Development of Novel Diagnostic Methods of Classical Swine Fever

（豚コレラの新しい診断法の開発）

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Contents

Abbreviations

ACE : 3-amino-9-ethylcarbazole
ALP : alkaline phosphatase
BES : N, N-Bis (2-hydroxyethyl)-2-aminoethanesulfonic acid
bp : base pair
BDV : border disease virus
BVDV : bovine viral diarrhea virus
BVDV-I : BVDV genotype-I
BVDV-Ia : BVDV-I subgroup a
BVDV-Ib : BVDV-I subgroup b
BVDV-II : BVDV genotype-II
cDNA : complementary DNA
cp : cytopathogenic
CPE : cytopathic effect
CPE-NT : CPE-neutralization test
CS : calf serum
CSF : classical swine fever
CSFV : classical swine fever virus
CSFV-1 : CSFV subgroup 1
CSFV-2 : CSFV subgroup 2
CSFV-3 : CSFV subgroup 3
DI : defective interfering
DIG : digoxigenin
dNTP : deoxyribonucleoside 5'-triphosphates
DTT : dithiothreitol
EDTA : ethylenediaminetetraacetic acid
ELISA : enzyme linked immunosorbent assay
END : exaltation of Newcastle disease virus
END$^-$: END phenomenon-negative
END$^+$: END phenomenon-positive
FBS : fetal bovine serum
FITC : fluorescein isothiocyanate

γ-GTP : gamma-glutamyltranspeptidase

HA : hemagglutination

IFA : immunofluorescence assay

kb : kilobase

MAb : monoclonal antibody

MEM : minimum essential medium

MOI : multiplicity of infection

ncp : noncytopathogenic

NDV : Newcastle disease virus

NPLA : neutralization peroxidase-linked assay

ORF : open reading frame

PBS : phosphate-buffered saline

PCiV : porcine circovirus

PCMV : porcine cytomegalovirus

PCR : polymerase chain reaction

RFLP : restriction fragment length polymorphism

PLA : peroxidase-linked assay

RT : reverse transcriptase

RT-PCR : reverse transcriptase-polymerase chain reaction

SDS : sodium dodecyl sulfate

ST : swine testicle

$TCID_{50}$: 50% tissue culture infectious doses

TdT : terminal deoxynucleotidyl transferase

Tris : tris(hydroxymethyl) aminomethane

TUNEL : terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

U : unit

UPGMA : unweighted pair group method using arithmetic average

UTR : untranslated region

VSV : vesicular stomatitis virus

WEEV : western equine encephalomyelitis virus

Preface

Classical swine fever virus (CSFV) is classified as a member of the genus *Pestivirus* within the family *Flaviviridae* (44). Pestiviruses also include the bovine viral diarrhea virus (BVDV) and border disease virus (BDV) of sheep. Although the current classification of pestiviruses refers to the host species from which they are isolated, numerous investigations have proven that pestiviruses are not highly host-specific (7, 33, 58, 69, 76). Pestiviruses are positive-sense single-stranded RNA viruses with a genome of approximately 12.3~12.5 kb (8, 37). The open reading frame (ORF), which is flanked by 5'-untranslated region (UTR) and 3'-UTR, encodes protein composed of about 4000 amino acids processed by viral and cellular enzymes. The first protein is a nonstructural autoprotease N^{pro} and the genes encoding structural proteins, capsid C and envelope glycoproteins E^{ms}, E1 and E2 have been mapped on the 5' part of the genome (41). The 3' part of the ORF encodes nonstructural proteins such as p7, NS2-3, NS4A, NS4B, NS5A and NS5B (41).

Hog cholera has been recognized as "classical swine fever (CSF)" to distinguish from African swine fever. CSF is a highly contagious viral disease of swine. The infection can run an acute, subacute, chronic or inapparent course, depending on the virulence of the virus (74). High-virulent virus causes acute disease and high mortality, whereas infections with low-virulent virus often go unrecognized. Pigs with acute hog cholera show, after incubation period of 2-6 days, high fever, with peaks above 42°C, anorexia, depression, conjunctivitis, nasal discharge, constipation preceding diarrhea, vomiting, weak hind legs and purplish discoloration of the skin. Concurrent with fever, a leukopenia and thrombocytopenia are observed. Most pigs that suffer from acute hog cholera die between 8 and 20 days postinfection. Pigs experimentally infected with strains of intermediate virulence show a great variation in clinical responses. They may die from acute or subacute disease, they may recover or survive the acute phase only to succumb later from chronic hog cholera, or they may develop only mild signs of disease. Chronic hog cholera is characterized by intermittent disease periods with anorexia, fever, leukopenia, diarrhea and dermatitis. Runt pigs are also found during chronic hog cholera. Such pigs are severely retarded in growth, have skin lesions, and often stand with arched backs. Pigs infected with low-virulent strains show mild signs of diseases

or remain apparently healthy.

One property of pestiviruses is the existence of two biotypes that are recognized by morphological changes of cells during virus growth in tissue culture system. Noncytopathogenic (ncp) pestiviruses replicate without clearly visible effects, while cytopathogenic (cp) viruses cause lysis of appropriate target cells. Some preparations of cp BVDV have insertion of cellular genome, duplication of the viral gene sequences in their genome or defective interfering (DI) particles (41, 46). In the case of CSFV, some cp isolates are composed of DI particles and ncp helper viruses (40, 42) although most CSFVs are ncp. Because of these noncytopathogenicity, the immunofluorescence method, immunoperoxidase staining, interference method, reverse transcriptase-polymerase chain reaction (RT-PCR), enzyme linked immunosorbent assay (ELISA) and the exaltation of Newcastle disease virus (END) method have been used for virus assays (30, 36, 62, 70). The END phenomenon is based on exaltation of the cytopathic effect (CPE) of Newcastle disease virus (NDV) by the growth of CSFV in porcine cell cultures (29) (Fig. 1). Since most of the Japanese CSFV isolates are END phenomenon-positive (END $^{+}$) viruses, the END method and END neutralization method (27, 61) were developed and used for the assays of CSFV and anti-virus antibodies. However, some virus strains do not show the END phenomenon but interfere with the growth of NDV, vesicular stomatitis virus (VSV) and western equine encephalomyelitis virus (WEEV) (12, 13, 62). We call them END phenomenon-negative (END^{-}) strains of CSFV (Fig. 2). The CSFV GPE^{-} strain is an attenuated END^{-} virus obtained by adaptation to guinea pig kidney cells and has been used as a live vaccine in Japan. Interference method was usually used for the virus titration of these END^{-} viruses (12).

The serum, which is free of antibodies against BVDV, is necessary for the assays of pestiviruses since CSFV is antigenically closely related to BVDV. However, calf serum (CS) and fetal bovine serum (FBS) usually used in laboratory studies contain antibodies against BVDV since bovine viral diarrhea is endemic in many countries (5). Furthermore, the serum prepared from the cattle persistently infected with BVDV contains the infectious virus and/or the viral RNA (5). Pestivirus contamination has been detected in the several cell lines used for basic research, vaccine preparation and propagation of viral stocks (3, 4). Contaminant viruses can be maintained during cell

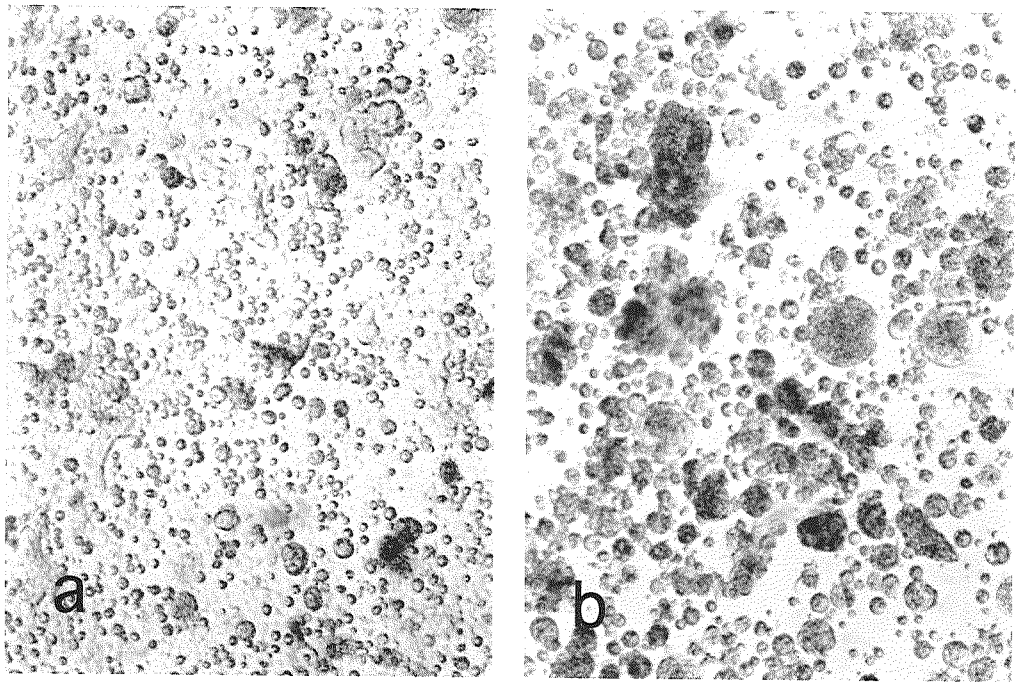

Fig. 1. END phenomenon. (a) CPK cells were infected with NDV Miyadera strain and incubated for 3 days. Weak cytopathic effect by NDV was observed. (b) CPK cells were infected with CSFV ALD strain and incubate for 4 days. Furthermore, cells were superinfected with NDV Miyadera strain and incubate for more 3 days. Cytopathic effect of NDV was exalted by the infection of CSFV ALD strain. Both magnifications, x 160.

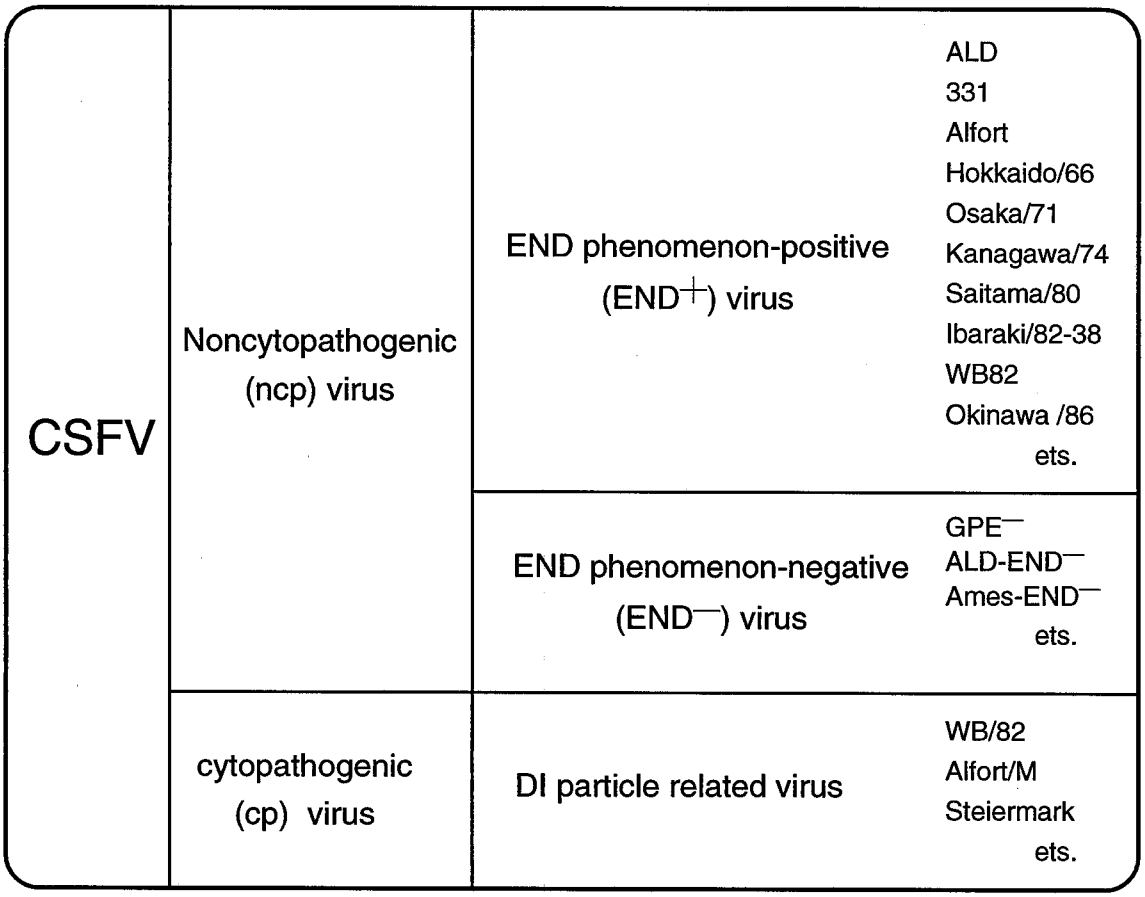

Fig. 2. Classification of CSFV strains based on biotypes in tissue culture cells. Most CSFV strains are noncytopathogenic and divided into two groups (END+ and END− viruses). Cytopathogenic viruses are composed of DI particles and noncytopathogenic helper viruses. Representative strains of each group were shown.

passages. Use of cell lines that harbor pestiviruses may result in contaminated vaccines, invalid diagnostic tests and unreliable research data (32, 34, 79). To prevent the contamination of the viruses and antibodies in the culture media, it is, therefore, indeed required to establish cell lines that could be cultured by serum-free media for reliable results in the biological production and diagnosis.

The present thesis consists of three chapters. In the first chapter, the establishment of porcine kidney cell lines in serum-free culture is described. In the second chapter, developments of virus titration and neutralization tests using these cell lines are stated. In the last chapter, the results of the genetic characterization of CSFV and other ruminant pestiviruses are shown.

Chapter I

Establishment and characterization of serum-free culture cell lines

Introduction

Since CSFV is antigenically closely related to BVDV, the serum free of antibodies against BVDV, is necessary for the assays of CSFV. However, most of CS and FBS used in laboratory studies contain antibodies against BVDV because bovine viral diarrhea is endemic in many countries (5). In addition, the serum prepared from the cattle persistently infected with BVDV has a high possibility of the existence of the infectious virus and/or the viral RNA (5). Several cell lines used in basic biological investigation, vaccine preparation and propagation of viral stocks have been tested for possible contamination with pestiviruses (3, 4). To prevent the contamination of the viruses and antibodies in the culture media, it is, therefore, indeed required to establish cell lines that could be cultured by serum-free media for reliable results in the biological production and diagnosis.

Porcine circovirus (PCiV) is already known as a contaminant of a porcine kidney cell line, PK-15, which was usually used for the assays of the porcine viral diseases (71). The ubiquity of infection by PCiV is further confirmed by the results of serological tests, indicating that infection of pigs is widespread and probably worldwide (11). There is also a high possibility of contamination in cell lines by porcine cytomegalovirus (PCMV), retrovirus and mycoplasma strains, since these pathogens are endemic all over the world and cause a latent infection (18, 65, 72).

In this chapter, the results of the establishment of stable porcine kidney cell lines that are cultured and passaged in simple serum-free media, is described. In addition, the results of the tests whether these cells were free from pestiviruses, other possible adventitious viruses and mycoplasmas are also shown.

Materials and Methods

Cells and media

The established porcine kidney cell lines, FS-L3 and CPK-NS were grown in monolayers in 25-cm^2 tissue culture flasks (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) at 37°C in serum-free medium. This serum-free medium was composed of Eagle's minimum essential medium (MEM) "Nissui" No. 1 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 0.295% tryptose phosphate broth (Difco Laboratories, Detroit, USA), 0.5% Bacto peptone (Difco Laboratories), 10mM N, N-Bis (2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) (Dojindo Laboratories, Kumamoto, Japan), 0.292 mg of L-glutamine per ml and 2.25 mg of sodium bicarbonate per ml. Sterilized L-glutamine and sodium bicarbonate were added after MEM, tryptose phosphate broth, Bacto peptone and BES were mixed and autoclaved.

The stable porcine kidney cell line, SK-L was established from the renal cortex of a healthy piglet in our laboratory 20 years ago. SK-L cells were the parental cells of FS-L3 cell line. The stable porcine kidney cell line, CPK was established by Komaniwa et al. (27). CPK cells were the parental cells of CPK-NS cell line. A fetal lamb lung cell line (FLL-YFT) was established in our laboratory 10 years ago. SK-L, CPK, FLL-YFT, Vero (simian kidney cell line), L929 (mouse fibroblast cell line), MDBK (bovine kidney cell line), BHK-21 (hamster kidney cell line), primary testicle cells and primary kidney cells in monolayers were grown in MEM containing 0.295% tryptose phosphate broth and 10% heat-inactivated FBS at 37°C.

Chromosome counts of the established cell lines

Chromosome slide preparation and conventional Giemsa staining were used for general chromosome counts as described by Doyle et al. (9). The chromosomes were counted in 200 metaphase samples.

Preparation of genomic DNA and dot blot hybridization

The trypsinized cells were treated with digestion buffer (100 mM NaCl, 10 mM Tris-HCl (pH 7.5), 25 mM EDTA (pH 7.5), 0.5% SDS, 0.1 mg of proteinase K per ml) at 50°C for 18 hr. The cellular genomic DNA was extracted and purified for use in

hybridization. The probe was prepared with a digoxigenin (DIG)-DNA labeling kit (Boehringer Biochemica, Mannheim, Germany) using PRE-I fragment from *Hinc* II-digested pBS/PRE-I plasmid (kindly provided by Dr. H. Yasue, National Institute of Animal Industry, Tsukuba, Japan). PRE-I sequence is the specific repetitive sequence of the porcine genomic DNA (66).

Purified cellular genomic DNA was denatured by alkaline treatment and dot-blotted on a positively charged nylon membrane (Boehringer Biochemica). The DNA on the membrane was hybridized with the DIG-labeled DNA probe. The hybridization was performed at 42°C for 16 hr with 10 ng of denatured DIG-labeled DNA probe per ml in the hybridization buffer (50% formamide, 5x SSC, 1% blocking reagent, 0.1% N-lauroylsarcosine, 0.02% SDS). After hybridization, the membrane was washed twice with 2x SSC, 0.1% SDS and 0.1x SSC, 0.1% SDS. After incubation with diluted anti-DIG alkaline phosphatase (ALP) conjugate solution in a DIG nucleic acid detection kit (Boehringer Biochemica), the membrane was incubated with color-substrate solution for detection of desired spots.

Histochemical characterization of serum-free cell lines

Histochemical characterization of FS-L3 and CPK-NS cells were performed as follows. Prepared cells were fixed with acetone for 5 min and stained with an AP-red substrate kit (Zymed Laboratories Inc., South San Francisco, USA) for detection of ALP. They were then rinsed with distilled water and counterstained with hematoxylin. For the detection of gamma-glutamyltranspeptidase (γ-GTP), fixed cells were incubated in substrate solution [12 mM β-glutamyl-α-naphthylamide (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 5 mM fast garnet GBC salt (Sigma chemical Co., St. Louis, USA) in 40 mM phosphate buffer (pH 6.7)] for 20 min, then rinsed with distilled water and counterstained with hematoxylin. Cells were also immunostained with anti-cytokeratin antibody (Chemicon International Inc., Temecula, USA) and Histofine SAB-PO (R) Kit (Nichirei Corp., Tokyo, Japan) to confirm the epithelial origin of these cells.

Direct immunofluorescence assay (IFA)

FS-L3 and CPK-NS cells were washed once with PBS and fixed with acetone at -20°C. Both cells were then incubated for 60 min at 37°C in the presence of FITC-

labeled hyperimmune anti-CSFV serum (Kyoto Biken Laboratories Inc., Kyoto, Japan). FS-L3 cells were also incubated for 60 min at 37°C in the presence of FITC-labeled hyperimmune anti-PCMV Ka strain serum (gift from Dr. H. Kawamura, Gen Corp., Tochigi, Japan). The cells were washed and then observed under a fluorescence microscope.

RNA extraction and RT-PCR amplification

The highly conserved 5'-UTR of pestiviruses was amplified by RT-PCR. An RNA polymerase chain reaction (PCR) Kit Ver. 2 (Takara Shuzo Co., Ltd., Shiga, Japan) was used for the RT-PCR procedure in accordance with the manufacturer's instructions. Cellular RNA was extracted from FS-L3 and CPK-NS cells with RNA zolB in accordance with the manufacturer's instructions (Tel-Test Inc., Friendswood, USA). Each cDNA reaction mixture consisted of 0.5 μg of extracted RNA, 10mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 1 mM dNTP mixture, 2.5 μM random 9 mers, 20 units (U) of RNase inhibitor and 5 U of reverse transcriptase. The mixtures were incubated for 10 min at 30°C and then 30 min at 42°C. Amplification of cDNA by the PCR was carried out in a total volume of 100 μl containing synthesized cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM dNTP mixture, 2.5 U of Takara Taq and 0.2 μmol of each primer. The previously described primers, 324 and 326 (75), which can detect a broad range of pestiviruses in pig, cattle and sheep, were used for this experiment. The sequences of oligonucleotides and their positions in the genome of CSFV Alfort strain (37) are as follows: 324, ATG CCC (T/A)TA GTA GGA CTA GCA (sense: 90-110); 326, TCA ACT CCA TGT GCC ATG TAC (antisense: 373-353). The reaction mixtures were heated in a thermocycler for 35 cycles of 95°C for 1 min, 56°C for 1 min and 72°C for 1 min. Electrophoretic analysis of the PCR product was carried out in 2% agarose gel in Tris-borate-EDTA buffer.

