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# Classical swine fever disease in Cheolwon

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## Abstract

Two cases of classical swine fever (CSF) disease have broken out in Cheolwon (7 April, 2002). The suspected pig herds were huddled together because of high fever (over 40℃) and showed remarkable decrease of the leukocytes. The staggering gait related to posterior weakness, constipation and lethargy, hyperemia, hemorrhagic lesions (on the skin, muzzle, ears, limbs, tail and inner part of legs) and conjunctivitis with dirty streaks below the eyes were observed. The inflammation in the lung, infarction in the spleen, swelling and hemorrhage in lymph nodes, kidney, intestine, heart and cheese like purulent inflammation of the tonsil were observed. The ulcers of the colon were also detected.

Several clinical and laboratory techniques including blood test, histo-pathological examinations, indirect fluorescent antibody (IFA) test and RT-PCR test were applied to diagnose the disease. Inoculation test on PK-15 cell was also performed. The necrosis of the lymphatic cells and infiltration of the vessel circumferential cells in the brain and lymph organs were commonly viewed. The proliferation of the glia cell (gliosis) in the lymph was particular. Cytopathogenic effect (CPE) and specific fluorescent-bright-green areas (with IFA) appeared in PK-15 cells inoculated with suspected blood plasma. The IFA test on the epithelial and mucous membrane cells of tonsil was positive. RT-PCR technique required more working hours and labor than other techniques in this examination but it was useful because of the sensitivity to the CSF viral gene.

Key words : Classical swine fever, Laboratory examination

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## Introduction

Classical swine fever (CSF) disease is a highly contagious viral disease of the swine caused by a lipid-enveloped RNA pathogen belonging to the family *Flaviv iridae*, genus *Pestivirus*[1,2]

The disease is also commonly known as 'hog cholera'. The organism has a close antigenic relationship with the 'bovine viral diarrhea virus (BVDV)' and the 'bor der disease virus (BDV)'. CSF is chara cterized by the symptoms including high fever, loss of appetite, diarrhea and constipation, red or purplish skin, stagg ering because of paralysis, high morbidity and mortality. The virulence of the strain or the susceptibility of the host influences the clinical signs of swine. Affected pigs release the virus during before or after showing the clinical signs and infected condition can be sustained to the last of lifelong. CSF was reported first in Amer ica by Hanson[3] and the disease has broken out in many countries. The first outbreak of the disease in Korea appeared probably in 1908. Since then, sporadic outbreaks have been reported every year. In 1997, the Korean government conducted nationwide to vaccinate swine against CSF and the vaccination stopped on December 1st, 2001. The Government notif ied OIE of its CSF free condition in 2002. However, unfortunately, two cases of CSF have broken out in Cheolwon (7 April, 2002).

This study was undertaken to identify the characteristic symptoms and histopa thological lesions of CSF in Cheolwon and to apply the results to diagnosis.

## Materials and Methods

### Clinical and pathological observation

The growing aspects, the appetite, vigor, walking and the skin color of pigs were carefully observed. The excrement was also closely observed and the body temperature was taken.

### Sample collection

The study used blood and tissue samples from 30 CSF-suspected pigs. Blood samples were collected in blood-collecting bottles (heparin added) from jugular vein of the suspected pigs. The number of leukocyte in collected blood samples was measured with automatic blood cell counter (CA570, Medonic Co. Sweden). The leukocytes were separated using Leucosep® tube (Greiner, Austria). The serum and plasma in the blood were also separated. The pigs were autopsied before or after death and internal organs were also carefully observed with the naked eye. Tissue samples were collected from 5 pigs' organs out of the 30 suspected swine. The half of each tissue samples was refrigerated and the rest were fixed in buffered formalin. The fixed tissue samples were embedded in paraffin and sectioned (5~7 ㎛ in thickness). For microscopic examinations, hematoxylin -eosin staining was used and examined by a microscope (magnifying power of 1,200).

### Cell culture

PK-15 cell line (distributed from NVRQS) was cultivated with α-minimal essential medium (α-MEM; virus culture kit. JNKit®, JBI, Korea), in which 10% of fetal calf serum was added. Antibiotic mycotic sol., non-essential acid (1%), L-glutamine (1%) and sodium pyruvic acid (1%) were also contained in the medium. The cultured cells were put in tissue culturing flask (media. Vol.: 30mℓ) set as 105 cell/mℓ in each flask. The flasks were incubated again under the atmosphere of 5% $CO_2$ at 37℃ for 3 days. Than, the cultured cells were observed by invert microscope and viewed the conditions such as contamination and cell activities. These cultured cells were washed 3 times and 3mℓ of trypsin-EDTA (JBI, Korea) was added to the cells. After incubation for about 10 minutes under the atmosphere of 5% $CO_2$ at 37℃, the cells were ripped off and succeeding cultivated.

