primers

The primers used in this study were described in Table 1. The primers were designed using computer software (Oligo 4.0 program, National Biosciences, Plymouth, MN, USA) and species-specific region was chosen. The size of PCR products and the references of primers are also shown in Table 1.

Table 1
Reverse transcription-polymerase chain reaction (RT-PCR) primers for detection of classical swine fever virus

| Primer set | Orientation | Sequences (5′–3′) | Position | Product size (bp) | Reference |
|---|---|---|---|---|---|
| A | Outer F[a] | AGACGGCCTGTACCATAATA | 1062–1081 | 610 | |
| | Outer R[b] | GTATAAGATGTCCTCCACGG | 1671–1652 | | Choi and Chae (2003a) |
| | Inner F | GACAACGGCACTAATGGTAT | 1201–1220 | 471 | Choi and Chae (2003a) |
| B | Outer F | GACAACGGCACTAATGGTAT | 1201–1220 | 471 | Choi and Chae (2003a) |
| | Outer R | GTATAAGATGTCCTCCACGG | 1671–1652 | | Choi and Chae (2003a) |
| | Inner F | ACATGGATGGTGTAACTGGT | 1407–1426 | 265 | This study |
| C | Outer F | GACAACGGCACTAATGGTAT | 1201–1220 | 405 | Choi and Chae (2003a) |
| | Outer R | AAAGTTTTTCCCTTTCTTGC | 1605–1586 | | This study |
| | Inner F | ACATGGATGGTGTAACTGGT | 1407–1426 | 199 | This study |

[a] F, Forward primer.
[b] R, Reverse primer.

### 2.8. Reverse transcription

For the first-strand cDNA synthesis, 1 μl of the CSFV RNA (5 ng/μl) was supplemented in a total reaction volume of 10 μl with 1× RT buffer (50 mM Tris–HCl, 8 mM $MgCl_2$, 30 mM KCl, 1 mM dithiothreitol (pH 8.3)), 0.5 mM (each) deoxynucleotide triphosphates (dNTPs), 2.5 μM random hexanucleotide mixture, 20 U of RNase inhibitor, and 50 U of Moloney murine leukemia virus reverse transcriptase. After incubation for 40 min at 42 °C, the mixture was incubated for 3 min at 94 °C to denature the products. The mixture was then chilled on ice.

### 2.9. Polymerase chain reaction

The composition of the PCR mixture (50 μl) was 10 μl of cDNA (5 ng/μl), 1 μl of each primer (250 nM), 5 μl of 10× PCR buffer (10 mM Tris–HCl, 40 mM KCl, 1.5 mM $MgCl_2$ (pH 8.3)), 1.2 μl of each dNTP (0.2 mM), 1 μl of 2.5 U of Taq polymerase, and 27.2 μl of distilled water. The PCR reaction for CSFV was done under the following conditions in a thermal cycler (Perkin–Elmer–Cetus, Norwalk, CT, USA): 1 cycle of 5 min at 94 °C; 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, and elongation at 72 °C for 1 min; and 1 cycle of 2 min at 72 °C. Seminested PCR was performed on 2 μl of cDNA product by using nested primers and the same reaction, step time and temperature, and numbers of cycles as in the first amplification.

The sensitivity of RT-PCR was carried out as described previously (Choi and Chae, 2003a). The primers were tested with 10-fold dilutions of RNA extracted from 100 μl of a purified CSFV suspension (preadjusted to $10^6$ viral particles/ml, as determined by negatively stained electron microscopy) and from PK-15 cells (adjusted to $10^6$ cells/ml).

### 2.10. Sequencing

RT-PCR products were purified using a 30-kD cutoff membrane ultrafiltration filter. Purified RT-PCR products were sequenced with automatic sequencer (ABI Prism Sequencer, Applied Biosystems, Foster City, CA, USA).

### 2.11. In situ hybridization

In situ hybridization was performed as previously described (Choi and Chae, 2003b).

## 3. Results

### 3.1. Clinical signs

Pigs became febrile at between 4 and 6 dpi, but no other clinical signs were observed. The negative control pigs remained clinically normal.