Reverse transcriptase activity assay

Reverse transcriptase (RT) activity was measured by the method described previously by Takase-Yoden and Watanabe (67). Namely, 10 μl of culture supernatants were disrupted with 14 μl of solution A containing 7 mM Tris-HCl (pH 7.6), 70 mM NaCl, 0.7 mM EDTA and 0.03% TritonX-100. The mixture was incubated with 16 μl

of solution B containing 125 mM Tris-HCl (pH 7.6), 150 mM NaCl, 25 mM DTT, 2.5 mM $MnCl_2$, 60 μg of poly (rA) p $(dT)_{12-18}$ (Pharmacia, Uppsala, Sweden) per ml and ^{3}H-dTTP (1 μCi) (DuPont/NEN Research Products, Boston, USA) for 1 hr at 37°C. The sample was spotted onto DE81 ion-exchange paper (Whatman, Maidstone, UK) and the incorporated radioactivity was determined with a scintillation counter.

DNA extraction and PCR amplification

For the detection of mycoplasmas associated with the cultured cells, the Mycoplasma PCR Primer Set (Stratagene, La Jolla, USA) and PCR Amplification Kit (Takara Shuzo Co., Ltd.) were used for the PCR in accordance with the manufacturer's instructions. Cells (5 x 10^3) were boiled for 10 min and the supernatants were used for the PCR as a template. The reaction mixture (50 μl) consisted of extracted DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM dNTP mixture, 1 U of Takara Taq and each primer. The reactions were heated in a thermocycler for 40 cycles of 95°C for 45 sec, 56°C for 45 sec and 72°C for 3 min. Electrophoretic analysis of the PCR product was carried out in 2% agarose gel in Tris-borate-EDTA buffer.

Results

Establishment and growth of FS-L3 cell line

To establish a stable cell line which is able to grow well in serum-free medium, the concentration of serum in the medium was reduced gradually from 10% to 0.1% in every passage of SK-L cells. Finally, the medium was completely replaced from the above-mentioned low serum medium (0.1%) to the serum-free medium supplemented with 0.5% Bacto peptone and 10mM BES instead. New cell line, which grows well in the serum-free medium, was named FS-L3. Before 40th passage in serum free medium, it was extremely difficult to maintain the cells. The medium was replaced with fresh one several times every 5 days until the cells grow in confluent monolayer. The cells at the 80th ~ 160th passage level can grow well in serum-free medium and be useful for various assays. The 100th passaged cells were used in all these experiments. For subculture, the cells have been passaged every 5 days. The trypsinized cells were washed twice with culture medium and seeded at 2.5 $x10^6$ per 25-cm^2 tissue culture flasks in 5 ml of culture medium.

The growth of the FS-L3 cells was observed day by day (Fig. 3a). The doubling time of the cells after 2 days in culture was approximately 24 hr. The cells reached confluence 4 days after cell seeding. Fluid-filled, multicellular domes were observed 3 days after cell seeding and the number of dome was increased day by day even after the cells became confluent (Fig. 4a, 4b, 4c). When the medium was changed 5 days after cell seeding, the domes grew substantially and appeared to form a three-dimensional structure. Finally the three-dimensional domes detached from the surface of the substrate and floated into the medium (Fig. 4d).

Establishment and growth of CPK-NS cell line

To establish a stable cell line which is able to grow well in serum-free medium, the concentration of serum in the medium was reduced gradually from 10% to 0.1% at every passage of CPK cells. Finally, a new cell line was established in serum-free culture and named CPK-NS. Before the 30th passage in serum-free medium, it was extremely difficult to maintain the cells. The medium was replaced with the fresh several times until the cells grew in a confluent monolayer. The cells at the 50th ~ 120th passage level

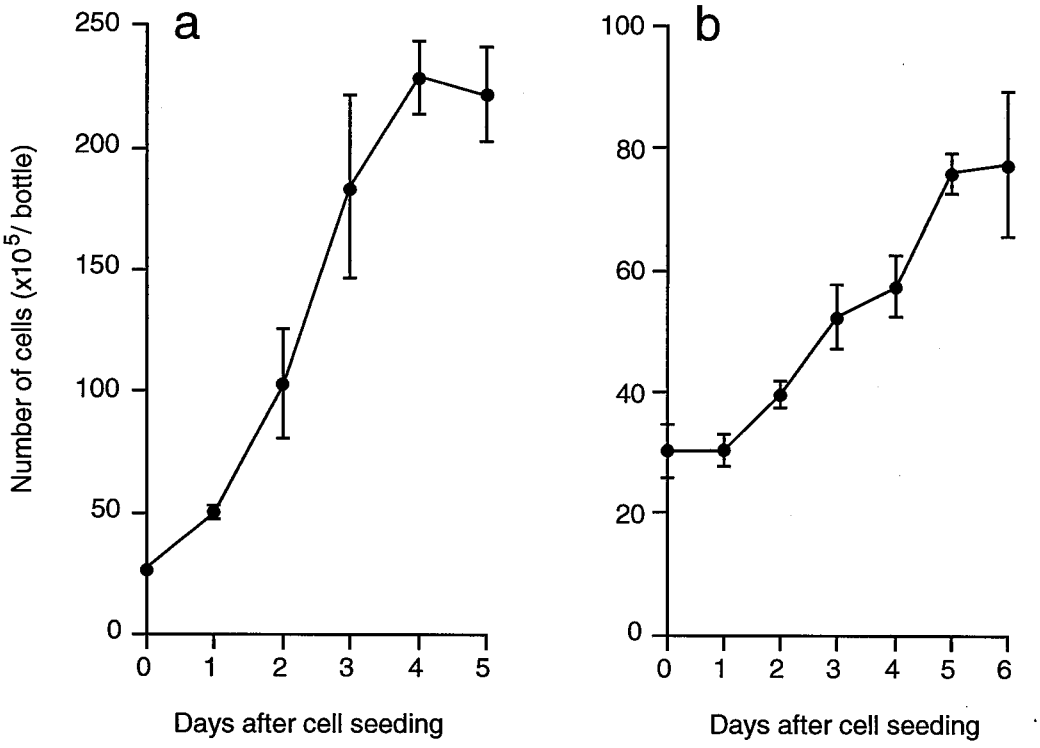

Fig. 3. Growth of FS-L3 cells (a) and CPK-NS cells (b) in serum-free medium. The cells (FS-L3; 2.5 x10^6 cells, CPK-NS; 3 x10^6 cells) were seeded in 25-cm^2 plastic tissue culture flasks. The cells were counted daily for 5 or 6 days with a hemocytometer. The data represent the average of four replicate flasks. Each bar represents the geometric mean ± standard deviation.

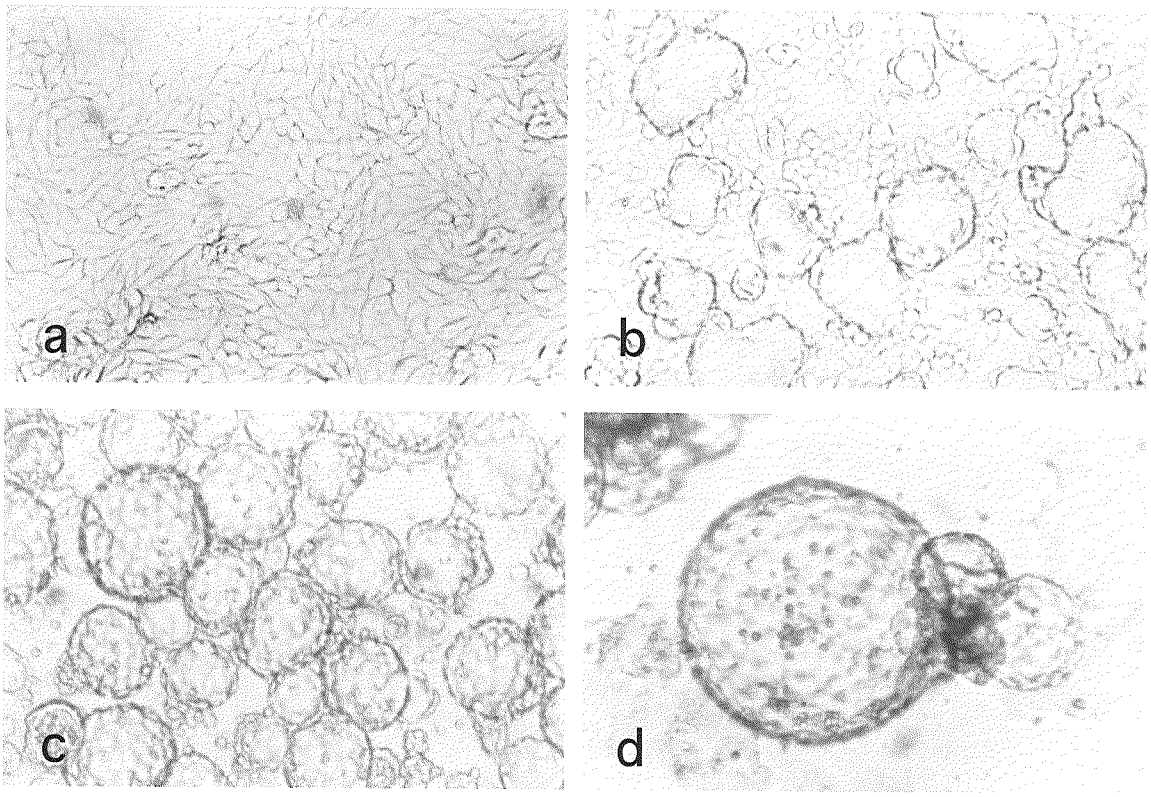

Fig. 4. Morphology of FS-L3 cells. The cells (2.5 x10^6) were seeded in plastic flasks and cultured at 37°C. (a) Monolayer of FS-L3 cells 2 days after cell seeding. (b) Appearance of dome development 5 days after cell seeding. (c) Soccer ball-like three-dimensional structure 7 days after cell seeding. (d) Floating morphology of soccer ball-like structure in the medium. a, b and c, magnification x 160; d, magnification x320.

can grow well in serum-free medium and the 60th passaged cells were used in all these experiments. For subculture, the cells have been passaged every 7 days. The trypsinized cells were washed twice with culture medium and seeded at 3 x10^6 per 25-cm^2 tissue culture flasks in 5 ml of culture medium (Fig. 5a). The medium was replaced with fresh medium 4 days after cell seeding and the culture were incubated for 3 more days until the next passage.

The growth of the CPK-NS cells was observed day by day (Fig. 3b). The cells reached confluence 2 days after cell seeding (Fig. 5b). The growth of CPK-NS cells was lower than that of the FS-L3 cells. A few and smaller domes than those of FS-L3 cells were observed 5 days after cell seeding (Fig. 5c). Their epithelial-like appearance was also confirmed with a phase contrast microscope.

Chromosome number and original species

Chromosome slide preparation and conventional Giemsa staining were carried out for general chromosome counts. The data show the average numbers of 200 metaphase samples. The mode of chromosome numbers of FS-L3 cells is 37 to 38 (Fig. 6a), whereas the diploid number of pigs is 38. Ninety percent of the FS-L3 cells are in a diploid range. In case of the CPK-NS, the mode of chromosome numbers is 55 (Fig. 6b).

In the experiment on dot blot hybridization, specific repetitive sequence in the porcine genome, PRE-I was detected in cellular DNA of porcine origin; i.e. FS-L3 cells, CPK-NS cells, primary porcine kidney cells, primary porcine testicle cells, CPK cells and SK-L cells but not for other species (bovine, ovine, simian and hamster) (Fig. 7).

Histochemical characterization of serum-free cell lines

Histochemical characterization of FS-L3 and CPK-NS cells were investigated (Table 1). ALP activity and cytokeratin were detected by histochemical experiments although γ-GTP activity was not detected in FS-L3 cells. Cytokeratin was detected in CPK-NS cells, although ALP and γ-GTP were not detected. Same results were obtained in CPK, parental cell line of CPK-NS. PK-15 cells contained ALP activity and cytokeratin, Vero cells showed γ-GTP activity and existence of cytokeratin. Mouse fibroblast cell line, L929 was practiced as the negative control for all detection.

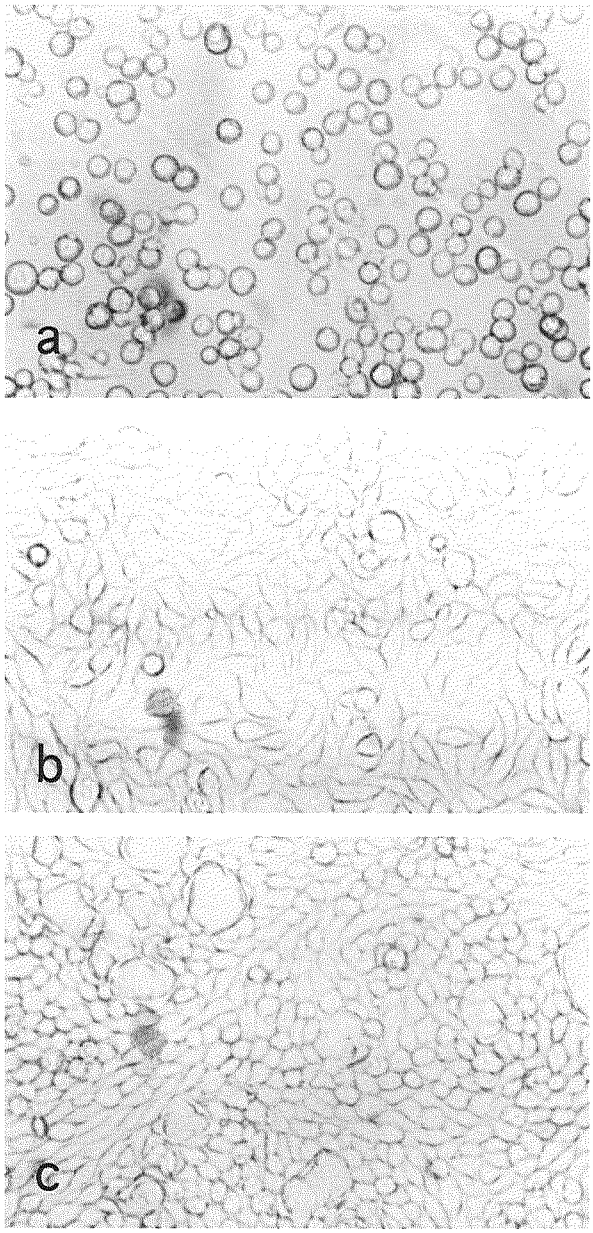

Fig. 5. Morphology of CPK-NS cells. The cells ($3x10^6$) were seeded in plastic flasks and cultured at 37°C. (a) CPK-NS cells suspended in plastic flask. (b) Monolayered cells 2 days after cell seeding. (c) Appearance of dome development 5 days after cell seeding. All magnifications, x 250.

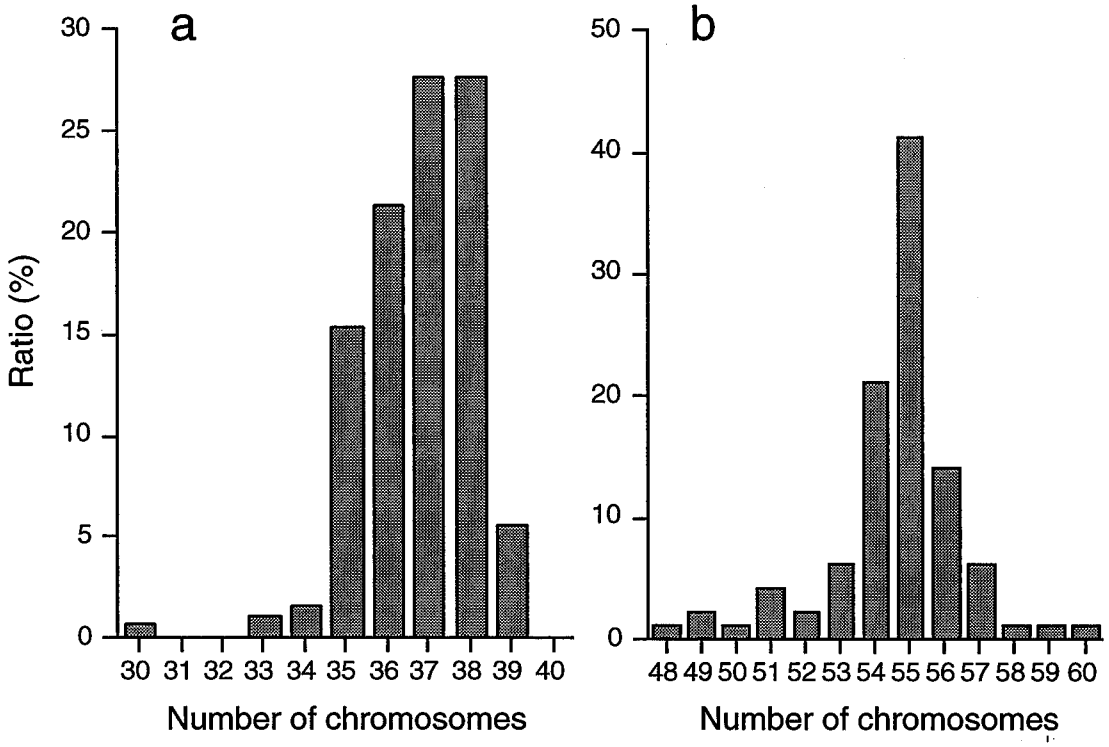

Fig. 6. Distribution patterns of chromosome number per cell. The data (diploid number) show the average numbers of 200 chromosome samples of FS-L3 cells (a) and CPK-NS cells (b).

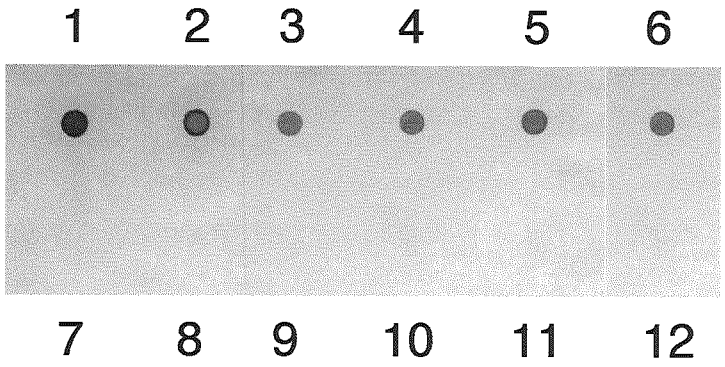

Fig. 7. Detection of the PRE-I gene in various cell lines by dot blot hybridization. Extracted cellular DNA (1, primary porcine kidney cells; 2, primary porcine testicle cells; 3, CPK cells; 4, SK-L cells; 5, FS-L3 cells; 6, CPK-NS cells; 7, primary bovine testicle cells; 8, MDBK cells; 9, Vero cells; 10, BHK-21 cells; 11, FLL-YFT cells; 12, culture medium) were hybridized with the PRE-I gene.

Table 1. Histochemical characteristics of FS-L3, CPK-NS and other cells

Cell line	Origin	ALP[a]	γ-GTP[b]	cytokeratin
FS-L3	swine kidney	+	−	+
CPK-NS	swine kidney	−	−	+
CPK	swine kidney	−	−	+
PK-15	swine kidney	+	−	+
Vero	simian kidney	−	+	+
L929	mouse fibroblast	−	−	−

[a] ALP; alkaline phosphatase

[b] γ-GTP; gamma-glutamyltranspeptidase

Denial of virus contamination

The FS-L3 and CPK-NS cells were investigated whether the cells are contaminated with viruses and mycoplasmas (Table 2). RT-PCR and direct IFA were used to check the contamination of pestiviruses. Amplified product by RT-PCR method was not detected from extracted RNA of FS-L3 and CPK-NS cells. A specific band (approximately 284 bp) was observed only in FS-L3 cells infected with CSFV, GPE^{-} strain. Furthermore, no antigen was detected in FS-L3 and CPK-NS cells with FITC-labeled hyperimmune anti-CSFV serum by direct IFA. RT activity of the supernatant of FS-L3 cells was measured to check the contamination of endogenous retroviruses. No RT activity was detected in the culture medium of FS-L3 cells. In addition, no PCMV was detected in FS-L3 cells with FITC-labeled hyperimmune anti-PCMV Ka strain serum. In the PCR procedure, specific bands of mycoplasmas were not detected in the DNA extracted from FS-L3 cells, although the positive control sample was amplified specific bands (approximately 180 and 600 bp).

Table 2. Denial of the contamination in FS-L3 and CPK-NS cells

Contaninants	Methods	Cell line	
		FS-L3	CPK-NS
Pestivirus	RT-PCR	Negative	Negative
	direct IFA	Negative	Negative
Retrovirus	RT assay	Negative	ND[a]
Cytomegalovirus	direct IFA	Negative	ND
Circovirus[b]	PLA[c]	Negative	ND
Mycoplasmas	PCR	Negative	ND

[a] ND; not determined.

[b] The data was reported by Edwards and Sands (11).