### IFA test on the tissue

A part of collected tonsil samples were refrigerated and sectioned (5~7㎛ in thick ness) for specimen slides. The specimens (slide) were stained with anti-CSF virus monoclonal antibody at 4℃ for 30~60 minutes in the moisture box. After washed in cold PBS, the specimens were stained with 2nd antibody (fluorescent-labeled anti-mouse IgG). And washed again and mounted with stock glycerin. Fluorescence microscope was used in the observation.

### IFA test on the cultured cell

The sub cultured PK-15 cell line was monolayered in the 48 wells of tissue culture plate. After the removal of the medium, serum less medium was added to the wells (0.2mℓ in each well). The cells in the wells were inoculated with suspected blood plasma (10μℓ, ×20 dilution) and activated at 37℃ for 2 hours. After the washing of the wells with PBS, a medium containing 5% serum was projected into the wells. The plates were incubated again for 48~72 hours under the atmosphere of 5% $CO_2$ at 37℃. The next procedures were the same as the manual of kit (JNKit® ICS set, Korea). Fluorescent microscope was used in observation.

### ELISA for antibody and antigen

Classical Swine Fever Virus specific Antibody ELISA Kit® (Jeno Biotech, Korea) was used in the test. The plates of the kit used in the test were adsorbed CSFV protein E2 (of recombinant CSF virus, gp55). The next procedures of the test were the same as the manual of the kit (JNKit® ICS set, Korea). The reading point of light wavelength for the antibody detection was 405nm (with Anthos Ⅲ ELISA reader, Austria). The positive control value of O.D was 1.0185 and the negative control value of that was 0.178 for antibody detection. Sample to positive (s/p) ratio was considered.

### RT-PCR test for antigen detection

To detect genotypic CSF virus in leuk ocyte, the reverse transcription-polymerase chain reaction (RT-PCR) technique was

preformed. The RNA of the leukocytes was extracted by using RNeasy Mini Kit® (QIAGEN, Germany).
The nucleotide sequence of the primer used in this test was produced by using ALFORT 187 strain[4] (Table 1).

## Results

### Clinical and autopsy signs

The suspected (sick) swine became gaunt and anorectic. The staggering gait related to posterior weakness, constipation and lethargy, hyperemia, hemorrhagic lesions (on the skin, muzzle, ears, limbs, tail and inner part of legs) were observed. Conjunctivitis with dirty streaks below the eyes caused by the accumulation of dust and feed particles was also observed. The herds were huddled together because of high fever (over 40℃) (Table 2, Fig 4).

### Autopsy signs

The atrophy was observed in some individuals. The swelling and hemorrhage in lymph nodes (of the mesentery, inguinal and pulmonary hilum) were viewed in 29 heads (96.7%). The inflammation in the lung (in 26 heads), hemorrhage in the intestine (in 22 heads), pinpoint (ecchymotic) hemorrhages on the surface of the kidney (in 24 heads), infarction in the spleen (in 15 heads), hemorrhage in the heart (in 6 heads, 20%) and cheese like purulent inflammation of the tonsil (in 3 heads) were observed. The inflammation in the stomach and the ulcer of the colon were also detected (Table 3).

Table 1. Nucleotide sequence of the primer used in RT-PCR

| Primer | Nucleotide sequence | Expected PCR size |
|---|---|---|
| NCR region | F : 5′-CTA GCC ATG CCC AYA GTA GG-3′<br>R : 5′-CGA CTT CAR YGT TGA TTG T-3′ | 421 bp |
| E2 region | F : 5′-AGR CCA GAC TGG TGG CCN TAY GA-3′<br>R : 5′-TTY ACC ACT TCT GTT CTC A-3′ | 671 bp |
| NS5B region | F : 5′-GCA ACG AGY GCA GGC AAC A-3′<br>R : 5′-AGY GGG TTC CAG GARTAC AT-3′ | 448 bp |

Table 2. The clinical signs and symptoms in CSF suspected swine

| Clinical signs | No. of observed | % |
|---|---|---|
| Diarrhea | 3 | 10.0 |
| Pyrexia, 40℃～41.5℃ | 28 | 93.3 |
| Anorexia | 30 | 100.0 |
| Depression (Reluctant to move) | 19 | 63.3 |
| Cyanosis | 30 | 100.0 |
| Ataxia | 5 | 16.7 |
| Weaving/Weak hind legs | 6 | 20.0 |
| Exudative conjunctivitis | 12 | 40.0 |