### 3.2. Histopathology

The predominant microscopic lesions observed in the lymph nodes was necrosis in the center of prominent lymphoid follicles. Necrotic foci of 1–10 cells with pyknosis and karryorrhexis were commonly observed in the center of prominent follicles and less often in the surrounding lymphoreticular tissues. No microscopic lesions were observed in sections from lymph nodes of negative control pigs.

### 3.3. Virus isolation

CSFV was isolated from 12 (100%) of 12 lymph node from the 12 experimentally infected pigs and 12 (100%) of lymph nodes from the 12 naturally infected pigs. No CSFV was isolated from negative control pigs.

### 3.4. Specificity and sensitivity of the RT-PCR amplification

Another pestivirus (bovine viral diarrhea virus) did not react with primers specific for the CSFV genome in

the RT-PCR. These primers also did not react with seven other swine viruses (namely porcine circovirus, porcine reproductive and respiratory syndrome virus, transmissible gastroenteritis virus, porcine respiratory coronavirus, rotavirus, porcine epidemic diarrhea virus, and pseudorabies virus). The sensitivity of detection of CSFV with three different primer sets was also examined. There was no difference in sensitivity identified among the three primer sets. A 10-fold dilution series was made in culture medium. Approximately, 73 viral particles per ml could be detected with the outer RT-PCR. In contrast, as few as 27 viral particles per ml could be detected by the seminested RT-PCR with the three primer sets.

### 3.5. Seminested RT-PCR

The sensitivity and specificity of seminested RT-PCR were calculated from the data in Table 2. Sensitivity was determined by dividing the number of CSFV seminested RT-PCR positive lymph nodes by the number of CSFV-inoculated pigs; 12/12 = 100%. The specificity was determined by dividing the number of control samples that were negative for CSFV seminested RT-PCR by the number of mock-infected control pigs: 12/12 = 100%.

Table 2 summarizes the lymph nodes results in which CSFV nucleic acid was detected in experimentally and naturally infected pigs. Tissues were fixed in formalin for 24 h before embedding. Each specific primer pair for CSFV yielded a seminested RT-PCR product of the expected size from RNA extracted from formalin-fixed, paraffin-embedded tissues.

As for the choice of digestion buffer, digestion buffer 1 worked best followed by digestion buffer 2 and buffer 3 (Table 2). Trizol LS purified RNA gave better results than PCI alcohol for all primer sets tested. To test the effect of amplicon size in the seminested RT-PCR, primer set C (405/199 base pair (bp)) worked best when the tissue was digested with buffer 1 and RNA extracted with Trizol LS (Fig. 1). With this combination, CSFV nucleic acid was detected in 12/12 and 12/12 of lymph nodes from experimentally and naturally infected pigs, respectively. Nine seminested RT-PCR products from lymph node from each experimentally and naturally infected pigs were sequenced, and their identity confirmed as CSFV (data not shown). This primer set C (405/199 bp) amplified both the outer and inner product well. For the other primer set B (471/265 bp), the inner product amplified more readily than the outer product. The primer set A (610/417 bp) yielded the poorest results. The inner product could be amplified but the outer product failed for all but one reaction. CSFV nucleic acid was not detected in the lymph nodes from negative control pigs.

### 3.6. Fixation time

Digestion with proteinase K, extraction with Trizol LS, and followed by seminested RT-PCR (primer set C)

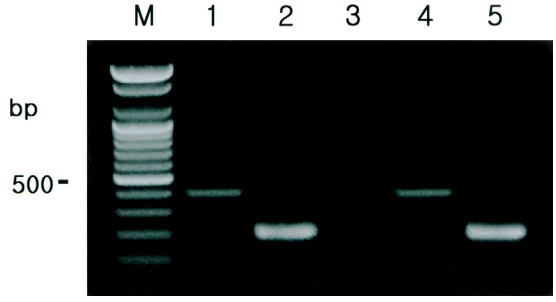


Fig. 1. Agarose gel electrophoresis of reverse transcription-polymerase chain reaction (RT-PCR) and seminested RT-PCR products of classical swine fever virus detected in formalin-fixed, paraffin-embedded lymph node tissues from experimentally infected pigs. Lane 1, RT-PCR from positive control; lane 2, seminested RT-PCR from positive control; lane 3, seminested RT-PCR from negative control; lane 4, RT-PCR from pig at 7 days post-inoculation (dpi); lane 5, seminested RT-PCR from pig at 7 dpi.