[c] PLA; peroxidase-linked assay

Discussion

The FS-L3 and CPK-NS cells, which originated from the renal cortex of a healthy piglet, have been established as a continuous cell line in simple serum-free culture. It has been reported that some mammalian cells grow in serum-free medium such as DM 160 medium, which is completely synthetic without any protein and lipid (24, 25). Our serum-free medium consists of MEM supplemented with only 0.295% tryptose phosphate broth, 0.5% Bacto peptone and 10mM BES. When FS-L3 cells were seeded at 5 $x10^5$ /ml but not at less than 1 $x10^5$ /ml, they proliferated logarithmically in the serum-free medium. It was not necessary for cell proliferation to use culture flasks coated with collagen or some other substrate. It is therefore suggested that FS-L3 cells might produce auto- or paracrine cell growth and adherence factors.

For identification of the FS-L3 and CPK-NS cell lines, hybridization using the DNA probe PRE-I was performed. The PRE-I sequence was reported as the specific repetitive sequence in the swine genome and belongs to the short interspersed sequence (SINEs) (84). The size of its consensus sequence is about 230 bp and its frequency in the swine genome was estimated to be 2 $x10^6$ per diploid genome (66). The detection of PRE-I gene in the established cell lines is useful as regards specificity and simplicity in comparison with isoenzyme analysis for the confirmation of porcine origin. The mode of the chromosome number was still in a diploid range of 37 to 38 in the FS-L3 cell population. Furthermore, FS-L3 cells were unable to grow in soft agar even when they were inoculated at a high density. These results suggest that the FS-L3 cells have not undergone malignant transformation.

The kidney is a complex organ containing numerous cell types. In the histochemical experiments, ALP and cytokeratin were detected in FS-L3 cells although γ-GTP activity was not detected. In the case of CPK-NS cells and its parental cell line CPK, cytokeratin was detected, although ALP and γ-GTP activity were not detected. These results and epithelial-like appearance with a phase contrast microscope confirm that FS-L3 and CPK-NS cells have originated from renal tubular epithelial cells. The lack of ALP and γ-GTP activity was suggested that long-term cultures lead to loss of functional characteristics, which is present in the *in vivo* situation.

As shown in Fig. 4, numerous fluid-filled, multicellular domes were developed on

the monolayer of FS-L3 cells. These domes became three-dimensional structures like soccer balls and detached from the surface of the cell monolayer. Dome formation of several renal cell lines (glomerular epithelial or tubular epithelial cells) have been reported (26, 31, 73). This phenomenon suggests that FS-L3 cells possess differentiated polarity and may have active secretory activity. Dome formation seems to be a result of fluid accumulation between the cell layer and the surface of the culture dish. SK-L cells, the parental cell line of FS-L3, produces fewer but larger domes (approximately 1 cm in diameter) in the medium with 10% serum in a plastic culture flask (data not shown). In contrast, FS-L3 cells showed numerous small domes (approximately 0.1 mm in diameter) and formed the three-dimensional structures mentioned above.

Some of the established porcine cell lines have been contaminated with porcine pestiviruses, retroviruses or mycoplasmas (3, 4, 18, 72). FS-L3 and CPK-NS cells were found free from pestiviruses. Furthermore, FS-L3 cells were also free from other adventitious viruses (retroviruses, PCMV and PCiV) and mycoplasma strains. Concerning the PCiV, it has been reported that the SK-L cell line, which is the parental cells of the FS-L3 cell line, was free of PCiV (11). These results show that the FS-L3 cell line is clean without any contamination.

Furthermore, the porcine kidney cell lines, which are known to form domes, were used for a comparative toxicity study of aminoglycoside antibiotics (19, 80). In conclusion, it is obvious that FS-L3 and CPK-NS cells will be widely used in various fields of study such as physiological experiments, production of biological products including genetic engineering, model analysis of renal function, *in vitro* evaluation for toxicity assay and further analysis of the mechanism of the three-dimensional formation.

Summary

A stable porcine kidney epithelial cell lines, FS-L3 and CPK-NS, were established and maintained in Eagle's minimum essential medium (MEM) containing 0.295% tryptose phosphate broth, 0.5% Bacto peptone and 10 mM N, N-Bis (2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) without any serum. The mode of chromosomes is 37-38 in FS-L3 cells and 55 in CPK-NS. The FS-L3 cells formed fluid-filled, multicellular three-dimensional domes on a single monolayer. The number of the domes increased markedly after further cultivation. The origin of these cell lines was confirmed as porcine by hybridization using PRE-I, which can be detected as a specific sequence in the porcine genome. It was also found that both cell lines were free from pestiviruses, furthermore FS-L3 cells were free from mycoplasmas and possible adventitious viruses like retrovirus, porcine cytomegalovirus (PCMV) and porcine circovirus (PCiV).

Chapter II

Development of virus and antibody assays for classical swine fever

Introduction

Generally, immunofluorescence method and immunoperoxidase staining have been used internationally for the detection of CSFV and its antibody (70) since most CSFVs are ncp. In Japan, END method developed by Kumagai et al. (29, 30) has been usually used for the titration of the END^+ CSFV strains and neutralization test. Interference method was also used for the virus titration of the END^- CSFV strains, especially CSFV vaccine strain GPE^- (12, 61) since these viruses did not show clear CPE in the usual tissue culture cells. For immunofluorescence and immunoperoxidase staining, hyperimmune polyclonal sera or specific monoclonal antibody (MAb) against CSFV is necessary for the detection of CSFV-proteins. Furthermore, virulent END^+ CSFV (e.g. ALD strain) and NDV (e.g. Miyadera strain) are needed for the END method, VSV or WEEV is necessary for the superinfection of the interference method. END and interference methods have a possibility to spread out these virulent and exotic viruses. From these points, new virus assay of CSFV is necessary to develop for safe, simple and reliable diagnosis.

In the present study, new cell lines, FS-L3 and CPK-NS cells were established from swine kidney epithelial cells. These cell lines are able to grow well in simple serum-free medium. In this chapter, development of the new assays of virus and antibody by using unique CPE in FS-L3 and CPK-NS cells infected with CSFV END^- strains is described. Furthermore, the correlation between these new methods and other usual methods is shown.

Materials and Methods

Cells and viruses

The FS-L3 and CPK-NS cell lines were grown as monolayers in serum-free medium explained in chapter I. The 100th passaged FS-L3 and 60th passaged CPK-NS cells in serum-free culture were used in all these experiments. PK-15 and CPK were grown as monolayers in serum-supplemented medium explained in chapter I. Primary swine testicle (ST) cells were grown as monolayers in MEM containing 10% inactivated FBS and 0.75 mg of sodium bicarbonate per ml (30).

CSFV isolates ALD, 331, Alfort, Osaka/71, Kanagawa/74 and Hokkaido/66 were kindly provided by Dr. M. Shimizu (National Institute of Animal Health, Tsukuba, Japan), CSFV Thai isolate NRSM/93 by Dr. S. Damrongwatanapokin (National Institute of Animal Health, Bangkok, Thailand), CSFV ALD-END$^-$ and Ames-END$^-$ strains by Dr. N. Ogawa (National Veterinary Assay Laboratory, Tokyo, Japan). The WB82 strain was isolated from a wild boar suffering from CSF in Tsukuba, Japan, in 1982. The CSFV vaccine strain GPE$^-$ and other CSFV strains were propagated in FS-L3 cells by incubation at 37°C for 3 or 4 days.

The VSV New Jersey strain was also grown in FS-L3 cells at 37°C for 2 days. NDV Miyadera strain was propagated in the allantoic cavity of 11-day-old embryonated hens' eggs at 38°C for 72 hr and then the allantoic fluids were harvested.

RNA isolation, gel electrophoresis and Northern blot hybridization

CSFV WB82, ALD and GPE$^-$ strains were inoculated onto FS-L3 cells at a multiplicity of infection (MOI) of one. Total RNA was extracted from FS-L3 cells by RNA zolB in accordance with the manufacturer's protocol (Tel-Test Inc.). Five micrograms of extracted RNA was separated on 1% agarose gels containing 5.5% formaldehyde and transferred to a positively charged nylon membrane (Boehringer Biochemica). An RNA ladder (GIBCO-BRL Life Technologies Inc., Gaithersburg, USA) served as a size standard.

A 2.1-kb cDNA fragment (NS3 region) of the CSFV GPE$^-$ strain was used as a DIG-labeled probe. The probe was labeled with a DIG-DNA labeling kit (Boehringer Biochemica). The hybridization was performed at 50°C for 16 hr with

10 ng of denatured DIG-labeled DNA probe per ml in DIG Easy Hyb (Boehringer Biochemica). After hybridization, the membrane was washed twice with 2x SSC, 0.1% SDS and 0.1x SSC, 0.1% SDS. After incubation with diluted anti-DIG ALP conjugate solution in a DIG nucleic acid detection kit (Boehringer Biochemica), the membrane was incubated with color-substrate solution for the detection of desired spots.

Analysis of apoptosis

CPK-NS cells infected with CSFV strain GPE$^-$ at an MOI of 0.1. The cells were incubated for 3 days, fixed with Carnoy's fixative (60% ethanol, 30% chloroform and 10% glacial acetic acid), and stained with Giemsa's stain solution (Wako Pure Chemical Industries, Ltd.). The morphology of the stained nuclei was observed with a microscope to detect chromatin condensation and fragmentation.

DNA fragmentation was detected by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) method with an Apop-Tag in situ apoptosis detection kit (Oncor, Gaithersburg, USA). CPK-NS cells infected with CSFV strain GPE$^-$ for 3 days were fixed with 10% formalin. Then DIG-labeled dUTP was added to the 3' termini of fragmented DNA by TdT. DIG-labeled fragmented DNA was incubated with peroxidase-labeled anti-DIG antibody and detected with staining buffer (0.05% diaminobenzidine in PBS).

Indirect IFA

FS-L3 cells infected with the GPE$^-$ strain were washed once with PBS and fixed with acetone at -20°C. The cells were then incubated for 60 min at 37°C in the presence of anti-CSFV E2 protein MAb, f48 (28), which was kindly provided by Dr. E. Weiland (Federal Research Center for Virus Diseases of Animals, Tübingen, Germany). The cells were washed and then incubated for 60 min at 37°C in the presence of FITC-labeled anti-mouse goat serum (Organon Teknika Corp., Durham, USA). The cells were washed again and then observed under a fluorescence microscope.

Titration of GPE$^-$ strain by the interference method in ST cells

This assay was carried out according to the method previously described (12, 62).

ST cell suspension and tenfold diluted virus (GPE^- strain) were transferred simultaneously to a 96-well microplate. After cultivation for 4 days at 37°C, the medium was discarded and cultures were challenged with 100 µl of 10^6 50% tissue culture infectious doses ($TCID_{50}$) of VSV New Jersey strain. The cultures without CPE were read as CSFV positive, and the tissue culture infectious dose was calculated by Kärber's method (22).

Peroxidase-linked assay (PLA) and neutralization peroxidase-linked assay (NPLA) in PK-15 cells

PLA and NPLA were performed by essentially following standard procedure (70) in flat-bottomed microplates. For the virus titration, PK-15 cells were infected with CSFV strains and incubated for 4 days. For the neutralization test, diluted sera and virus suspension (CSFV GPE^- strain; 100 $TCID_{50}$/ 25 µl) were mixed and incubated for 1 hr at 37°C. Then the mixture was added to the PK-15 cells and incubated for 4 days. The plates were washed once with PBS and fixed at 80°C for 1 hr. The cells were then incubated for 60 min at 37°C in the presence of anti-CSFV MAb, A425 (47), which was kindly provided by Dr. S. Yamada (National Institute of Animal Health, Tsukuba, Japan). The cells were washed and then incubated for 60 min at 37°C in the presence of goat anti-mouse IgG (H+L) horseradish peroxidase conjugate (Bio-Rad Laboratories, Hercules, USA). The cells were washed again and then stained with an ACE (3-amino-9-ethylcarbazole) staining kit (Sigma chemical Co., St. Louis, USA).

Virus titration and neutralization test by the END method in CPK cells

The END method in CPK cells was described previously (27). Tenfold diluted END^+ CSFV strain (ALD strain) and CPK cell suspensions were added to a 96-well microplate and incubated in a 5% CO_2 incubator for 4 days. Then the culture fluid was removed from each well, and 150 µl of a 1 hemagglutination (HA) U /ml suspension of NDV, Miyadera strain, was added. After incubation at 37°C for 3 days, the plates were examined for enhanced CPE of NDV by CSFV under a microscope. The cells with strong CPE were read as CSFV positive.

The END neutralization test in CPK cells was also described previously (27). Test sera were inactivated at 56°C for 30 min before use. Twenty-five microliters of twofold

diluted serum was mixed with an equal volume of 100 $TCID_{50}$ ALD strain and incubated at 37°C for 1 hr. Fifty microliters of this mixture and the CPK cell suspension were transferred to 96-well microplate and incubated in a 5% CO_2 incubator for 4 days. Then the culture fluid was removed from each well, and 150 µl of a 1 HA U /ml suspension of NDV Miyadera strain was added. After incubation at 37°C for 3 days, the plates were examined for enhanced CPE of NDV under a microscope. The cells without strong CPE were read as CSFV-antibody positive.

Serum samples for neutralization test

An 8-weeks-old CSFV antibody-free pig was immunized experimentally by subcutaneous injection with 1 ml of 10^4 $TCID_{50}$ CSFV GPE^- strain. Serum was collected for the neutralization test at 0~10, 13 and 18 weeks after inoculation. These sera and 93 serum samples collected from domestic pigs in the field were determined by END neutralizing method and newly developed method using FS-L3 cells (dome disappearance method). Furthermore, serum samples of the breeding pigs (n=633) and fattening pigs (n=484) were collected from several prefectural Livestock Hygiene Service Centers. Neutralizing antibody titers were surveyed by dome disappearance method with FS-L3 cells. Neutralizing antibody titers were also determined by NPLA in PK-15 cells, dome disappearance method in FS-L3 cells and newly developed method using CPK-NS cells (CPE-NT method) for 68 serum samples collected from vaccinated or non-vaccinated pigs in the field.

Results

Cytopathogenicity of CSFV END⁻ strains in FS-L3 and CPK-NS cells

CSFV vaccine strain GPE⁻ was inoculated to the FS-L3 cells. The fluid-filled, multicellular domes on a single monolayer of the FS-L3 cells were disappeared and clear CPE was observed (Fig. 8a) although control mock-infected cells were not observed any changes on the cells (Fig. 8b). In the case of the CPK-NS cells, strong CPE was observed on the surface of cells infected with GPE⁻ strain and thin, single monolayer still remained even after the virus growth (Fig. 8c). Mock-infected CPK-NS cells were not observed any changes on the cells (Fig. 8d).

CSFV END⁺ and END⁻ strains were inoculated onto FS-L3, CPK-NS, CPK, PK-15 and ST cells, then observed for their cytopathogenicity (Table 3). All END⁺ viruses (ALD, 331, Alfort, Osaka/71, Kanagawa/74 and Hokkaido/66) were ncp in all cell lines. On the other hand, all END⁻ strains (GPE⁻, ALD-END⁻, Ames-END⁻ and NRSM/93) showed CPE in FS-L3 cells and the fluid-filled, multicellular domes on a single monolayer were also disappeared. In the case of the CPK-NS cells, strong clear CPE was observed on the surface of cells infected with all END⁻ strains. Furthermore, weak CPE was also observed in CPK and PK-15 cells infected with END⁻ CSFV strains although no change was observed in ST cells. WB82 strain showed strong CPE in all five cell lines. It has been found that the strong cytopathogenicity of strain WB82 is caused by DI particles.

To characterize the genomic RNA of the GPE⁻ strain, total RNA of FS-L3 cells infected with strains WB82, ALD and GPE⁻ was isolated and hybridized by Northern blotting with the DIG-labeled CSFV-specific cDNA probe of the NS3 region (Fig. 9). The genomic CSFV RNA with a length of about 12.3 kb was detected in FS-L3 cells infected with the ALD (Fig. 9 lane 3) and GPE⁻ strain (Fig. 9 lane 4). Internal deleted RNA with a length of about 7.5 kb was detected only in the cells infected with the WB82 strain (Fig. 9 lane 2). The internal deleted RNA was not detected in cells infected with the GPE⁻ strain at MOIs of 0.01, 0.1 and 10 (data not shown).

Condensation and fragmentation of chromatin are morphological hallmarks of apoptosis. In order to observe the general morphology in the nuclear induced by END⁻ CSFV infection, CPK-NS cells were infected with the GPE⁻ strain and stained with

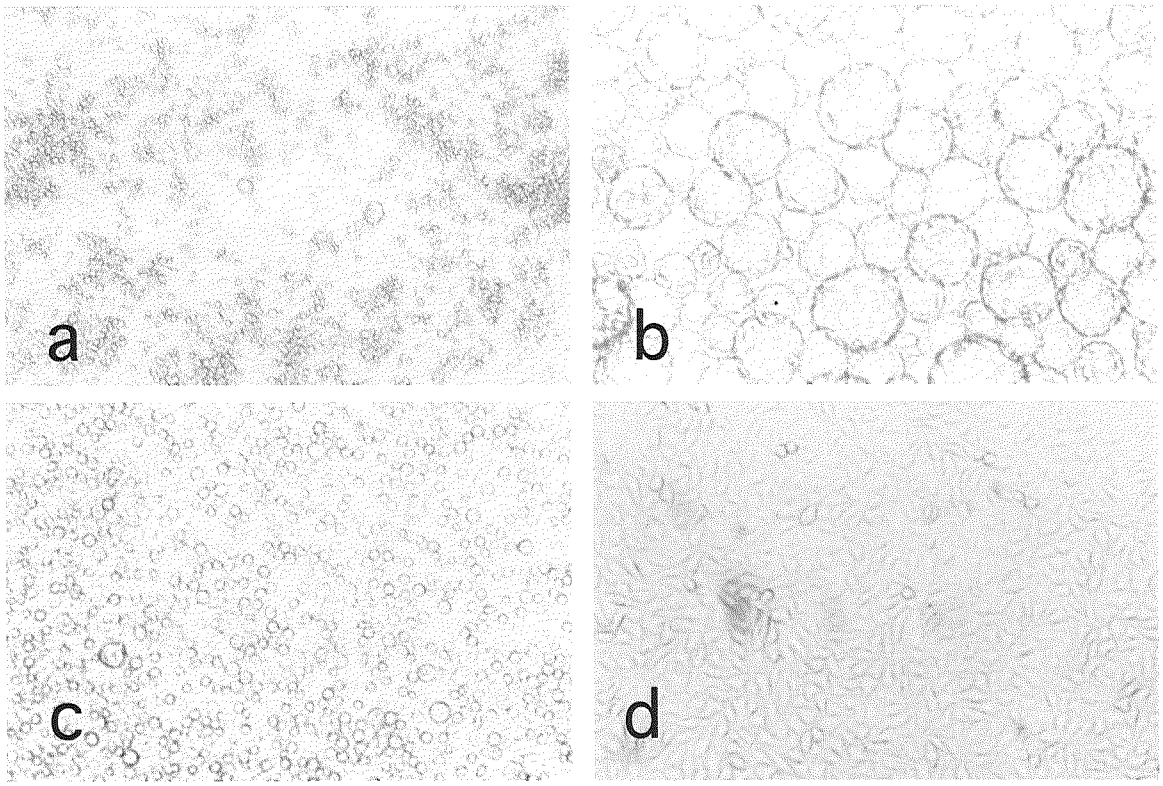

Fig. 8. Morphology of the cells infected with CSFV strain GPE^{-}. (a) Dome disappearance of FS-L3 cells at 3 days after virus inoculation. (b) Dome formation of mock-infected FS-L3 cells 5 days after the previous passage. (c) Cytopathic effect of CPK-NS cells 3 days after virus inoculation. (d) Mock-infected CPK-NS cells 3 days after the previous passage. Magnifications are x160 (a, b) and x200 (c, d).

Table 3. Cytopathogenicity of CSFV strains

CSFV strains[a]	Cytopathogenicity[b] on the following cells				
	FS-L3	CPK-NS	CPK	PK-15	ST
END^{+} viruses ALD, 331, Alfort, Osaka/71, Kanagawa/74, Hokkaido/66	-	-	-	-	-
END^{-} viruses GPE^{-}, ALD-END^{-}, Ames-END^{-}, NRSM/93	++[c]	++	+	+	-
DI particle related virus WB82	++	++	++	++	++

a FS-L3, CPK-NS, CPK, PK-15 and ST cells were infected with END phenomenon-positive (END^{+}) or -negative (END^{-}) viruses in 96-well microplate and examined for CPE 4 days postinfection.

b ++, clear CPE; +, weak CPE; -, no effect on the cell

c Disappearance of the domes was also observed.