Table 3. Autopsy views in suspected swine

| Lesions | No. observed (N=30) | (%) |
|---|---|---|
| Lymphatic peripheral hemorrhage | 29 | 96.7 |
| Tonsillitis | 5 | 16.7 |
| Cardial hemorrhage | 6 | 20 |
| Pneumonia | 26 | 86.7 |
| Gastritis-hemorrhage | 1 | 3.3 |
| Enteritis-hemorrhage | 22 | 73.3 |
| Ulceration of colon | 1 | 3.3 |
| Spleen infection | 15 | 50 |
| Renal petechiation | 24 | 80 |
| Urinar bladder-petechia | 1 | 3.3 |
| Skin - cyanosis and hyperemia | 30 | 100 |

Table 4. The number of the leucocytes in collected blood samples

| | Normal | Abnormal |
|---|---|---|
| | Leukopenia (WBC count 〈 $7\times10^6$cells/㎖, OIE ) | |
| Samples | 3 | 27 |
| Range | 7 ~ $10\times10^6$cells/㎖ | 1.2 ~ $6\times10^6$cells/㎖ |

### Aspects of blood samples

The leukocytes in the collected blood samples (a total of 30) were scanty. The 27 samples out of 30 showed remarkable decrease of the leukocyte and the rest 3 samples showed comparatively small (Table 4).

### Microscopic findings

The blood vessels in each tissue were enlarged or closed. The epithelial cells in inner part of the vessels were swelled due to the extension of the nucleus. The necrosis of the lymphatic cells and the infiltration of the vessel circumferential cells in the brain and lymph organs were commonly viewed. The observation on proliferation of the glia cell (gliosis) in the lymph was particular.

### Results of IFA test and ELISA

Cytopathogenic effect (CPE) appeared in PK-15 cells inoculated with suspected blood plasma (30 samples, 100%, Fig 4). The specific fluorescent-bright-green areas were observed on the inoculated PK-15 cells, epithelial cells and mucous membrane of tonsil slides (in 5 heads).

Three samples (out of 30 samples) showed S/P ratio above 0.14 in ELISA and were diagnosed as positive. The plates used for the antigen detection were adsorbed protein E2 (of recombinant CSF virus) specific monoclonal antibody. The positive and negative control value of O.D was respectively 0.829 and 0.095. The doubtful positive control value was 0.282. (Table 5).

Table 5. Comparative analysis of CSF disease by used methods

| Sample No. | IFA Tonsil | IFA Cell | ELISA Ab** | ELISA Ag*** | PCR | Sample No. | IFA Tonsil | IFA Cell | ELISA Ab | ELISA Ag | PCR |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | NT* | + | − | ± | + | 16 | NT | + | − | + | + |
| 2 | NT | + | − | + | + | 17 | NT | + | − | + | + |
| 3 | NT | + | − | + | + | 18 | NT | + | − | + | + |
| 4 | NT | + | − | + | + | 19 | NT | + | − | + | + |
| 5 | NT | + | + | + | + | 20 | NT | + | − | + | + |
| 6 | NT | + | − | ± | + | 21 | + | + | − | + | + |
| 7 | NT | + | − | + | + | 22 | NT | + | − | + | + |
| 8 | NT | + | − | + | + | 23 | NT | + | − | − | + |
| 9 | + | + | + | + | + | 24 | NT | + | − | + | + |
| 10 | NT | + | − | + | + | 25 | + | + | − | + | + |
| 11 | NT | + | − | + | + | 26 | NT | + | − | + | + |
| 12 | + | + | + | + | + | 27 | NT | + | − | + | + |
| 13 | + | + | − | + | + | 28 | NT | + | − | + | + |
| 14 | NT | + | − | − | + | 29 | NT | + | − | − | + |
| 15 | NT | + | − | + | + | 30 | NT | + | − | + | + |

*:Not test **:Antibody ***:Antigen,
RT-PCR positive/sample = 30/30, ELISA antigen positive/sample = 26/30
ELISA antibody positive/sample = 3/30, IFA(tonsil) positive/sample = 5/5
IFA cell positive/sample = 30/30

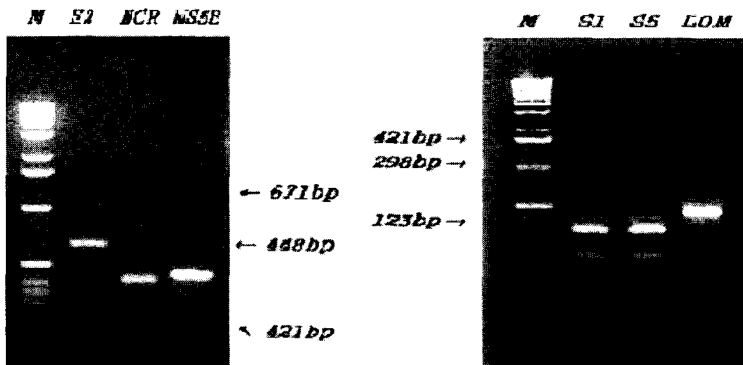


Fig. 1 Fig. 2

Fig 1. Fragments of the RT-PCR amplified viral envelope protein E2.
M : molecular marker (1 kbp RNA ladder); E2 : Viral envelope protein 671 bp; NCR : 5′noncording region 421 bp; NS5B : RNA polymerase 448 bp