Table 2
Comparison of RNA extraction techniques and seminested reverse transcription-polymerase chain reaction (RT-PCR) for the detection of classical swine fever virus in formalin-fixed, paraffin-embedded lymph nodes from experimentally and naturally infected pigs

| Digestion buffer | RNA extraction | Amplicon size of seminested RT-PCR ($n = 12/12$) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 610/471 bp | | 471/265 bp | | 405/199 bp | |
| | | Outer | Inner | Outer | Inner | Outer | Inner |
| 1 | Trizol LS | 1[a]/0[b] | 3/3 | 4/4 | 6/6 | 6/5 | 12/12 |
| | PCI alcohol[c] | 0/0 | 1/3 | 2/1 | 4/5 | 3/2 | 10/9 |
| 2 | Trizol LS | 0/0 | 2/2 | 2/2 | 3/2 | 2/2 | 8/8 |
| | PCI alcohol | 0/0 | 1/1 | 2/0 | 2/2 | 2/3 | 5/4 |
| 3 | Trizol LS | 0/0 | 1/1 | 1/1 | 2/3 | 2/1 | 6/5 |
| | PCI alcohol | 0/0 | 0/2 | 1/1 | 2/4 | 1/1 | 3/3 |

[a] Number of positive samples from experimentally infected pig.
[b] Number of positive samples from naturally infected pig.
[c] Phenol–chloroform–isoamyl alcohol.

was use to determine whether prolonged fixation of tissues in formalin affected the functionality of the seminested RT-PCR, 12 lymph node samples from 12 pigs inoculated with CSFV were examined following fixation for 1–180 days and subsequent embedment. CSFV nucleic acid was detected in 84 (87.5%) out of 96 fixed tissues. Acceptable PCR signals were detected from all tissues fixed for periods up to 60 days. Thereafter, the number yielding positive signals declined. By 180 days in formalin, positive signals were only detected from 7 of 12 blocks of lymph nodes (58.3%).

### 3.7. In situ hybridization

Hybridization signals for CSFV were detected in lymph nodes from 12 experimentally and 12 naturally infected pigs. The tissue morphology was well preserved despite the relatively high temperatures required in the incubation procedure. CSFV positive cells typically exhibited a dark reaction product in the cytoplasm without any observable background staining. CSFV nucleic acids were consistently found in the cytoplasm of mononuclear cells and lymphocytes in the medullary sinuses and lymphoid follicles of lymph nodes (Fig. 2). Within lymph nodes, CSFV nucleic acid was occasionally found in lymphoid follicles within the cortices.

The sensitivity of seminested RT-PCR compared with in situ hybridization for CSFV was 100%. The specificity compared with virus isolation was also 100%. No hybridization signal was consistently seen in tissue sections treated with RNase A prior to in situ hybridization. Sections from negative control pigs and BVDV-infected calves showed no hybridization signal for CSFV.

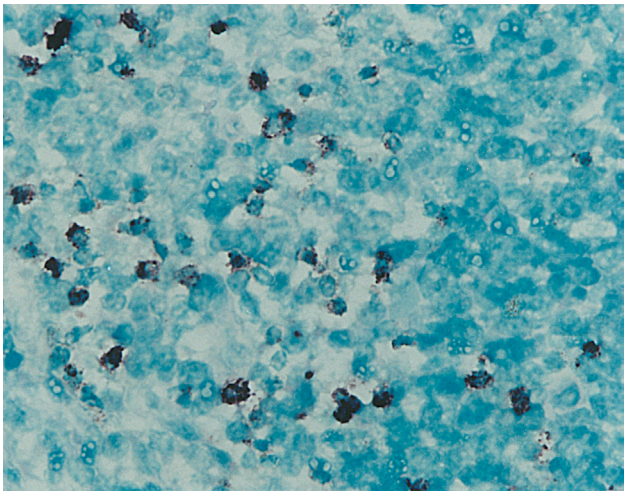

Fig. 2. Lymph node; pig experimentally infected with classical swine fever virus (CSFV) at 7 days post-inoculation. CSFV RNA (black reaction) is detected in mononuclear cells. In situ hybridization; nitroblue tetrazolium and 5-bromocresyl-3-indolylphosphate, methyl green counterstain.