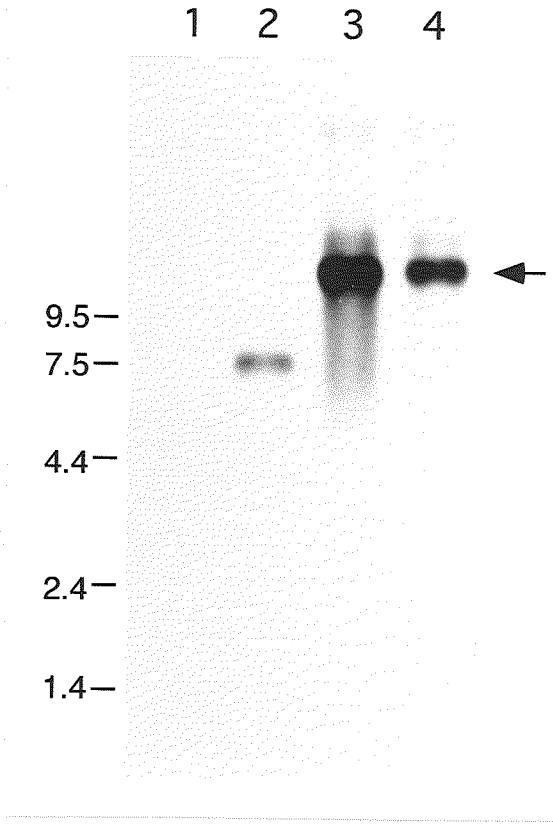

Fig. 9. Northern blot analysis of RNA from FS-L3 cells infected with CSFV strain WB82, ALD and GPE$^-$ at an MOI of one. Hybridization was performed with a CSFV GPE$^-$-derived probe (NS3 region). Numbers on the left indicate the size of an RNA ladder in kilobases. The genomic CSFV RNA with a length of about 12.3 kb (indicated in arrow) were detected in FS-L3 cells infected with the ALD (lane 3), GPE$^-$ (lane 4) strains. Internally deleted RNA with a length of about 7.5 kb was detected only in the cells infected with the WB82 strain (lane 2). Lane 1 is RNA purified from mock-infected FS-L3 cells.

Giemsa 3 days after infection. The detached cells showing CPE were collected from culture medium and stained. The nuclei of cells were observed the blebbing of the nucleus, rounded subnuclear masses of chromatin (Fig. 10a). In the case of mock-infected cells, neither condensation nor fragmentation of chromatin was observed (Fig. 10b). The TUNEL method was also used for the detection of DNA fragmentation of the cells. The cells showing CPE by the infection with the GPE^- strain were collected and used for TUNEL method. A specific red signal confirmed the DNA fragmentation was detected from the cells showing CPE (Fig. 10c), although no signal was detected in the mock-infected CPK-NS cells (Fig. 10d).

Virus propagation in FS-L3 and CPK-NS cells

FS-L3 cells were cultured in 25 cm^2 plastic flasks and inoculated with CSFV strain GPE^- at an MOI of 0.1. After 2 hr's adsorption, the cells were washed twice with MEM and then maintained in 5 ml of serum-free medium. The supernatant and cells were harvested at 24-hr intervals. Strain GPE^- grew very well, to about 10^7 $TCID_{50}$/ml in the cellular phase of FS-L3 cells (Fig. 11). In the supernatant phase, the virus titer was lower than that in the cellular phase, approximately 10^6 $TCID_{50}$/ml. The CPE was observed only on the surface of the cell monolayer 2 days postinfection and caused the dome disappearance on the single cell monolayer. Finally, no dome was observed 3 days postinfection.

Virus growth of GPE^- strain in CPK-NS cells was also observed (Fig. 12). In the serum-free culture, GPE^- strain did not grow so much as FS-L3 cells, about $10^{4.5}$ $TCID_{50}$/ml in the cellular phase (Fig. 12a). However, clear strong CPE was observed on the surface of the cell monolayer 3 days after inoculation. Furthermore, CPK-NS cells were infected with GPE^- strain in the medium supplemented with 5% FBS (Fig. 12b). By addition of 5% FBS, strain GPE^- grew very well again, to about 10^5 $TCID_{50}$/ml in the cellular phase and 10^7 $TCID_{50}$/ml in the supernatant phase. The titer of the supernatant phase was higher than that of the cellular phase in the serum-supplemented culture of CPK-NS cells, although the titers of both phases were opposite in serum-free culture of CPK-NS and FS-L3 cells. The same clear strong CPE as the serum-free culture of CPK-NS was observed on the surface of the cell monolayer 3 days after inoculation.

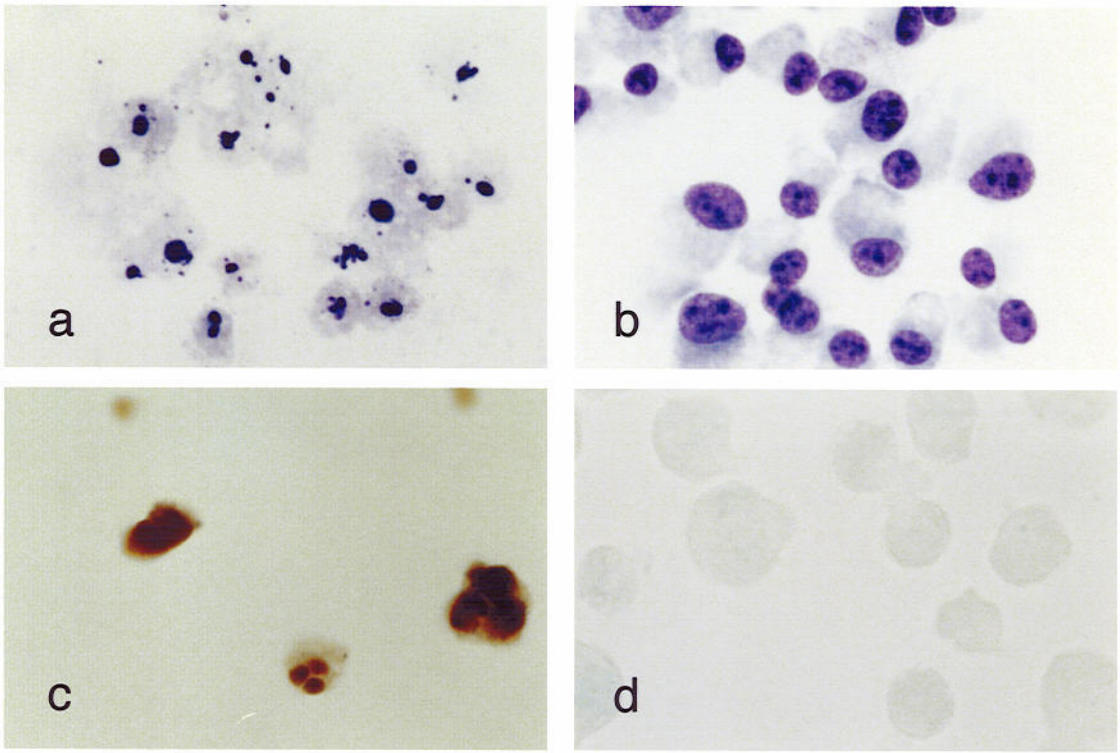

Fig. 10. Changes in nuclear morphology and DNA fragmentation induced by GPE^{-} strain. CPK-NS cells were infected with CSFV GPE^{-} strain at an MOI of 0.1 (a, c) or mock-infection (b, d). The cells were fixed with Carnoy's fixative and stained with Giemsa (a, b). DNA fragmentation was detected by the TUNEL method in the CPK-NS cells (c, d). Magnifications are x300 (a, b) and x700 (c, d).

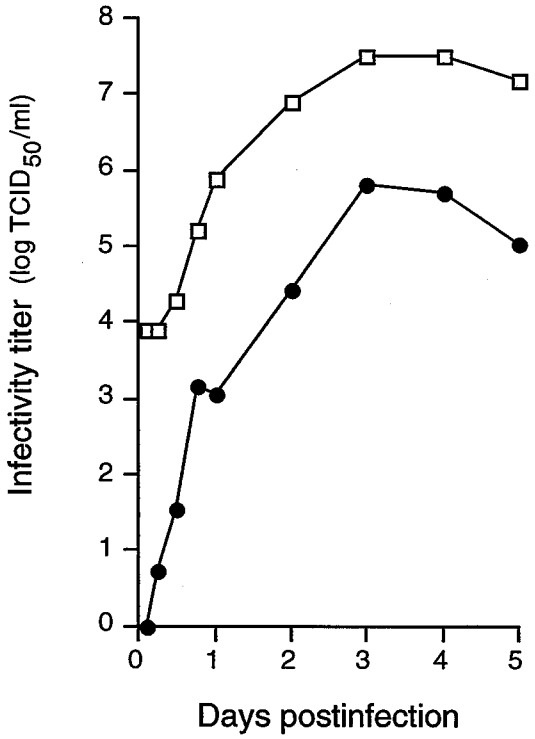

Fig. 11. The growth of CSFV strain GPE$^-$ in FS-L3 cells. FS-L3 cells were inoculated with CSFV vaccine strain GPE$^-$ at an MOI of 0.1 for 2 hr. After that, cells were washed with serum-free medium 3 times. Fresh serum-free medium was added to the cells for further cultivation. The virus titers in the cells and the supernatant were titrated by the interference method in ST cells.
—□— ; cellular phase, —●— ; supernatant phase.

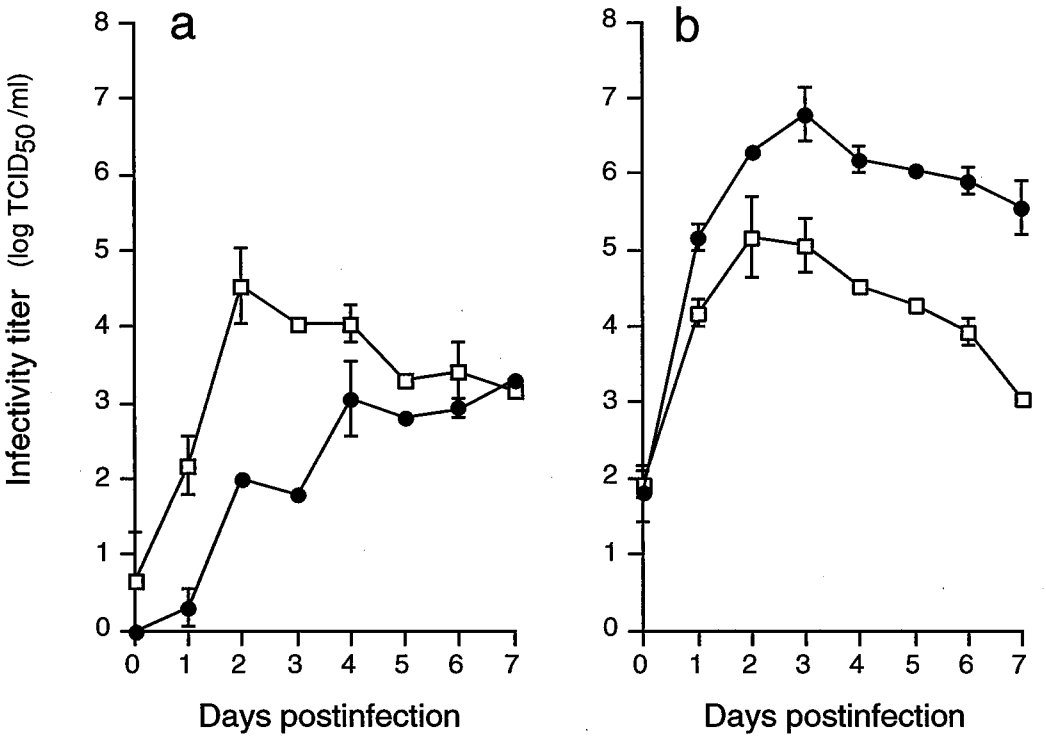

Fig. 12. The growth of CSFV strain GPE⁻ in CPK-NS cells. CPK-NS cells were inoculated with CSFV vaccine strain GPE⁻ at an MOI of 0.1 for 2 hr. After that, cells were washed with serum-free medium 3 times. Fresh serum-free medium (a) or the medium supplemented with 5% FBS (b) was added to the cells for further cultivation. The virus titers in the cells and the supernatant were titrated by the interference method in ST cells. —□— ; cellular phase, —●— ; supernatant phase.

Development of virus titration methods in FS-L3 cells (dome disappearance method) and CPK-NS cells (direct and indirect CPE methods)

To develop the dome disappearance method in FS-L3 cells, various assay conditions such as seeded cell number and volume of culture medium were examined. Consequently, the method described below was decided. Tenfold diluted CSFV GPE^- strain in serum-free culture medium was inoculated at 50 µl per well into a 96-well microplate. Forty microliters of a suspension of FS-L3 cells (containing 5 x 10^4 cells) was added to each well at the same time. The plate was incubated for 7 days at 37°C in a 5% CO_2 incubator, and then virus was titrated by the presence of domes on the monolayer. The wells, in which domes were disappeared, were read as CSFV positive. The best total volume of the medium was 90 µl per well of a 96-well microplate. If the volume was over 90 µl, maintenance of the cells and dome formation were not so good. In contrast, the dome formation was good in less than 90 µl of medium, but it was difficult to maintain the cells for 7 days.

For the titration of END^- viruses, direct CPE method was carried as follows in CPK-NS cells. Tenfold diluted END^- virus in serum-free culture medium was inoculated at 50 µl per well into a 96-well microplate. One hundred microliters of a suspension of CPK-NS cells (containing 5 x 10^4 cells) was added to each well at the same time. The plate was incubated for 7 days at 37°C in a 5% CO_2 incubator, and then virus titer was judged by clear CPE on the monolayer.

For the titration of END^+ viruses, indirect CPE method was carried as follows in CPK-NS cells. Tenfold diluted END^+ virus and cell suspensions were added to a 96-well microplate. The plate was incubated at 37°C for 4 days. Then the culture fluid was removed from each well and the cells were superinfected with 100 µl of the 10^5 $TCID_{50}$ GPE^- strain. After incubation at 37°C for 3 days, the plates were examined for clear CPE of the GPE^- strain under a microscope. The cells, which interfered with the growth of the GPE^- strain and did not show clear CPE of the GPE^- strain, were read as CSFV END^+ strain positive.

The virus titers obtained by these new methods were compared with those of the indirect IFA in FS-L3 cells, interference method in ST cells, PLA in PK-15 cells and END method in CPK cells (Table 4). From the average of three independent experiments, it was found that the sensitivity of these newly developed methods (dome disappearance,

Table 4. Comparison of the virus titers by the new methods and the usual methods

Methods	Cells	Virus titer[a] ($TCID_{50}$ /ml)	
		GPE⁻ strain	ALD strain
Dome disappearance	FS-L3	$10^{6.9}$	—[b]
Direct CPE	CPK-NS	$10^{7.0}$	—
Indirect CPE	CPK-NS	—	$10^{7.1}$
Indirect IFA	FS-L3	$10^{6.8}$	ND[c]
Interfernce	ST	$10^{6.8}$	—
PLA	PK-15	$10^{7.1}$	$10^{7.2}$
END	CPK	—	$10^{7.3}$

[a] The same virus stocks were used for the titration of each strain. The titers are the average of three independent experiments.

[b] Test is not available for the titration.

[c] ND; not determined.

direct CPE and indirect CPE methods) was almost the same as that of usual methods.

Neutralization test by the dome disappearance method in FS-L3 cells

Test sera were inactivated at 56°C for 30 min. Twenty-five microliters of twofold diluted sera was mixed with an equal volume of 100 $TCID_{50}$ GPE^- strain and the mixture was incubated at 37°C for 1 hr. Fifty microliters of this mixture and FS-L3 cell suspension (5 x 10^4/ 40 μl) were inoculated into a 96-well microplate at the same time and the plate was incubated in a 5% CO_2 incubator for 7 days. By using disappearance of domes as an infectious marker, the neutralizing antibody was titrated.

The sera of pig were used for the neutralization test at 0~10, 13 and 18 weeks after inoculation with GPE^- strain (Fig. 13a). Neutralizing antibodies were detected 2 weeks after inoculation by the dome disappearance method, and 3 weeks after inoculation by the END neutralizing method. There is a high correlation between the titers in the two methods, whereas most of the titers in the dome disappearance method were higher than those in the END neutralizing method. Neutralizing antibody titers were also determined for 93 serum samples collected from domestic pigs in the field by using the two methods mentioned above (Fig. 13b). The titers of 26 serum samples in the dome disappearance method were higher than those in the END neutralizing method, on the other hand the titers of 15 serum samples in the dome disappearance were lower than those in the END neutralizing. However, this data clearly shows a high correlation between the titers determined the two methods. The coefficient of correlation was r = +0.971 at a probability rate of below 1%.

Furthermore, more than 1000 serum samples collected from Livestock Hygiene Service Center were surveyed by dome disappearance method in FS-L3 cells (Fig. 14). Histograms of the neutralizing antibody titers displayed a normal distribution in breeding and fattening groups although nine percent of the sera in both groups were negative (less than 1:2). The peaks of the antibody titers in both distribution curves were 1:128 in breeding pigs and 1:256 in fattening pigs.

CPE-neutralization test (CPE-NT) in CPK-NS cells

By the use of the clear CPE of CPK-NS cells infected with END^- CSFV, a neutralizing test was also developed. The CPE-neutralization test (CPE-NT) was carried

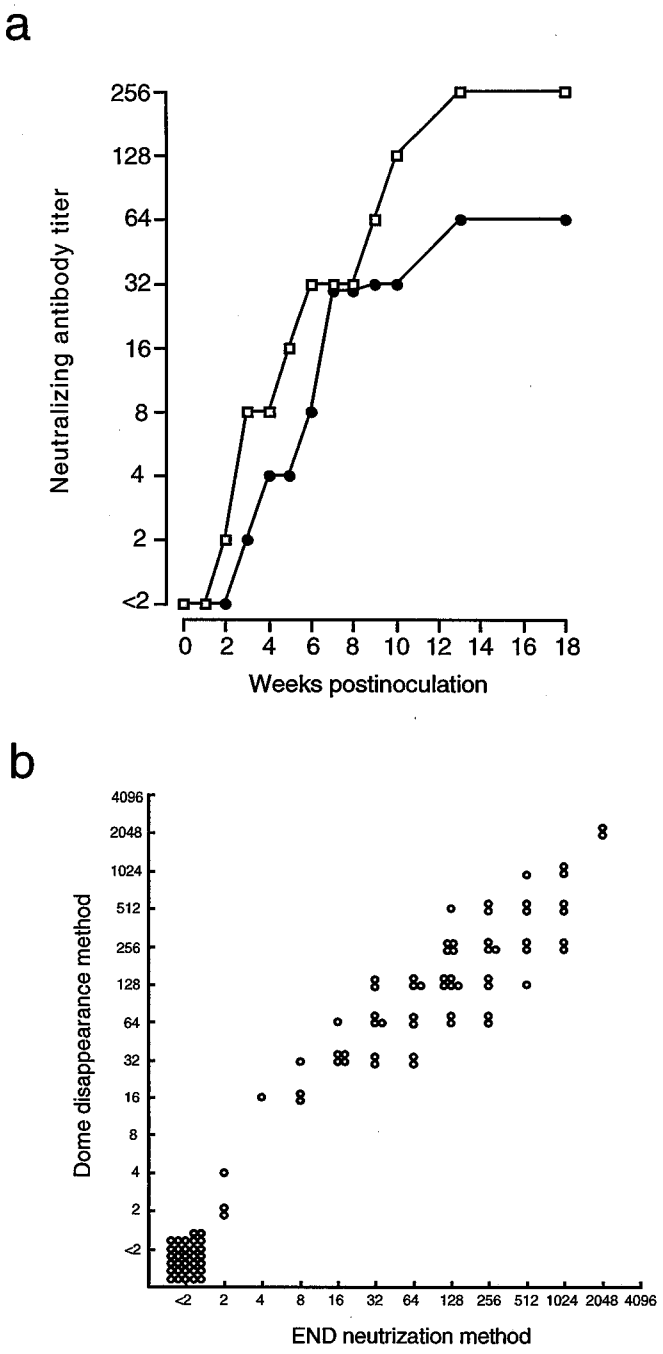

Fig. 13. (a) Neutralizing antibody titer of pig inoculated with the CSFV GPE^{-} strain. Neutralization tests were carried out on the pig serum of 0~10, 13 and 18 weeks after inoculation by dome disappearance (—□—) and END neutralization (—●—) methods. (b) Correlation of neutralizing antibody titers obtained by the dome disappearance method and the END neutralization method. The antibody titers of 93 serum samples collected from domestic pigs in the field were determined by the two methods. The coefficient of correlation was r = +0.971 at a probability rate of below 1%.