Fig 2. Fragments of the RT-PCR amplified CSFVcompared with vaccine CSFV strain using RT-PCR.
M : molecular marker (1 kb RNA ladder); S1, S5 : Samples;
LOM : Vaccine CSF virus strain

Fig 3. Fragments of RT−PCR amplified field CSF viral envelope protein.
M : molecular marker (1kb RNA ladder); S1~S4 : samples; NC : negative control; PC : positive control (NCR region 421 bp, E2 region 671 bp)

Fig. 4. Clinical and immunological signs of CSF

Left above : Pigs huddled together.

Left lower : The staggering gait related to posterior weakness.

Right above : The specific fluorescent-bright green areas on PK-15 cells.

## Results of RT−PCR

See Fig 1, Fig 2 and Fig 3 for the results of the reverse transcription polym erase chain reaction test.

## Discussion

CSF has become known well worldwide during several decade years, but the disease has broken out continually and remained as one of the most difficult disease to diagnose. Multiple infections of the disease with the porcine reproductive and respiratory syndrome (PRRS) or porcine dermatitis and nephropathy syndrome (PDNS) is very likely to go misdiagnosis. It is also very difficult to distinguish between acute or chronic type of the disease and particularly in case of

transplacental infection form. Therefore, it is necessary to clarify the origin of outbreak and the transmission of the disease[5]. To control CSF, the rapid diagnosis is essential. The fluorescent antibody test has been performed gener ally in laboratory to detect the CSFV since Solorzano et al[6]. Mengeling et al[7,8]. Stair et al[9]. and Ailen et al[10]. have established this technique. And OIE recommends this technique as one of the standard method. The present RT-PCR technique was useful as a rapid and convenient method to detect infective viral particles from asymptomatic carriers or samples with low virus levels. The results of IFA test in this examination were identical to the results of RT-PCR, and more identical than the first (78%) and the second report (99%).. Meanwhile, the CSFV inoculation test on the PK-15 cell line showed high sensitivity so that the plaque (cytopathogenicity) and antigen were detected in all 30 samples. Gim et al[11]. obtained nearly identical results with END method, though neutralized antibody titer dropped remarkably during the process of non-specific exclusion. The blocking ELISA method using whole blood[12] and complex-trapping blocking ELISA method using two CSFV-specific monoclonal antibodies[13] have been repo rted. Recently an improved method using baculovirus-expressed viral envelope prot ein E2[14] has also been reported. Another fast and simple method named Ceditest ELISA for CSFV-Ab has also been reported. It makes use of an automated system[15] and showed identical results (97%) with those of CTB ELISA method. The diagnosis kits (manufactured on the basis of the method) are being used commonly by domestic animal disease prevention agencies and the present study also used the kit though it confirmed discrepancy with ELISA or RT-PCR method.

According to the OIE health standards, serological test is useful for detection of low virulent CSF virus-specific antibody, especially in breeding pigs and latent pigs. ELISA (antigen-capture enzyme-linked immunosorbent assay) is useful for early diagnose on living pigs and has been recently developed for the newly infected herds (OIE). Fenton et al.[16] have developed monoclonal antibody capture ELISA technique for the detection of *pestivirus* in ovine and bovine. Depner et al.[17] reported the double-antibody sandwich type ELISA technique using mono- or polyclonal antibody. The results in this examination using ELISA plate in which anti-CSFV E2 protein was adsorbed were identical (98%) with the results of RT-PCR and 98.7%.

The gene analysis techniques have been developed in recent years and the RT-PCR method is so sensitive that it is regarded as the method for the isolation of the virus. Especially, the actual value of 3 regions in CSFV gene namely NS5B[18-20], 5′NCR[21-23] and E2 glycopr otein[22,24] was practically used in RT-PCR technique to estimate the pathogen and to distinguish between field virus and vaccine[18,19,22,23]. Because the virus could be detected a few days after the vaccination, we must ascertain necessarily whether (if) the pig was vaccinated recently. Song[25] et al. reported that the LOM strain (be used as vaccine virus) could be distinguished from field-isolated CSF virus by treatm ent of restricted enzyme. RT-PCR

technique required more working hours and labor than other techniques in this examinations but it was useful because of the sensitivity to the CSFV gene.

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