## 4. Discussion

In this study, a sensitive and reproducible method was developed for the detection of CSFV in formalin-fixed, paraffin-embedded tissues by seminested RT-PCR. The results obtained show that the use of deparaffinization with xylene, digestion with proteinase K, extraction with Trizol LS, and then seminested RT-PCR is very reliable. Comparison of conventional RT-PCR with seminested RT-PCR clearly demonstrated the greater sensitivity of the seminested RT-PCR approach. After tissue digestion, only a few copies of viral RNA were likely to be present in the samples. The ability to amplify specific regions of RNA from formalin-fixed, paraffin-embedded tissue by RT-PCR may have a profound impact on diagnostic pathology. This technique allows pathologists to better correlate histopathologic changes in tissues with specific virus identification.

The detection of RNA from fixed tissues, in comparison to DNA, is more difficult because of the fragility of RNA. The lack of reproducibility of RT-PCR RNA detection is a serious obstacle to the further use of RT-PCR-based diagnostic methods. Technical problems to overcome include RNA fragmentation, formalin-induced protein cross-linking, the addition of monomethyl groups to nucleotides, dimerization of adenine (which interferes with subsequent PCR), ubiquitous RNase enzymes, and paraffin interference with RNA extraction by guanidinium hydrochloride (Akyol et al., 1992; Bresters et al., 1994; Guerrero et al., 1997; Mizuno et al., 1998; Lewis et al., 2001). Therefore, an optimized CSFV RNA extraction method was devised to circumvent these problems.

CSFV RNA could not be extracted from formalin-fixed, paraffin-embedded tissues with guanidinium thiocyanate alone in this study. However, initial digestion with proteinase K released the RNA from the formalin-fixed, paraffin-embedded tissues. Furthermore, proteinase K is known to solubilize tissue proteins and reverse monomethyl nucleotide modifications of RNA (Jackson et al., 1990). The use of a digestion buffer containing proteinase K was found to yield viral RNA suitable for RT-PCR amplification. Despite the optimized RNA extraction protocol, RNA obtained from formalin-fixed, paraffin-embedded tissues was still highly degraded and, as such, one would expect smaller amplicon sizes to provide better sensitivity. It has been shown previously that the success of RT-PCR was limited in attempts to amplify large target fragments and that most success was obtained when target amplicons were small (Bresters et al., 1994; Guerrero et al., 1997). When this was tested in the present study, it was found that there was an increase in sensitivity as amplicon size decreased.

Another problem that affects the ability to produce an amplificable cDNA from RNA isolated from

formalin-fixed, paraffin-embedded tissues is the presence of ribonucleases (RNases). RNA is prone to breakdown due to the ubiquitous presence of both endogenous and exogenous RNases, therefore, its degradation is usually more severe than DNA (Mizuno et al., 1998). Although RNases are very stable and highly active and do not require cofactors to function, endogenous RNases will have been totally inactivated by cross-linking during formalin fixation. Extraction with proteinase K ensures that these fixed cross-linked RNases are then totally destroyed (Lewis et al., 2001), thus avoiding any potential reactivation during the reversal of the RNA fixation in aqueous buffers.

Although the ability of the RT-PCR procedures to amplify target sequences is particularly powerful for the detection of viral RNA from formalin-fixed, paraffin-embedded tissues, it does not provide any cellular detail and histological architecture (Cheon and Chae, 2000). In contrast to RT-PCR, the great value of in situ hybridization lies in the possible association of the signal with cells of particular types or with observable histological changes (Cheon and Chae, 2000; Kim and Chae, 2001, 2002). Its major disadvantage is the greater technical complexity and expense compared with RT-PCR.

The use of seminested RT-PCR in tissues subjected to prolonged formalin fixation yielded falsely negative CSFV RNA results, like that previously observed for other viruses (Chung et al., 2002; Jung et al., 2003). When tissue is stored for archival purposes, prolonged bulk storage in formalin is likely to be of limited use for studies involving RNA detection. Rather, early formalin fixation and storage in paraffin-embedded block are more likely to provide informative material for later use. The specific optimization of protocols permits the extension molecular analyses to even smaller amounts of archival tissue. The development of optimized semi nested RT-PCR protocol for the detection of CSFV RNA in formalin-fixed, paraffin-embedded tissue is a useful tools for the diagnosis of CSFV.

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