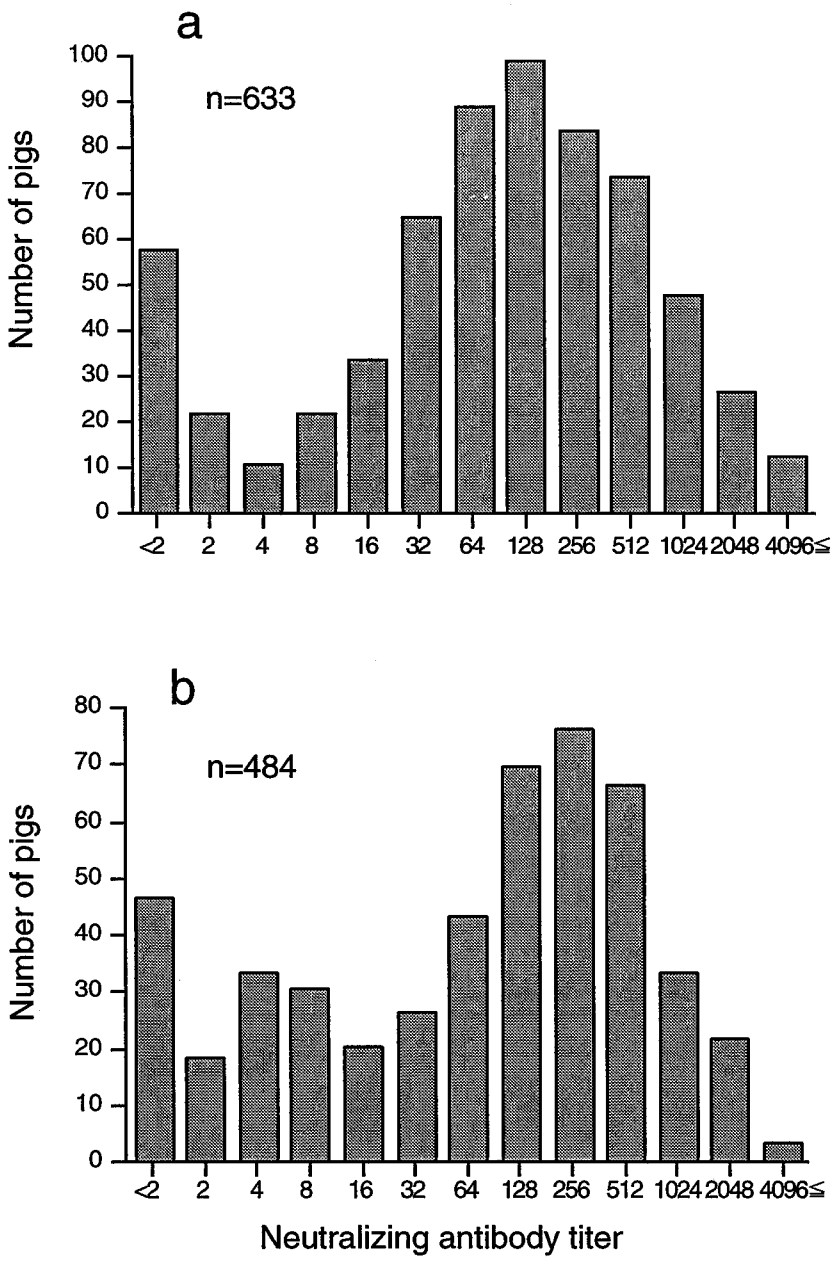

Fig. 14. Surveillance of neutralizing antibody titers of the breeding (a) and fattening (b) pigs. Serum samples collected from several prefectural Livestock Hygiene Service Centers were titrated neutralizing antibody titer by dome disappearance method with FS-L3 cells.

as follows. Twenty-five microliters of twofold diluted sera were mixed with an equal volume of 100 $TCID_{50}$ GPE^- strain and the mixture was incubated at 37°C for 1 hr. This mixture and CPK-NS cell suspension (5 x 10^4/ 100 μl) were inoculated into a 96-well microplate at the same time and the plate was incubated in a 5% CO_2 incubator for 7 days. The neutralizing antibody was titrated by using clear CPE as an infectious marker.

Neutralizing antibody titers were also determined for 68 serum samples collected from vaccinated or non-vaccinated pigs in the field. The neutralizing antibody titers in CPK-NS cells were compared with the NPLA in PK-15 cells (Fig. 15a) and the dome disappearance method in FS-L3 cells (Fig. 15b). The coefficient of correlation between the CPE-NT in CPK-NS cells and NPLA in PK-15 cells was r = +0.947, and between the CPE-NT in CPK-NS cells and the dome disappearance method in FS-L3 cells was r = +0.966.

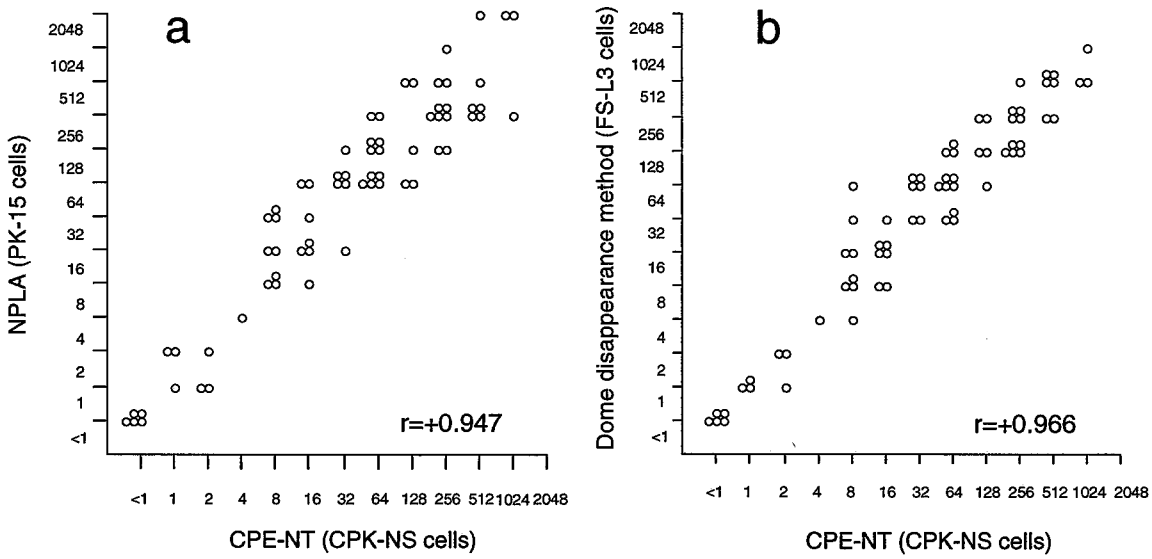

Fig. 15. The correlation of neutralizing antibody titers obtained by the newly developed method (CPE-NT) in CPK-NS cells and the usual methods. Sixty-eight serum samples were collected from vaccinated and non-vaccinated pigs in the field of Japan for the neutralization test. (a) The correlation between CPE-NT in CPK-NS cells and NPLA in PK-15 cells. (b) The correlation between CPE-NT in CPK-NS cells and the dome disappearance method in FS-L3 cells.

Discussion

All END$^-$ strains showed clear CPE in FS-L3 and CPK-NS cells and weak CPE in CPK and PK-15 cells although no change was observed in ST cells. Furthermore, fluid-filled, multicellular domes on a single monolayer were also disappeared in FS-L3 cells infected with the END$^-$ viruses. On the other hand, all END$^+$ viruses were ncp in all cell lines. WB82 strain, which contained the DI particle showed strong CPE in all five cell lines. Several types of viral cytopathogenicity of pestiviruses have been reported. In the case of BVDV, insertion of cellular sequence and the duplication of the viral genome have been detected in the genome of cp strains (38, 39). The full nucleotide sequences of GPE$^-$ strain and its parental strain ALD were already reported (20). From these findings, there is no possibility to explain that the CPE of strain GPE$^-$ is due to the insertion or duplication of cellular or other sequence. Another type of CPE was shown to be due to DI particles (40, 68). Internal deletion encompassed the complete viral structural proteins (C, E^{ms}, E1 and E2) and some non-structural proteins (N^{pro}, p7 and NS2). To check whether strain GPE$^-$ produces DI particles, Northern blot analysis was performed. Defective RNA was not found in the viral gemone of the GPE$^-$ strain by Northern blotting analysis (Fig. 9). Defective RNA was not found either in the CPK-NS cells infected with the GPE$^-$ strain by Northern blot analysis (data not shown). Internally deleted RNA with a length of about 7.5 kb was detected only in the cells infected with the WB82 strain containing DI particles. The CPE by the infection of DI particle related virus was observed only in the cells infected at a high MOI (68). In contrast, this CPE caused by GPE$^-$ could be observed even at the end point of virus titration. In other case, distinct CPE in porcine bone marrow stroma cell infected with END$^+$ CSFVs (ALD and Alfort strain) were also reported (60). However, it seems that the mechanism of the unique cytopathogenicity of END$^-$ viruses in FS-L3 and CPK-NS cells is a different type from some mechanisms reported already.

I studied here this END$^-$ strain-induced cell death was due to the apoptosis. The nuclei of cells infected with the GPE$^-$ strain exhibited extreme condensation and margination of the chromatin. DNA fragmentation was also detected in the detached CPK-NS cells infected with END$^-$ strains by the TUNEL method. Moreover, nonrandom oligonucleosomal length fragmentation of DNA induced by END$^-$ CSFV

were detected by electrophoresis on an agarose gel (data not shown). It has been reported that the CPE of BVDV is mediated by apoptosis (16, 85). In the case of CSFV, it was also shown that E^{ms} inhibits the protein synthesis of lymphocytes, and that apoptosis of lymphocytes was detected after incubation with E^{ms} (6). Our reported apoptosis was induced only by infection with END$^-$ CSFV strains. Further study on the correlation between the specific CPE by END$^-$ CSFV strains and the mechanism of the apoptosis in these cells is needed.

The virus titration methods were developed by using FS-L3 cells (dome disappearance method) and CPK-NS cells (direct and indirect CPE methods) in serum-free culture. The CPE by END$^-$ strains was used as marker of infection in all methods. For the indirect CPE method, homologous interference between CSFV strains was used. The cells infected with END$^+$ strains did not show CPE, but interfere the growth of superinfected END$^-$ strains. Consequently, the infections of END$^+$ strains were visible. The titers determined by these methods show a high correlation with those determined by the usual methods (indirect IFA, PLA, END method and interference method).

In the case of the neutralization test, the titers obtained by the dome disappearance method in FS-L3 cells and END neutralization method clearly show a high correlation in the sera from not only the experimentally infected pig but also the field set. However, most of the titers by the dome disappearance method were higher than those by the END neutralization method in the experimental infection (Fig. 13a) and it is also considered that there is the tendency like that in the field samples (Fig. 13b). Serum antibodies against CSFV were induced by vaccine strain (GPE$^-$ strain) in the experimental infection. Furthermore, there is no outbreak of CSFV since 1993 in Japan, it seems that all antibodies against CSFV in the field are also due to the vaccination of GPE$^-$ strain. In the neutralization test, GPE$^-$ strain was used as marker virus for the dome disappearance method, and ALD strain was used for END neutralization method. Since the difference of antigenicity between GPE$^-$ and ALD strains were previously reported (12, 62), the most likely reason for the difference of antibody titers between the two methods is the difference in the antigenicity of these strains (GPE$^-$ and ALD) used as marker viruses. Although dome disappearance method in FS-L3 cells is useful for the neutralization test of CSFV, there are some difficult cases to titrate the low antibody sera (1:1~1:8 antibody

titers) because of some inhibitory factors in porcine sera for the dome formation of FS-L3 cells. To develop a more stable neutralization test in serum-free culture, the new neutralization method, CPE-NT, in CPK-NS cells was established. The CPE by the infection with END$^-$ CSFV was also used for infectious marker. This CPE-NT in CPK-NS cells shows a high correlation with NPLA and dome disappearance method. Furthermore, this CPE-NT method was able to titrate the sera with low antibody titer more clearly. It was shown here that this serum-free assay method in CPK-NS is simple, reliable and stable.

For the titration of the virus and antibodies, these methods in FS-L3 and CPK-NS cells needs 7 days as a cultivation time. In the case of the conventional assays (immunofluorescence and immunoperoxidase staining methods) using serum-supplemented medium, 3~4 days are enough time for the detection of the antigen. In the case of serum-free culture, viruses grow more slowly, more cultivation time is needed for their complete growth and cytopathogenicity at the virus end point. Virus antigen is able to be detected by immunoperoxidase staining method as small focuses at the virus end point after 4 days cultivation, but not enough for dome disappearance, direct CPE and CPE-NT methods. The sensitivity of CPK-NS in PLA after 4 days cultivation is almost same as that of the direct and indirect CPE methods after 7 days cultivation (data not shown). In spite of the necessity of more cultivation time, these serum-free culture systems have great benefits. It is the greatest advantage to prevent the contamination of the BVDV and antibody against it from FBS. Hyperimmune porcine sera and specific MAbs for the detection of CSFV-proteins in immunofluorescence and immunoperoxidase staining are not necessary, all we have to do is checking the CPE by microscope after 7 days in this novel system. Furthermore, virulent CSFV, NDV, VSV and WEEV used in the END method or interference method are not necessary since the vaccine GPE$^-$ strain is used for the marker of infection in dome disappearance method, CPE method and CPE-NT method.

Establishment procedures of these serum-free cell lines are almost the same; CPK-NS and FS-L3 were established from stable porcine kidney cell lines (CPK and SK-L) which needed the serum for their growth. I suggest here that establishment of serum-free culture cell lines will be necessary for basic studies and biological production of pestiviruses in the near future. Some bovine cell lines for BVDV assays and PK-15 cells

for the usual diagnosis of CSFV might be able to grow well in serum-free medium by this procedure and will be more useful for many situations.

Summary

Clear cytopathic effect (CPE) was observed in FS-L3 and CPK-NS cells infected with exaltation of Newcastle disease virus (END) phenomenon-negative (END^-) strains of classical swine fever virus (CSFV). This CPE result in the disappearance of the unique fluid-filled multicellular domes on a single monolayer of FS-L3 cells. Chromosome condensation and DNA fragmentation, a marker for apoptosis, were detected in cells infected with END^- CSFV strains. By using this CPE as a marker of infection, virus assays of CSFV were established in FS-L3 and CPK-NS cells. The virus titer determined in FS-L3 and CPK-NS cells shows a high correlation with the usual immunofluorescence assay (IFA), interference method, peroxidase-linked assay (PLA) and END method. Furthermore, the antibody titer by neutralizing test in FS-L3 and CPK-NS cells also correlated with that measured by the usual END neutralizing method and neutralization peroxidase-linked assay (NPLA). These FS-L3 and CPK-NS cells have a great advantage that clear CPE was caused by the infection with END^- CSFV strains and any sera are not necessary for the cell culture of virus assays.

Chapter III

Genetic characterization of classical swine fever virus and ruminant pestiviruses

Introduction

Whereas the current classification of pestiviruses refers to the host species from which they were recovered, numerous investigations have proven that pestiviruses are not highly host-specific. It has been reported that BVDV can infect not only cattle but also sheep (7) and pigs (69); BDV infects pigs (76). CSFV infects mainly pigs but experimentally also cattle (33) and goats (58). In rare cases, pestiviruses have been isolated from buffalo, deer and giraffe (2). The detection and characterization of pestiviruses are mainly carried out with polyclonal antibodies (21, 59) and MAbs (47, 49) although all members cross-react serologically to various extents. Differentiation of the pestiviruses is now accomplished by the use of CSFV-specific or BDV-specific MAbs (10, 48). Considering the epizootiological importance and difficulties in diagnosis of pestiviruses, there is a need to develop novel methods of rapid detection, identification and classification of the virus.

The use of molecular genetic methods for the laboratory detection and characterization of viruses has significantly improved. Various assays by RT-PCR have been developed for the general detection of pestiviruses or for the specific recognition of CSFV and BVDV (54, 64, 75, 81). The genetic characterization of viruses has also been improved by determination of the phylogenetic relationship among the various strains. The phylogenetic analyses of pestiviruses have been mostly based on small fragments derived from 5'-UTR (17, 78), N^{pro} (2, 78) or E2 (35, 77) regions.

In this chapter, all porcine pestiviruses mainly isolated in Japan were propagated with FS-L3 cells in serum-free culture and detected by RT-PCR method. Furthermore, genetic characterization of pestiviruses isolated from domestic pigs, wild boars, cattle and cell line contaminated with pestivirus was shown by nucleotide sequence determination, investigation of their phylogenetic relationships and restriction enzyme analysis.

Materials and Methods

Viruses and cells

CSFV isolates; Osaka/51, Ibaraki/66, Nakamura/66, Hokkaido/66, Yamanashi/69, Osaka/71, Fukuoka/72, Shizuoka/73, Kanagawa/74, Ibaraki/81-115, Miyazaki/81, Saitama/81, Ibaraki/82-20-1, Ibaraki/82-40-5, Ibaraki/82-38, Okinawa/86, ALD and 331 were kindly provided by Dr. M. Shimizu and Dr. S. Yamada (National Institute of Animal Health, Tsukuba, Japan). BVDV-isolates; T-20, T-40, NM87-1-cp, NM87-1-ncp, TK87-2-cp, TK87-2-ncp, 190-cp, 190-ncp, 419, 420, 437, 438, 443, 763, 799, Shiribeshi-2, Shiribeshi-4 and Shiribeshi-129 were kindly provided by Dr. H. Sentsui (National Institute of Animal Health, Tsukuba, Japan). In the case of NM87-1, TK87-2 and 190 strains, cp and ncp strains were isolated from cattle with mucosal disease. BVDV Nasu/97 and Chiba/98 were kindly provided by Dr. O. Yamaguchi (Tochigi Animal Hygiene Laboratory, Tochigi, Japan) and T. Nakane (Chiba Prefectural Institute of Animal Health, Chiba, Japan). CSFV WB82 strain was isolated from a wild boar suffering from CSF in Tsukuba, Japan, in 1982 (Ishikawa et al., in preparation). The WB82-helper strain is the helper virus for DI particle causing cytopathogenicity. WB82-DI is the DI particle of the WB82 strain. The CSFV strain GPE$^-$ and BVDV strain No.12 are the vaccine strains, which have been used as live vaccines in Japan. ALD-END$^-$ and Ames-END$^-$, which did not show the END phenomenon (29), were cloned from strain ALD or Ames by the reverse plaque formation method (13). An unknown V/FLL strain was isolated as a contaminant from a fetal lamb lung cell line (FLL-YFT cell) established in Japan. All virus strains examined in this study were listed in Table 5.

All CSFV strains were propagated in FS-L3 cells at 37°C for 3 or 4 days in serum-free culture described in chapter II. All bovine isolates had been propagated with primary bovine testicle cells at 37°C for 3 or 4 days in the medium supplemented with 10% FBS described in chapter I. V/FLL strain was prepared by freezing and thawing of FLL-YFT cells.

Peroxidase-linked assay (PLA)

PLA were performed essentially by the procedure explained in chapter II. For the

Table 5. Pestivirus strains used in this study

	Strain	Isolation	Origin		Strain	Isolation	Origin
Porcine				**Porcine**			
(Field isolate)	Osaka/51	1951	Japan	(other)	NRSM/93	1993	Thailand
	Ibaraki/66	1966	Japan		KPP/93	1993	Thailand
	Nakamura/66	1966	Japan		CBR/93	1993	Thailand
	Hokkaido/66	1966	Japan				
	Yokoba/68	1968	Japan	**Bovine**			
	Ogatamura/68	1968	Japan	(Field isolate)	Nose	1974	Japan
	Yamanashi/69	1969	Japan		T-20	1978	Japan
	Osaka/71	1971	Japan		T-40	1978	Japan
	Fukuoka/72	1972	Japan		HS-86	1986	Japan
	Shizuoka/73	1973	Japan		NM87-1-cp	1987	Japan
	Kanagawa/74	1974	Japan		NM87-1-ncp	1987	Japan
	Kanagawa/75	1975	Japan		TK87-2-cp	1987	Japan
	Chiba/80	1980	Japan		TK87-2-ncp	1987	Japan
	Fukushima/80	1980	Japan		190-cp	1987	Japan
	Saitama/80	1980	Japan		190-ncp	1987	Japan
	Aichi/80	1980	Japan		SY-89	1989	Japan
	Yamagata/80	1980	Japan		MS-1	1989	Japan
	Shimane/80	1980	Japan		763	1989	Japan
	Miyazaki/80	1980	Japan		KA-91	1991	Japan
	Ibaraki/80	1980	Japan		MS-2	1991	Japan
	Ibaraki/81-115	1981	Japan		MS-3	1991	Japan
	Miyazaki/81	1981	Japan		TR-91	1991	Japan
	Saitama/81	1981	Japan		419	1991	Japan
	Ibaraki/82-20-1	1982	Japan		420	1991	Japan
	Ibaraki/82-40-5	1982	Japan		437	1991	Japan
	Ibaraki/82-38	1982	Japan		438	1991	Japan
	WB82-helper	1982	Japan		443	1991	Japan
	WB82-DI	1982	Japan		Shiribeshi-2	1991	Japan
	Okinawa/86	1986	Japan		Shiribeshi-4	1991	Japan
	Okinawa/86-2	1986	Japan		Shiribeshi-129	1991	Japan
					799	1993	Japan
(Laboratory)	LOA	1969	Japan		Nasu/97	1997	Japan
	LOM	1969	Japan		Chiba/98	1998	Japan
	GPE$^-$	1970	Japan				
	ALD-END$^-$	1981	Japan	(Laboratory)	No.12	1967	Japan
	Ames-END$^-$	1981	Japan				
				(other)	Oregon C24V	1960	USA
(other)	ALD	1950	USA				
	PAV-1	1965	USA				
	331	1966	France	**Unknown**			
	Ames	1975	USA	(Laboratory)	V-FLL	1997	Japan

porcine pestiviruses, FS-L3 cells were infected with each strain and incubated for 4 days. For the ruminant pestiviruses, primary bovine testicle cells were infected with each strain and incubated for 4 days. The plates were washed once with PBS and fixed at 80°C for 1 hr. The cells were then incubated for 60 min at 37°C in the presence of pestivirus-specific MAb CF10 (JCU Tropical Biotechnology, Queensland, Australia). The cells were washed and then incubated for 60 min at 37°C in the presence of goat anti-mouse IgG (H+L) horseradish peroxidase conjugate. The cells were washed again and then stained with an ACE staining kit.

RNA extraction and RT-PCR amplification

Virus RNA was extracted from the cell cultures (100 µl) with RNA zolB (Tel-Test Inc.) in accordance with the manufacturer's instructions. Procedure of RT-PCR method described essentially in chapter I was carried out with an RNA PCR Kit Ver. 2. (Takara Shuzo Co., Ltd.) Electrophoretic analysis of the PCR product was carried out in 2% agarose gel in Tris-borate-EDTA buffer.

Nucleotide sequencing and phylogenetic tree analysis

Nucleotide sequences were determined by direct sequencing from PCR products. Sequencing was carried by an Auto Load Solid Phase Sequencing Kit (Pharmacia) and an ALF II automated sequencer (Pharmacia) in accordance with the manufacturer's instructions. All sequences were confirmed by sequencing both strands and the primer region (42 bp) were removed for further analysis.

The nucleotide sequences (241 ~ 246 bp) of 5'-UTR were aligned by the computer program GENETYX-MAC Ver. 9.0 (Software Development Co., Ltd., Tokyo, Japan) and Clustal W program in Info-SINCA system (Fujitsu Kyushu System Engineering Co., Ltd., Japan). Consensus phylogenetic trees constructed by unweighted pair group method using arithmetic average (UPGMA) were evaluated by the bootstrap test (n=200) in the Info-SINCA system. For this analysis, the sequences of reference strains; CSFV, Brecia (43), Alfort (37), Alfort/187 (55), Switzerland 3/93/2 and p97 (63); BDV, BD31 (53); BVDV genotype-I (BVDV-I), NADL (8) and Osloss (51); BVDV genotype-II (BVDV-II), 890 (50) were taken from the nucleotide database.

Restriction fragment length polymorphism (RFLP) method

All RT-PCR products were digested with restriction endonucleases as follows. Amplified PCR products were incubated for 60 min at 37°C in a digestion mixture [5 mM Tris-HCl (pH 7.5), 10 mM NaCl, 1 mM $MgCl_2$, 0.1 mM DTT and 5 U of two restriction endonucleases (*Bgl* I and *Pst* I)]. Samples were analyzed by electrophoresis using 3% agarose gel in Tris-borate-EDTA buffer.

Results

Virus propagation and gene detection of pestiviruses

All porcine pestiviruses were propagated in FS-L3 cells and virus titration was performed by PLA. Each virus was able to grow well in serum-free culture, about 10^5 ~ 10^7 $TCID_{50}$/ml. Most of strains were ncp in FS-L3 cells except END^- strains (GPE^-, ALD-END^-, Ames-END^-, KPP/93 and NRSM/93 strain) and DI particle related virus (WB82 strain). Strain LOA and LOM, which were mixed END^+ and END^- viruses, also showed slight CPE in FS-L3 cells. All ruminant pestiviruses were propagated in primary bovine testicle cells. Each virus was also able to grow well in the medium supplemented with 10% FBS, about 10^5 ~ 10^7 $TCID_{50}$/ml.

The 5'-UTR of all porcine and ruminant isolates was detected by RT-PCR with the primers, 324 and 326. The PCR products (283 ~ 288 bp) were visualized clearly in ethidium bromide-stained agarose gels (Fig. 16).

Nucleotide sequencing and phylogenetic analysis of porcine isolates

Nucleotide sequences of the 5'-UTR were determined by direct sequencing from RT-PCR products. The alignments of the nucleotide sequences obtained from the 42 isolates and reference strains are performed. Sequence alignment of 24 representative strains was shown in Fig. 17. In about 240 bases of 5'-UTR, homology between these isolates was from 92.5 to 100 percent. Especially, Japanese Kanagawa/74, Okinawa/86 and Okinawa/86-2 were the most variable isolates. Homology between these isolates and reference strains (Brecia and Alfort) was very low (93.0~94.2%). However, these three Japanese isolates have high homology with Taiwan isolate p97 (p97 and Kanagawa/74, 96.7%; p97 and Okinawa/86, 86-2, 98.8%). Thai CBR/93 strain was also distinct from isolates used in this study (homologies, 94.2-96.7%). The phylogenetic tree on Fig. 18 was constructed from the nucleotide sequence data, and all porcine strains were divided all strains to the CSFV group. Most of these isolates were subdivided into two major subgroups, CSFV subgroup 1 (CSFV-1, represented by the Brecia strain) and subgroup 2 (CSFV-2, represented by the Alfort strain). The Japanese Kanagawa/74, Okinawa/86, Okinawa/86-2 and Thai CBR/93 strains were the most distinct variants and constituted another new disparate group, CSFV-3. Especially, two

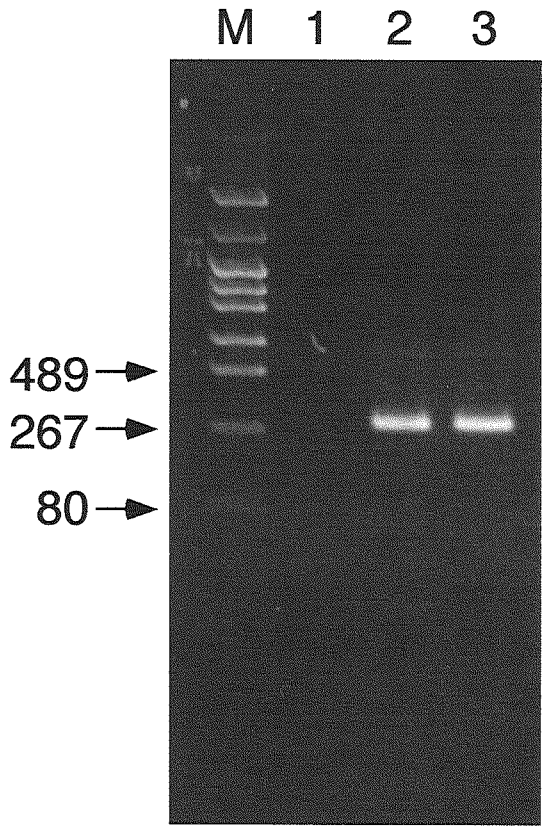

Fig. 16. Gel electrophoresis of RT-PCR products of pestiviruses. PCR products amplified with primers (324 and 326) were detected in 2% agarose gel electrophoresis. Lane 1, negative control; lane 2, CSFV Alfort strain; lane 3, BVDV-I No.12 strain; M, pHY marker.

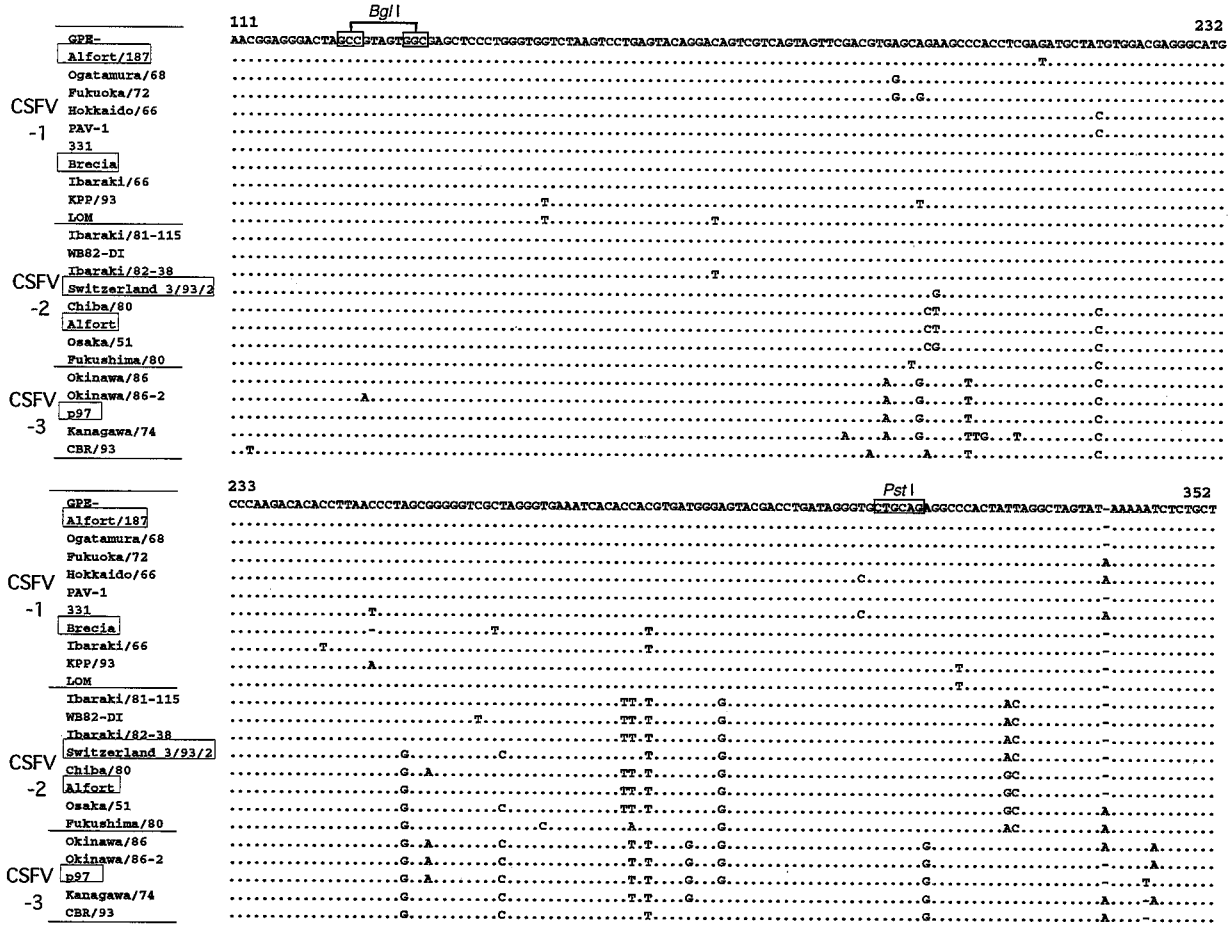

Fig. 17. Representative alignment of nucleotide sequences from the 5'-UTR of 24 porcine pestiviruses. The squares indicate the sequences of reference strains (Brecia, Alfort, Alfort/187, Switzerland 3/93/2, p97). Numbers above the nucleotide sequence corresponded to those of Alfort sequences. Dots show identity of the nucleotides.

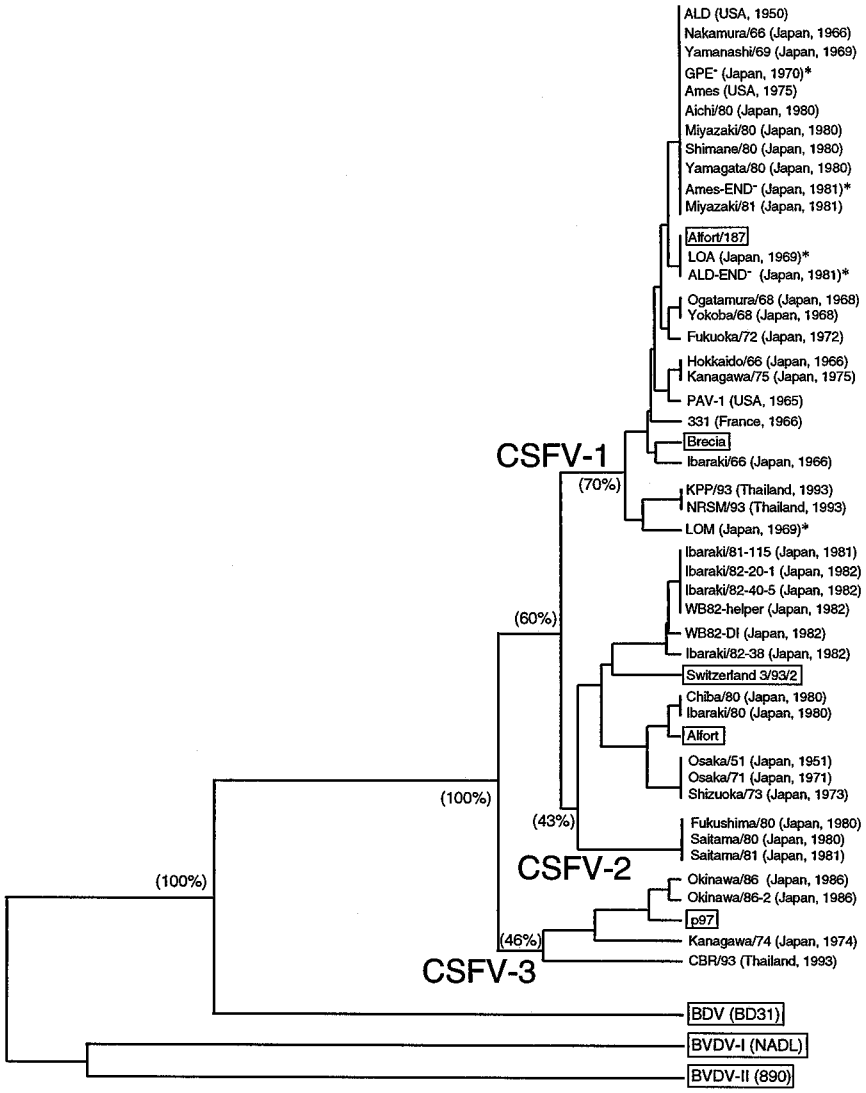

Fig. 18. Phylogenetic tree of porcine pestiviruses. Consensus phylogenetic tree were constructed by the UPGMA method and bootstrap test (n=200) from the sequence dataset of 5'-UTR. The length of the horizontal lines connecting one sequence to another is proportional to the estimated genetic distance between the sequences. Main bootstrap values (shown in percentage) are indicated in brackets. The squares indicate the reference strains (Brecia, Alfort, Alfort/187, Switzerland 3/93/2, p97, BD31, NADL, 890). The strains with an asterisk (*) were for the laboratory use, and no-asterisk strains are isolated from the filed.

Okinawa isolates were most related virus as reference strain p97. Sixteen field isolates of 1980~82, period of the latest biggest outbreaks of CSFV in Japan, were classified to CSFV-1 (Aichi/80, Shimane/80, Yamagata/80, Miyazaki/80 and Miyazaki/81) and CSFV-2 (Ibaraki/81-115, Ibaraki/82-20-1, Ibaraki/82-40-5, Ibaraki/82-38, WB82-helper, WB82-DI, Chiba/80, Ibaraki/80, Fukushima/80, Saitama/80 and Saitama/81).

Nucleotide sequencing and phylogenetic analysis of ruminant isolates

Nucleotide sequences of the 5'-UTR were determined by direct sequencing from RT-PCR products. The alignments of the nucleotide sequences obtained from the 31 isolates and reference strains are performed. Sequence alignment of 28 representative strains was shown in Fig. 19. In the 5'-UTR, homology between these isolates was from 73.8 to 100 percent. Especially, MS-1, SY-89 and V/FLL were the distinct variant from major isolates and have high identities with BVDV-II reference strain, 890 (MS-1; 91.9%, SY-89; 92.3% and V/FLL; 95.1%). The phylogenetic tree on Fig. 20 was constructed from the nucleotide sequence data, and all ruminant isolates were divided to the BVDV-I and BVDV-II. Twenty-eight strains assigned to the group BVDV-I were subdivided into two subgroups, BVDV-Ia (represented by the NADL strain) and Ib (represented by the Osloss strain). In the case of NM87-1, TK87-2 and 190 strains, both cytopathic and noncytopathic strains were isolated from each cattle and they show high homologies in each isolate. Two bovine isolates (MS-1, SY-89) and the cell-contaminated strain (V/FLL) isolated from ovine cell line were classified as BVDV-II. MS-1 and SY-89 were subdivided into the same cluster of BVDV-II.

RFLP analysis of amplified PCR products

From nucleotide sequences analysis of the porcine and ruminant pestiviruses, two restriction endonuclease sites were selected for the discrimination of CSFV, BVDV-I and BVDV-II genogroup. *Pst* I site (CTGCAG, positions in the genome of CSFV Alfort strain; 312-317) was conserved in CSFV and BVDV-I groups (Fig. 17 and Fig. 19). *Bgl* I site (GCCNNNNNGGC, positions in the genome of CSFV Alfort strain; 124-134) was conserved in only CSFV groups (Fig. 17 and Fig. 19). PCR products were digested with these two restriction endonucleases and observed the size of the digested PCR fragment (Fig. 21). All PCR products from CSFV group were digested with both

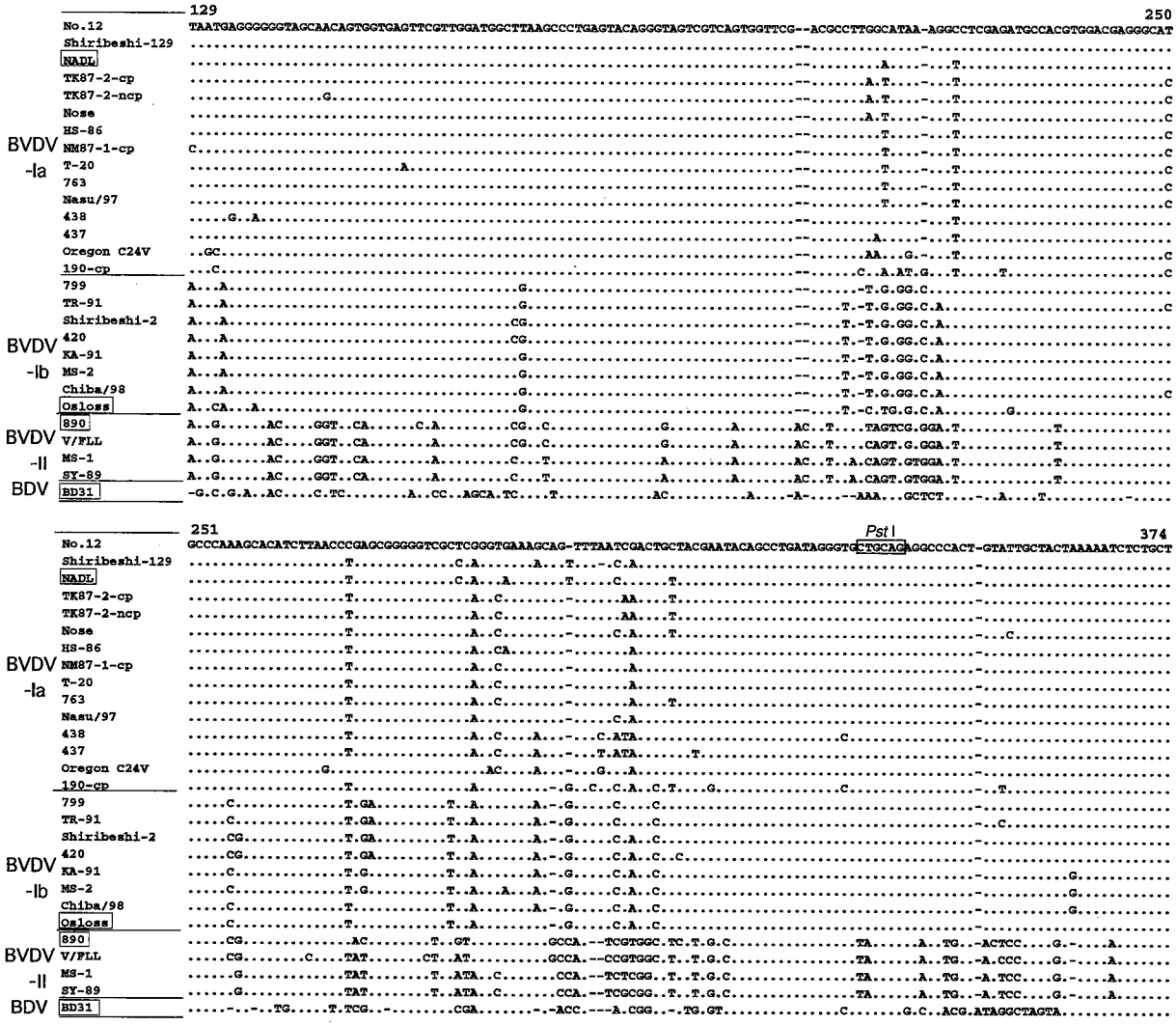

Fig. 19. Representative alignment of nucleotide sequences from the 5'-UTR of 28 ruminant pestiviruses. The squares indicate the sequences of reference strains (BD31, NADL, Osloss, 890). Numbers above the nucleotide sequence corresponded to those of NADL sequences. Dots show identity of the nucleotides.

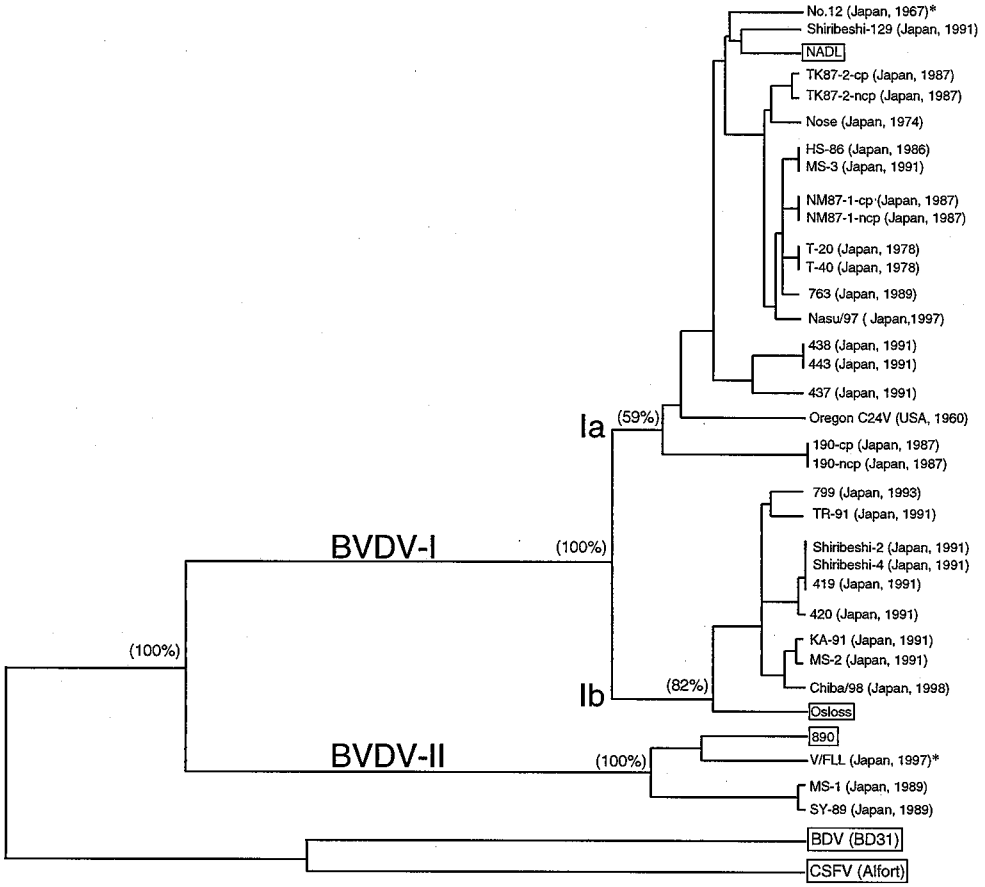

Fig. 20. Phylogenetic tree of ruminant pestiviruses. Consensus phylogenetic tree were constructed by the UPGMA method and bootstrap test (n=200) from the sequence dataset of 5'-UTR. The length of the horizontal lines connecting one sequence to another is proportional to the estimated genetic distance between the sequences. Main bootstrap values (shown in percentage) are indicated in brackets. The squares indicate the reference strains (Alfort, BD31, NADL, Osloss, 890). The strains with an asterisk (*) were for the laboratory use, and no-asterisk strains are isolated from the filed.

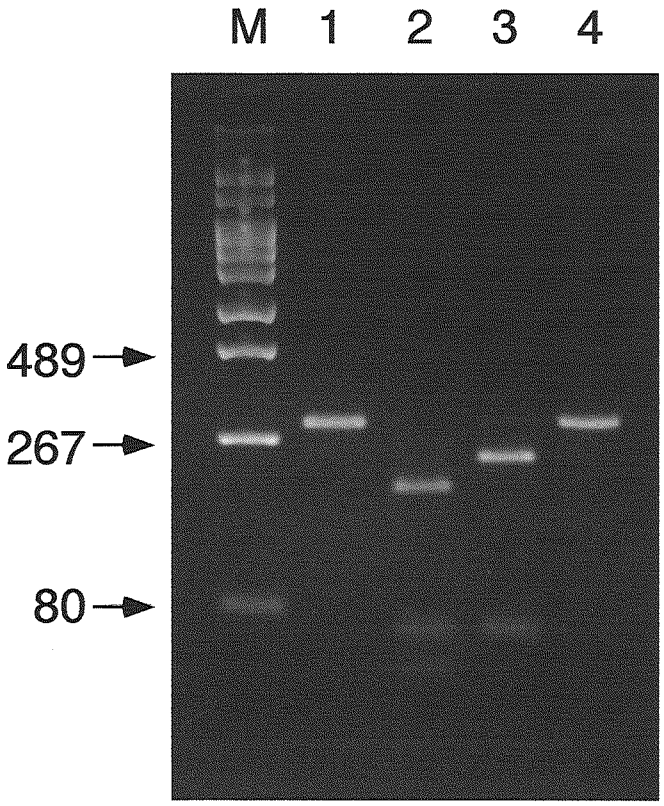

Fig. 21. RFLP analysis of PCR products of pestiviruses. PCR products amplified with primers (324 and 326) were digested with two restriction endonucleases (*Pst* I and *Bgl* I) and detected in 3% agarose gel electrophoresis. Lane 1, Control PCR products of CSFV Alfort strain without restriction endonucleases treatment. Lane 2, Digested PCR fragment of CSFV Alfort strain. Lane 3, Digested PCR fragment of BVDV-I No.12 strain. Lane 4, Digested PCR fragment of BVDV-II V/FLL strain. M, pHY marker.

enzymes, digested PCR fragment (approximately 190 bp) were detected in 3% agarose gel electrophoresis (Fig. 21, lane 2). In the case of the BVDV-I groups, all PCR product were digested with only *Pst* I, digested PCR fragment (approximately 230 bp) were detected (Fig. 21, lane 3). PCR products of BVDV-II were not digested with these enzymes, the size of the treated PCR products were same size as that of control PCR products without restriction endonucleases reaction (Fig. 21, lane 1 and lane 4).

Discussion

All pestiviruses examined in this present study were detected by the RT-PCR method. In our previous research (83), variant CSFV isolates like Kanagawa/74, Okinawa/86 and Thai isolates could not be detected by some specific primers, which were used for the detection of CSFV (81). Furthermore, some BVDV-II isolates were not detected by the primers of NS3 region, which is also highly conserved region like 5'-UTR in the pestiviruses. Our results confirm that these primers (324 and 326) as described by Vilcek et al. (75) are useful for detection of a broad range of pestiviruses, even in the case of the Japanese isolates. Genetic variability of porcine and ruminant pestiviruses was studied by comparative nucleotide sequence analysis of the 5'-UTR and Japanese pestivirus isolates were divided into three major genotypes: CSFV, BVDV-I, BVDV-II, but not BDV. In addition, rapid discrimination of CSFV and BVDV was developed easily by restriction endonuclease (*Bgl* I and *Pst* I) analysis by using same PCR fragments. These methods have an advantage for the eradication program of CSFV in Japan for the rapid characterization and surveillance of pestiviruses isolated from the field, especially unknown distinct variants.

Most of porcine isolates were divided into two major subgroups, CSFV-1 and CSFV-2. These main subgroups (CSFV-1 and CSFV-2) correlated with the genetic classification by Lowings et al. (35) (CSFV groups 1 and 2) and Vilcek et al. (77) (CSFV groups I and II). CSFV subgroup 1.1 (reference strain, Alfort/187) and 1.2 (reference strain, Brecia) by Lowings et al. (35) were classified to CSFV-1, subgroup 2.1 (reference strain, Switzerland 3/93/2) and subgroup 2.3 (reference strain, Alfort) to CSFV-2 in our division. Three isolates (LOM, KPP/93 and NRSM/93) of CSFV-1 were distinct from most of CSFV-1 isolates. Furthermore, they do not have a high homology with subgroup 2.2 by Lowings et al. (35) [e.g. Tübingen 1626/90 and Austria 128-90; described by Hofmann et al. (17)]. LOM strain was attenuated virus from a low virulence CSFV Japanese Miyagi strain (56). Thai isolates (KPP/93 and NRSM/93) were isolated from clonic pig or aborted fetus in the field. These three strains show CPE *in vitro*, but not END phenomenon, although most Japanese isolates from the field were ncp and show END phenomenon. Further investigation about the biotypes and genetic characterization is necessary for these isolates. Four CSFV strains

of subgroup CSFV-3 (Kanagawa/74, Okinawa/86, Okinawa/86-2 and CBR/93) were the most distinct variants and formed another, disparate new group with a reference strain p97. Strain p97 was also described as a distinct isolate from Alfort and Brecia strains (63). Furthermore, Kanagawa/74 strain was already reported as a variant virus by sequence comparison of E2, NS5B and 5'-UTR by Lowings et al. (35) and Vilcek et al. (77). The virulence of CSFV-3 is not special relationship between these isolates (Kanagawa/74, low virulence; Okinawa/86, Okinawa/86-2 and CBR/93, high virulence). Okinawa prefecture is the island of the southern part of Japan, close to the Taiwan main land. Complete genome analysis of these isolates and further genetic characterization of variants as these isolates are necessary to determine the origin, geographical relation of these distinct variants.

The outbreaks of CSFV were reduced by the use of live vaccine (GPE$^-$) since 1969, no outbreak was happened from 1976 to 1979 in Japan. However, numerous outbreaks were happened again all over the country from 1980 to 1982. From the antigenic analysis of these isolates, it was reported that several (at least two) kinds of CSFVs were circulating simultaneously in that period (57). Our genetic analysis was also confirmed that CSFV-1 and CSFV-2 were prevalent in Japan simultaneously in 1980~1982 since sixteen isolates were classified to several clusters. These results lead to the presumption that several genotypes of CSFV existed for four years in the field as small outbreaks or clonic pattern.

In the case of bovine isolates, most strains were classified as group BVDV-I and subdivided into two major subgroups, BVDV-Ia and BVDV-Ib. These main subgroups of BVDV-I correlated with the genetic classification of foreign strains by Ridpath et al. (52) and Baule et al. (1). Two bovine strains (MS-1, SY-89) isolated from persistently infected cattle at the same farm in Japan were shown to have high homology with reference strain 890 and were classified as the BVDV-II. Hemorrhagic syndrome of BVDV-II reported in North America was characterized by leukopenia, fever, diarrhea, thrombocytopenia, hemorrhaging and death (50). Respiratory disease and leukopenia were observed as a clinical symptom, cytopathic and noncytopathic strains were mixed in MS-1 isolate. In the case of the SY-89, fever, leukopenia and arthritis were observed and noncytopathic strain was isolated. Some isolates of BVDV-II were reported from cattle with mucosal disease or persistent infection in Japan (45). Our bovine isolates

(SY-89 and MS-1) and other Japanese isolates (KZ-91-CP, KZ-91-NCP, OY-89 and SW-90) were classified different clusters in BVDV-II group from Northern American BVDV-II (50), German BVDV-II (82) and other BVDV-II strains (14, 78) (data not shown). Further epidemiological surveillance of BVDV-II is necessary for the characterization of pathogenicity and genomic heterogeneity. The V/FLL strain which was isolated from an ovine fetal lung cells as contaminant, was also identified as BVDV-II based on sequence data. Sheep may be naturally infected not only with BDV, but also BVDV-I and BVDV-II (78), the origin of this virus may be due to the infection of sheep with BVDV-II in the field since no outbreak of BDV happened in Japan until now. Otherwise, BVDV-II was contaminated from calf serum used for the culture of FLL-YFT cells since the calf serum, which was prepared from the persistently infected cattle with BVDV has a high possibility of the existence of the virus (5).

The resulting consensus tree showed that the individual isolates and reference strains were segregated into major genogroups (CSFV, BVDV-I and BVDV-II and BDV) and further several clusters in each group. However, the bootstrap value of the clustering is not sufficiently because of the sequence identity of their 5'-NC, especially in the case of division of CSFV-1 and CSFV-2. Comparison of some other regions like E2, NS5B (35) and N^{pro} (2), which are variable regions of the viral genomes, would be needed for further detailed genetic characterization of each genotype. The secondary structure and the 5' terminus sequence analysis of 5'-UTR were performed (23), furthermore, a simple and practical method for genotyping on the basis of the palindromic nucleotide substitutions were reported at the secondary structural level in the 5'-UTR (15). Further analysis in 5'-UTR and other region would be get more in detail information for genetic diversity of our isolates.

Especially for porcine pestiviruses, newly developed FS-L3 cells were used for the propagation of the viruses. Infectious virus and/or inactivated viral RNA of BVDV were usually contained in FBS, which was prepared from the cattle persistently infected with BVDV. In addition, FBS contained the antibody against BVDV were not suitable for propagation of the pestiviruses. FBS, which is free from infectious BVDV, inactivated BVDV RNA and antibody against it, is necessary even for the RT-PCR experiment of pestivirus. Therefore, virus propagation in serum-free culture with FS-L3 cells was greatly useful for reliable preparation of virus stocks in RT-PCR experiment.

Establishment of the bovine cell lines in serum-free culture by the same procedure as FS-L3 will be more useful for propagation, virus assays and gene detection of BVDV.

Summary

The 5'-untranslated region (UTR) of 42 porcine and 31 ruminant pestiviruses was detected by reverse transcriptase-polymerase chain reaction (RT-PCR) method. The nucleotide sequence of 5'-UTR was determined by direct sequencing and phylogenetic analysis was performed from these nucleotide sequence data. Most porcine isolates were divided into two major subgroups, classical swine fever virus (CSFV) subgroup 1 (CSFV-1, represented by Brecia strain) and subgroup 2 (CSFV-2, represented by Alfort strain). However, the Japanese Kanagawa/74, Okinawa/86, Okinawa/86-2 and Thai CBR/93 strains were the most distinct variants and these were assigned to another new disparate subgroup, CSFV subgroup 3 (CSFV-3, represented by p97 strain). Most ruminant isolates were classified as the bovine viral diarrhea virus (BVDV) genotype-I (BVDV-I) and subdivided into two subgroups, BVDV-I subgroup a (BVDV-Ia, represented by the NADL strain) and BVDV-I subgroup b (BVDV-Ib, represented by the Osloss strain). Two bovine isolates (MS-1 and SY-89) and contaminated strain (V/FLL) in the ovine cell line were classified as BVDV genotype-II (BVDV-II) with the genetic characteristics. In addition, rapid discrimination of CSFV and BVDV was developed easily by restriction endonuclease (*Bgl* I and *Pst* I) analysis by using same PCR fragments. These data suggest that the detection, phylogenetic analysis and restriction fragment length polymorphism (RFLP) analysis of 5'-UTR are useful for the rapid characterization of field isolates.

Conclusion

A stable porcine kidney epithelial cell lines, FS-L3 and CPK-NS, were established and maintained in Eagle's minimum essential medium (MEM) containing 0.295% tryptose phosphate broth, 0.5% Bacto peptone and 10 mM N, N-Bis (2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) without any serum. The FS-L3 cells formed unique fluid-filled, multicellular three-dimensional domes on a single monolayer. The number of the domes increased markedly after further cultivation. It was also found that both cell lines were free from pestiviruses, furthermore FS-L3 cells were free from possible adventitious viruses and mycoplasmas. Clear cytopathic effect (CPE) was observed in FS-L3 and CPK-NS cells infected with exaltation of Newcastle disease virus (END) phenomenon-negative (END^-) strains of classical swine fever virus (CSFV). This CPE result in the disappearance of the unique fluid-filled multicellular domes on a single monolayer of FS-L3 cells. Chromosome condensation and DNA fragmentation, a marker for apoptosis, were detected in cells infected with END^- CSFV strains. By using this CPE as a marker of infection, virus assays of CSFV were established in FS-L3 and CPK-NS cells. The virus and neutralizing antibody titers determined in FS-L3 and CPK-NS cells show a high correlation with those measured by usual methods. These FS-L3 and CPK-NS cells have the great advantage that a clear CPE was caused by the infection with END^- CSFV strains and any sera are not necessary for the cell culture of virus assays.

To characterize the genetic relations of porcine and ruminant pestiviruses, 5'-untranslated region (UTR) of porcine and ruminant pestiviruses was detected by reverse transcriptase-polymerase chain reaction (RT-PCR) method and the nucleotide sequence was determined by direct sequencing. Phylogenetic analysis was performed from these nucleotide sequence data. Most porcine isolates were divided into two major subgroups, CSFV subgroup 1 (CSFV-1) and CSFV subgroup 2 (CSFV-2). However, the Japanese Kanagawa/74, Okinawa/86, Okinawa/86-2 and Thai CBR/93 strains were the most distinct variants and these were assigned to another new disparate subgroup, CSFV subgroup 3 (CSFV-3). Most ruminant isolates were classified as bovine viral diarrhea virus (BVDV) genotype-I (BVDV-I) and subdivided into two subgroups, BVDV-I subgroup a (BVDV-Ia) and BVDV-I subgroup b (BVDV-Ib). Two bovine isolates and

unknown pestivirus in the ovine cell line were classified as BVDV genotype-II (BVDV-II) with the genetic characteristics. In addition, rapid discrimination of CSFV and BVDV was developed easily by restriction endonuclease analysis by using same PCR fragments. The RT-PCR detection, phylogenetic analysis and restriction fragment length polymorphism (RFLP) analysis of 5'-UTR are useful for the rapid characterization of field isolates.

The FS-L3 cells have a great advantage that it was found free of possible adventitious contaminants and CSFV strains grow well in serum-free culture. Infectious virus and/or inactivated RNA of BVDV were usually contained in fetal bovine serum (FBS), which was prepared from the cattle infected with BVDV persistently. In addition, FBS contained the antibody against BVDV were not suitable for propagation of the pestiviruses. From these points, virus propagation in serum-free culture with FS-L3 cells was greatly useful to prepare reliable virus stocks for virus assays and RT-PCR experiments. Furthermore, it will be also useful for practical purposes as biological vaccine production. By using newly established FS-L3 and CPK-NS cells, simple and reliable titration methods of the virus and its antibody were developed. Furthermore, rapid genetic characterization of pestiviruses by RT-PCR, phylogenetic analysis and RFLP analysis were also investigated to determine the phylogenetic relationship among the various strains, especially discrimination of CSFV and other ruminant pestiviruses. These methods developed in this study will be more useful for diagnosis of CSFV, especially for epizootiological surveillance of CSFV in the eradication program.

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References

1) Baule, C., vanVuuren, M., Lowings, J. P. and Belak, S., 1997. Genetic heterogeneity of bovine viral diarrhoea viruses isolated in Southern Africa. *Virus Res.*, 52: 205-220.

2) Becher, P., Orlich, M., Shannon, A. D., Horner, G., Konig, M. and Thiel, H. J. 1997. Phylogenetic analysis of pestiviruses from domestic and wild ruminants. *J. Gen. Virol.*, 78: 1357-1366.

3) Bolin, S. R., Black, J. W., Frey, M. L., Katz, J. B., Ridpath J. F. and Roblin, R. O. 1994a. Detection of a cell line contaminated with hog cholera virus. *J. Am. Vet. Med. Assoc.*, 205: 742-744.

4) Bolin, S. R., Ridpath, J. F., Black, J., Macy, M. and Roblin, R. 1994b. Survey of cell lines in the American Type Culture Collection for bovine viral diarrhea virus. *J. Virol. Methods*, 48: 211-221.

5) Bolin, S. R. and Ridpath, J. F. 1998. Prevalence of bovine viral diarrhea virus genotypes and antibody against those viral genotypes in fetal bovine serum. *J. Vet. Diagn. Invest.*, 10: 135-139.

6) Bruschke, C. J. M., Hulst, M. M., Moormann, R. J. M., van Rijn, P. A. and van Oirschot, J. T. 1997. Glycoprotein E^{ms} of pestiviruses induces apoptosis in lymphocytes of several species. *J. Virol.*, 71: 6692-6696.

7) Carlsson, U. 1991. Border disease in sheep caused by transmission of virus from cattle persistently infected with bovine virus diarrhoea virus. *Vet. Rec.*, 128: 145-147.

8) Collett, M. S., Larson, R., Gold, C., Strick, D., Anderson, D. K. and Purchio, A. F. 1988. Molecular cloning and nucleotide sequence of the pestivirus bovine viral diarrhea virus. *Virology*, 165: 191-199.

9) Doyle, A., Griffiths, J. B. and Newell, D. G. 1995. In: *Cell and Tissue Culture: Laboratory procedures*, pp.9A: 1.4-1.10, John Wiley and Sons Inc., New York.

10) Edwards, S., Moennig, V. and Wensvoort, G. 1991. The development of an international reference panel of monoclonal antibodies for the differentiation of hog cholera virus from other pestiviruses. *Vet. Microbiol.*, 29: 101-108.

11) Edwards, S. and Sands, J. J. 1994. Evidence of circovirus infection in British pigs. *Vet. Rec.*, 134: 680-681.

12) Fukusho, A., Ogawa, N., Yamamoto, H., Sawada, M. and Sazawa, H. 1975. Interference method with vesicular stomatitis virus for detection of GPE⁻ strain of hog cholera virus. *Annu. Rep. Nat. Vet. Assay Lab.*, 12: 9-14.

13) Fukusho, A., Ogawa, N., Yamamoto, H., Sawada, M. and Sazawa, H. 1976. Reverse plaque formation by hog cholera virus of the GPE⁻ strain inducing heterologous interference. *Infect. Immun.*, 14: 332-336.

14) Harasawa, R. and Mizusawa, H. 1995. Demonstration and genotyping of pestivirus RNA from mammalian cell lines. *Microbiol Immunol.*, 39: 979-985.

15) Harasawa, R. and Giangaspero, M. 1998. A novel method for pestivirus genotyping based on palindromic nucleotide substitutions in the 5' - untranslated region. *J. Virol. Methods.*, 70: 225-230.

16) Hoff, H. S. and Donis, R. O. 1997. Induction of apoptosis and cleavage of poly (ADP-ribose) polymerase by cytopathic bovine viral diarrhea virus infection. *Virus Res.*, 49: 101-113.

17) Hofmann, M. A., Brechtbuhl, K. and Stauber, N. 1994. Rapid characterization of new pestivirus strains by direct sequencing of PCR-amplified cDNA from the 5' noncoding region. *Arch. Virol.*, 139: 217-229.

18) Hopert, A., Uphoff, C. C., Wirth, M., Hauser, H. and Drexler, H. G. 1993. Specificity and sensitivity of polymerase chain reaction (PCR) in comparison with other methods for the detection of mycoplasma contamination in cell lines. *J. Immunol. Methods*, 164: 91-100.

19) Hori, R., Yamamoto, K., Saito, H., Kohno, M. and Inui, K.-I. 1984. Effect of aminoglycoside antibiotics on cellular functions of kidney epithelial cell line (LLC-PK_1): A model system for aminoglycoside nephrotoxicity. *J. Pharmacol. Exp. Therap.*, 230: 742-748.

20) Ishikawa, K., Nagai, H., Katayama, K., Tsutsui, M., Tanabayashi, K., Takeuchi, K., Hishiyama, M., Saitoh, A., Takagi, M., Gotoh, K., Muramatsu, M. and Yamada, A. 1995. Comparison of the entire nucleotide and deduced amino acid sequences of the attenuated hog cholera vaccine strain GPE⁻ and the wild-type parental strain ALD. *Arch. Virol.*, 140: 1385-1391.

21) Kamijo, Y., Ohkuma, S. I., Shimizu, M. and Shimizu, Y. 1977. Differences in pathogenicity and antigenicity among hog cholera virus strains. *Natl. Inst. Anim.*

Health Q. (Tokyo), 17: 133-140.

22) Kärber, G. 1931. Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. *Arch. Exp. Pathol. Pharmacol.*, 162: 480.

23) Katayama, K., Kurihara, C., Fukushi, S., Hoshino, F. B., Ishikawa, K., Nagai, H., Ando, T. and Oya, A. 1995. Characterization of the hog cholera virus 5' terminus. *Virus Genes,* 10: 185-187.

24) Katsuta, H. and Takaoka, T. 1973. Cultivation of cells in protein- and lipid-free synthetic media. *Methods Cell Biol.*, 6: 1-42.

25) Katsuta, H. and Takaoka, T. 1976. Improved synthetic media suitable for tissue culture of various mammalian cells. *Methods Cell Biol:*, 14: 145-158.

26) Kawaguchi, M., Okigaki, T. and Wada, H. 1993. Studies of glomerular epithelial cells in culture I. Establishment and characterization. *Tiss. Cult. Res. Commun.*, 12: 13-22.

27) Komaniwa, H., Fukusho, A. and Shimizu, Y. 1981. Micro method for performing titration and neutralization test of hog cholera virus using established porcine kidney cell strain. *Natl. Inst. Anim. Health Q. (Jpn.)*, 21: 153-158.

28) Kosmidou, A., Ahl, R., Thiel, H. J. and Weiland, E. 1995. Differentiation of classical swine fever virus (CSFV) strains using monoclonal antibodies against structural glycoproteins. *Vet. Microbiol.*, 47: 111-118.

29) Kumagai, T., Shimizu, T. and Matsumoto, M. 1958. Detection of hog cholera virus by its effect on Newcastle disease virus in swine tissue culture. *Science*, 128: 366.

30) Kumagai, T., Shimizu, T., Ikeda, S. and Matsumoto, M. 1961. A new in vitro method (END) for detection and measurement of hog cholera virus and its antibody by means of effect of HC virus on Newcastle disease virus in swine tissue culture. I. Establishment of standard procedure. *J. Immunol.*, 87: 245- 256.

31) Lever, J. E. 1979. Induction of mammalian cell differentiation stimulates dome formation in a differentiated kidney epithelial cell line (MDCK). *Proc. Natl. Acad. Sci. USA*, 76: 1323-1327.

32) Levings, R. L. and Wessman, S. J. 1990. Bovine viral diarrhea virus contamination of nutrient serum, cell cultures and viral vaccines. *Dev. Biol. Stand.*, 75: 177-181.

33) Loan, R. W. and Storm, M. M. 1968. Propagation and transmission of hog cholera virus in nonporcine hosts. *Am. J. Vet. Res.*, 29: 807-811.

34) Loken, T., Krogsrud, J. and Bjerkas, I. 1991. Outbreaks of border disease in goats

induced by a pestivirus-contaminated orf vaccine, with virus transmission to sheep and cattle. *J. Comp. Pathol.*, 104: 195-209.

35) Lowings, P., Ibata, G., Needham, J. and Paton, D. 1996. Classical swine fever virus diversity and evolution. *J. Gen. Virol.*, 77: 1311-1321.

36) Matsumoto, M., Kumagai, T., Shimizu, T. and Ikeda, S. 1961. A new in vitro method (END) for detection and measurement of hog cholera virus and its antibody by means of effect of HC virus on Newcastle disease virus in swine tissue culture. II. Some characteristics of END method. *J. Immunol.*, 87: 257-268.

37) Meyers, G., Rumenapf, T. and Thiel, H. J. 1989. Molecular cloning and nucleotide sequence of the genome of hog cholera virus. *Virology*, 171: 555-567.

38) Meyers, G., Tautz, N., Dubovi, E. J. and Thiel, H. J. 1991. Viral cytopathogenicity correlated with integration of ubiquitin-coding sequences. *Virology*, 180: 602-616.

39) Meyers, G., Tautz, N., Stark, R., Brownlie, J., Dubovi, E. J., Collett, M. S. and Thiel, H. J. 1992. Rearrangement of viral sequences in cytopathogenic pestiviruses. *Virology*, 191: 368-386.

40) Meyers, G. and Thiel, H. J. 1995. Cytopathogenicity of classical swine fever virus caused by defective interfering particles. *J. Virol.*, 69: 3683-3689.

41) Meyers, G. and Thiel, H. J. 1996. Molecular characterization of Pestiviruses. In: *Advances in Virus Research*: 47, pp.53-118., Academic Press, Inc., San Diego.

42) Mittelholzer, C., Moser, C., Tratschin, J. D. and Hofmann, M. A. 1997. Generation of cytopathogenic subgenomic RNA of classical swine fever virus in persistently infected porcine cell lines. *Virus Res.*, 51: 125-137.

43) Moormann, R. J., Warmerdam, P. A., van der Meer, B., Schaaper, W. M., Wensvoort, G. and Hulst, M. M. 1990. Molecular cloning and nucleotide sequence of hog cholera virus strain Brescia and mapping of the genomic region encoding envelope protein E1. *Virology,* 177: 184-198.

44) Murphy, F. A., Fauquet, C. M., Bishop, D. H. L., Ghabrial, S. A., Jarvis, A. W., Martelli, G. P., Mayo, M. A. and Summers, M. D., eds. 1995. Flaviviridae, In: *Virus Taxonomy*, pp.415-427., Springer, Vienna.

45) Nagai, M., Sato, M., Nagano, H., Pang, H., Kong, X., Murakami, T., Ozawa, T. and Akashi, H. 1998. Nucleotide sequence homology to bovine viral diarrhea virus 2 (BVDV 2) in the 5' untranslated region of BVDVs from cattle with mucosal disease or

persistent infection in Japan. *Vet. Microbiol.*, 60: 271-276.

46) Nakamura, S., Sakamoto, K., Sakoda, Y., Shimazaki, T., Inoue, Y., Ogawa, N. and Fukusho, A. 1997. Variation from cytopathogenic biotype to non-cytopathogenic biotype is correlated with the deletion of cellular sequence from bovine viral diarrhea viruses. *J. Vet. Med. Sci.*, 59: 361-370.

47) Nishimori, T., Yamada, S. and Shimizu, M. 1996. Production of monoclonal antibodies against classical swine fever virus and their use for antigenic characterization of Japanese isolates. *J. Vet. Med. Sci.*, 58: 707-710.

48) Paton, D. J., Sands, J. J. and Edwards, S. 1994. Border disease virus: delineation by monoclonal antibodies. *Arch. Virol.*, 135: 241-252.

49) Paton, D. J., Sands, J. J., Lowings, J. P., Smith, J. E., Ibata, G. and Edwards, S. 1995. A proposed division of the pestivirus genus using monoclonal antibodies, supported by cross-neutralization assays and genetic sequencing. *Vet. Res.*, 26: 92-109.

50) Pellerin, C., van den Hurk, J., Lecomte, J. and Tijssen, P. 1994. Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities. *Virology,* 203: 260-268.

51) Renard, A., Schmetz, D., Guiot, C., Brown, S. S., Dagenais, L., Pastoret, P. P., Dina, D. and Martial, J. A. 1987. Molecular cloning of the bovine viral diarrhea virus genomic RNA. *Ann. Rech. Vet.*, 18: 121-125.

52) Ridpath, J. F., Bolin, S. R. and Dubovi, E. J. 1994. Segregation of bovine viral diarrhea virus into genotypes. *Virology,* 205: 66-74.

53) Ridpath, J. F. and Bolin, S. R. 1997. Comparison of the complete genomic sequence of the border disease virus, BD31, to other pestiviruses. *Virus Res.*, 50: 237-243.

54) Ridpath, J. F. and Bolin, S. R. 1998. Differentiation of types 1a, 1b and 2 bovine viral diarrhoea virus (BVDV) by PCR. *Mol. Cell. Probe,* 12: 101-106.

55) Ruggli, N., Tratschin, J. D., Mittelholzer, C. and Hofmann, M. A. 1996. Nucleotide sequence of classical swine fever virus strain Alfort/187 and transcription of infectious RNA from stably cloned full-length cDNA. *J. Virol.*, 70: 3478-3487.

56) Sato, U., Hanaki, T. and Nobuto, K. 1969. Attenuation of the hog cholera virus by continuous cell-virus propagation. 3. Growth interference of Newcastle disease virus

by attenuated hog cholera virus and its application to virus titration and the neutralization test. *Arch. Gesamte Virusforsch.*, 26: 1-10.

57) Shimizu, M. and Shimizu, Y. 1983. Properties of hog cholera viruses recently isolated in Japan. *Natl. Inst. Anim. Health Q. (Tokyo)*, 23: 103-104.

58) Shimizu, M. and Kumagai, T. 1989. Experimental infection of pregnant goats with swine fever virus. *Vet. Microbiol.*, 20: 207-214.

59) Shimizu, M., Watanabe, H., Satou, K. and Murakami, S. 1989. Antigenic diversity of bovine viral diarrhea-mucosal disease (BVD-MD) viruses recently isolated from persistently infected cattle and mucosal disease, and serologic survey on bovine sera using antigenically different BVD-MD viruses. *Jpn. J. Vet. Sci.*, 51: 1115-1122.

60) Shimizu, M., Yamada, S. and Nishimori, T. 1995. Cytocidal infection of hog cholera virus in porcine bone marrow stroma cell cultures. *Vet. Microbiol.*, 47: 395-400.

61) Shimizu, T., Kumagai, T., Ikeda, S. and Matsumoto, M. 1964. A new in vitro method (END) for detection and measurement of hog cholera virus and its antibody by means of effect of HC virus on Newcastle disease virus in swine tissue culture. III. END neutralization test. *Arch. Gesamte Virusforsch.*, 14: 215-226.

62) Shimizu, Y., Furuuchi, S., Kumagai, T. and Sasahara, J. 1970. A mutant of hog cholera virus inducing interference in swine testicle cultures. *Am. J. Vet. Res.*, 31: 1787-1794.

63) Shiu, J. S., Chang, M. H., Liu, S. T., Ho, W. C., Lai, S. S., Chang, T. J. and Chang, Y. S. 1996. Molecular cloning and nucleotide sequence determination of three envelope genes of classical swine fever virus Taiwan isolate p97. *Virus Res.*, 41: 173-178.

64) Sullivan, D. G. and Akkina, R. K. 1995. A nested polymerase chain reaction assay to differentiate pestiviruses. *Virus Res.*, 38: 231-239.

65) Tajima, T., Hironao, T., Kajikawa, T. and Kawamura, H. 1993. Application of enzyme-linked immunosorbent assay for the seroepizootiological survey of antibodies against porcine cytomegalovirus. *J. Vet. Med. Sci.*, 55: 421-424.

66) Takahashi, H., Awata, T. and Yasue, H. 1992. Characterization of swine short interspersed repetitive sequences. *Anim. Genet.*, 23: 443-448.

67) Takase-Yoden, S. and Watanabe, R. 1994. Accumulation of unintegrated viral DNA in rat glial cells infected with neurotropic Friend murine leukemia virus. *Virus Res.*,

32: 1-12.

68) Tautz, N., Thiel, H. J., Dubovi, E. J., and Meyers, G. 1994. Pathogenesis of mucosal disease: a cytopathogenic pestivirus generated by an internal deletion. *J. Virol.*, 68: 3289-3297.

69) Terpstra, C. and Wensvoort, G. 1988. Natural infections of pigs with bovine viral diarrhoea virus associated with signs resembling swine fever. *Res. Vet. Sci.*, 45: 137-142.

70) Terpstra, C. 1996. Classical swine fever. In: *Manual of Standards for Diagnostic Tests and Vaccines*, 3rd ed., pp.145-154., Office International Des Epizooties, Paris.

71) Tischer, I., Gelderblom, H., Vetterman, W. and Koch, M. A. 1982. A very small porcine virus with circular single-stranded DNA. *Nature (London)*, 295: 64-66.

72) Todaro, G. J., Benveniste, R. E., Lieber, M. M. and Sherr, C. J. 1974. Characterization of a type C virus released from the porcine cell line PK(15). *Virology*, 58: 65-74.

73) Tomono, Y., Moritoh, C., Yasuda, T. and Okigaki, T. 1995. Fatty acid composition of ganglioside GM3 of renal glomerular epithelial SGE1 cells during spontaneous dome formation in vitro. *Cell Struct. Funct.*, 20: 269-274.

74) van Orischot, J. T. 1994. Hog cholera virus, In: *Encyclopedia of Virology*, Vol.2, pp.649-654., Webster, R. G and Granoff, A., eds., Academic Press Inc., San Diego.

75) Vilcek, S., Herring, A. J., Herring, J. A., Nettleton, P. F., Lowings, J. P. and Paton, D. J. 1994. Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis. *Arch. Virol.*, 136: 309-323.

76) Vilcek, S. and Belak, S. 1996. Genetic identification of pestivirus strain Frijters as a border disease virus from pigs. *J. Virol. Methods,* 60: 103-108.

77) Vilcek, S., Stadejek, T., Ballagipordany, A., Lowings, J.P., Paton, D. J. and Belak, S. 1996. Genetic variability of classical swine fever virus. *Virus Res.*, 43: 137-147.

78) Vilcek, S., Nettleton, P. F., Paton, D. J. and Belak, S., 1997. Molecular characterization of ovine pestiviruses. *J. Gen. Virol.*, 78: 725-735.

79) Wensvoort, G. and Terpstra, C. 1988. Bovine viral diarrhea virus infections in piglets born to sows vaccinated against swine fever with contaminated vaccine. *Res.*

Vet. Sci., 45: 143-148.

80) Williams, P. D., Laska, D. A. and Hottendorf, G. H. 1987. Comparative toxicity of aminoglycoside antibiotics in cell cultures derived from human and pig kidney. *In Vitro Toxicol.*, 1: 23-32.

81) Wirz, B., Tratschin, J. D., Muller, H. K. and Mitchell, D. B. 1993. Detection of hog cholera virus and differentiation from other pestiviruses by polymerase chain reaction. *J. Clin. Microbiol.*, 31: 1148-1154.

82) Wolfmeyer, A., Wolf, G., Beer, M., Strube, W., Hehnen, H. R., Schmeer, N. and Kaaden, O. R. 1997. Genomic (5' UTR) and serological differences among German BVDV field isolates. *Arch. Virol.*, 142: 2049-2057.

83) Yamaguchi, O., Sakoda, Y., Sato, M., Nakamura, S., Fukusho, A. 1997. Gene detection and discrimination of pestivirus strains by RT-PCR and restriction endonuclease analysis. *J. Jpn. Vet. Med. Assoc.*, 50: 639-644.

84) Yasue, H., Takahashi, H., Awata, T. and Popescu, P. C. 1991. Uneven-distribution of short interspersed repetitive sequence, PRE-I, on swine chromosomes. *Cell Struct. Funct.*, 16: 475-479.

85) Zhang, G., Aldridge, S., Clarke, M. C. and McCauley, J. W. 1996. Cell death induced by cytopathic bovine viral diarrhoea virus is mediated by apoptosis. *J. Gen. Virol.*, 77: 1677-1681.

Summary in Japanese

和文要旨

豚コレラウイルス（CSFV）はフラビウイルス科、ペスチウイルス属の＋鎖RNAウイルスである。CSFVは同属の牛ウイルス性下痢ウイルス（BVDV）と共通抗原性を有するので、試験に用いる細胞の培養液に添加する牛血清は抗BVDV抗体陰性でなければならない。しかし、牛ウイルス性下痢病は世界中に蔓延しているため、抗BVDV抗体陰性血清の入手はきわめて難しい。また、BVDV持続感染牛から採取された血清には感染性BVDVを含むことが多いので、診断やワクチン製造のための細胞の培養に用いることはできない。また、このような血清には加熱非働化して感染性ウイルスを不活化しても、BVDV RNA断片は残存するので、誤った遺伝子検出成績を得るおそれがある。

ほとんどのCSFV株は、培養細胞に対して明らかな細胞変性効果（CPE）を示さない。そのため、ウイルスと抗体の検出には酵素抗体法、蛍光抗体法、END［ニューキャッスル病ウイルス（NDV）のCPEを増強させる］法および干渉法などが用いられていた。酵素抗体法と蛍光抗体法は抗CSFV特異抗体を用いてウイルス抗原を検出するもので、煩雑な作業を要する。また、END法および干渉法は強毒のCSFVやNDV、および海外悪性伝染病である水胞性口炎ウイルスや西部馬脳炎ウイルスなどをそれぞれ使用するので、ウイルス拡散を防止することができる施設において実施しなければならない。

本研究ではまず、無血清培地で増殖可能な豚腎臓由来株化細胞を樹立した。次にこれを用いて安全、簡便かつ信頼性の高い豚コレラ診断法を確立した。

1．無血清培地で増殖可能な細胞株の樹立とその性状解析

豚腎上皮由来の株化SK-L細胞およびCPK細胞を培養液中の血清濃度を徐々に減少させて継代した。継代を約５０代繰り返した結果、最終的にイーグルMEMに0.5% Bacto Peptone、0.295% Tryptose Phosphate Broth、10 mM N, N-Bis (2-hydroxyethyl)-2-aminoethanesulfonic acidのみを加えた無血清培養液で増殖可能な細胞株、FS-L3細胞およびCPK-NS細胞を樹立した。FS-L3細胞は継代３日目から単層形成後に本細胞特有なドームを形成し、この数は培養時間とともに増加した。さらにこのドームは、長期間培養すると球状の形態をとって培養液中に浮遊し、増殖した。これらの両細胞にはペスチウイルス属のすべてのウイルスの迷入がないことを確認した。さらにFS-L3細胞には、豚サイトメガロウイルス、豚レトロウイルス、豚サーコウイルス、マイコプラズマが存在しないことも確認した。

2．新しい豚コレラウイルスの感染価と中和抗体価の定量法の確立

FS-L3 細胞に CSFV ワクチン株（GPE⁻株）を感染させたところ、ウイルス接種３日後より CPE を認め、ウイルスの増殖に伴って FS-L3 細胞に特有な球状のドームが消失した。CPK-NS 細胞もまた、GPE⁻株の感染によって明瞭な CPE を示した。両細胞は END 現象を示さない（END⁻）すべての CSFV 株の感染により明瞭な CPE を示し、END 現象を示す（END⁺）CSFV 株の感染では CPE を示さなかった。この END⁻株の感染による CPE は、アポトーシス誘導の結果と想定された。

FS-L3 細胞におけるドームの消失と CPK-NS 細胞における明瞭な CPE をウイルス感染のマーカーとして、END⁻ CSFV 株の感染価定量法を確立した。また、このウイルス感染マーカーと豚コレラウイルス株間の干渉現象を利用して、単独では CPE を示さない END⁺ CSFV 株の感染価の定量法を確立した。さらに、GPE⁻株をマーカーウイルスとして用いた中和抗体価測定法を確立した。これらの方法で求めたウイルス感染価と中和抗体価は、従来法による値と高い相関を示した。

3．豚コレラウイルスと反芻動物のペスチウイルスの遺伝子解析による分類

RT-PCR 法により、豚ならびに反芻動物由来のペスチウイルスの 5'非翻訳領域のウイルス遺伝子を増幅し、PCR 産物を検出した。さらにこれらのウイルス遺伝子核酸の塩基配列を決定し、分子系統樹解析を行った。豚由来ペスチウイルス４２株はすべて CSFV グループに分類され、大半は２つのサブグループ (CSFV-1 と CSFV-2）に区分された。日本の分離株を含む４株は塩基配列が他の CSFV 株とは大きく異なり、新しいサブグループ（CSFV-3）に区分された。

反芻動物由来ペスチウイルス３１株はその大半が BVDV 遺伝子型 I（BVDV-I）に分類され、さらに２つのサブグループ（BVDV-Ia と BVDV-Ib）に分かれた。それ以外の反芻動物由来３株は BVDV 遺伝子型 II（BVDV-II）として分かれた。また、制限酵素を用いた PCR 産物の切断片長解析から CSFV、BVDV-I、BVDV-II の遺伝子グループを容易に区別することが可能となった。