FULL PAPER *Virology*

Identification and Isolation of Foot-and-Mouth Disease Virus from Primary Suspect Cases in Korea in 2000

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ABSTRACT. The Republic of Korea had been free from foot and mouth disease (FMD) since 1934, until a recent outbreak in 2000. From March to April 2000, a total of 15 FMD outbreaks due to the serotype O virus were recorded. Coincidental outbreaks of FMD in cattle or pigs by the serotype O virus were reported in the region, including Taiwan, China, Japan, Russia and Mongolia. In this report, the results of emergency investigations of FMD cases on a dairy farm located approximately 5-km from the demilitarized zone in Korea are described. The causative agent of the disease was identified as the FMD virus O by reverse transcription-polymerase chain reaction (RT-PCR) assays using primers derived from the 3D polymerase, internal ribosome entry site (IRES), 1D/2B regions, enzyme-linked immunosorbent assay (ELISA) for antigen detection and typing. Sequence data of the partial 1D/2B region obtained from vesicular fluid showed close similarity (98% sequence identity) to the Kinmen isolate of the FMD virus O in Taiwan. The causative virus was isolated using black goat fetal lung cells following propagation in unweaned mice.
KEY WORDS: foot and mouth disease, serotype O, virus isolation.

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Foot and mouth disease (FMD) is one of the most significant diseases of livestock because the occurrence of this disease can create severe economic loss for the livestock industry and can have a devastating impact on the international trade of animals and animal products. The causative agent of FMD is *Foot-and-mouth disease virus* (FMDV), a member of the genus *Aphthovirus*, family *Picornaviridae*. There are seven serotypes of FMDV, including O, A, C, Asia-1, South African Territory (SAT)-1, -2, and -3.

The Republic of Korea had been free from the disease since 1934, until a recent outbreak in 2000. From March to April 2000, 15 outbreaks of FMD by the serotype O virus were recorded. Coincidental outbreaks of FMD in cattle or pigs were reported in the region, including Taiwan, Japan, Russia and Mongolia [17], and the causative agents were determined and characterized in Taiwan and Japan [9, 10, 22].

In Korea, primary suspect cases on a dairy farm located in Paju City, Gyeonggi Province, about 5-km from the demilitarized zone (DMZ), were officially notified to the National Veterinary Research and Quarantine Service (NVRQS), Anyang, Korea, on 25 March 2000. The affected Holstein cows showed excessive salivation, anorexia, depression, lameness, vesicles and ulcers on the teats, mouth, tongue, or feet, and a sudden drop of milk production. An immediate laboratory investigation was performed using reverse transcription-polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assay (ELISA) for antigen detection and typing, ELISA for structural or nonstructural antibody detection, and transmission electron microscopy (TEM) to determine whether the animals were affected by FMDV. Following the initial laboratory findings, nucleotide sequences and cell culture isolates of the causative agent were obtained, and the etiology of the disease was confirmed as of 2 April 2000 [16].

Here, we report the genetic and antigenic identification, and cell culture isolation of FMDV from primary suspect cases of the 2000 spring FMD epidemic in Korea.

MATERIALS AND METHODS

Collection and transportation of clinical specimens: Vesicular fluid samples were collected from the unruptured teat vesicles of affected cattle using a 3-m*l* syringe. Saliva was collected in a 50-m*l* conical tube. Clinical specimens were packaged in a pre-chilled plastic box. The outside surface of the box was thoroughly cleaned and disinfected before leaving each farm. The specimen box was directly transported to the Maximum Containment Research Laboratory, Foreign Animal Disease Research Division, NVRQS, Anyang, Korea.

RNA isolation from clinical specimens: The guanidium isothiocyanate (GTC)/silica method was used for the isolation of RNA from clinical specimens [1]. Briefly, 0.9 m*l* of lysis buffer (5 M GTC, 0.05 M Tris, 25 mM EDTA, 2% Triton X-100, pH 6.4) was mixed with 0.1 m*l* of vesicular fluid or saliva. Size-fractionated silica suspension (40 μl) was added to the mixture. After incubation for 10 min at room temperature, the tube was centrifuged at × 12,000 *g* for 30 sec. The supernatant was poured into a 500-m*l* chemical waste container containing 60 g of NaOH. The silica-nucleic acid pellet was washed once with washing buffer (5 M GTC, 0.05 M Tris, pH 6.4), once with 70% ice-cold ethanol, and once with ice-cold acetone. After proper disposal of the acetone, the pellet was dried at 56°C for 10 min. The RNA was eluted with 0.1 m*l* of 0.1% diethyl pyrocarbonate

Table 1. FMDV-specific primer sequences

Primer	Orientation	Sequence* (5'→3')	Serotype specificity	Reference
3DPoF	forward	CCT ATG AGA ACA AGC GCA TC	All serotypes	Unpublished
3DPoR	reverse	CAA CTT CTC CTG TAT GGT CC	All serotypes	
IRES 2	forward	CCT CCT TGG TAA CAA GGA CCC	All serotypes	[6]
IRES 3	reverse	CCT TCT CAG ATC CCG AGT GT	All serotypes	
IRES 4	reverse	CCT ATT CAG GCG TAG AAG CTT	All serotypes	
2BF (P32)	forward	CAG ATG CAG GAG GAC ATG TC	All serotypes	[24]
2BR (P33)	reverse	AGC TTG TAC CAG GGT TTG GC	All serotypes	
O1DF (P38)	forward	GCT GCC TAC CTC CTT CAA	O	
A1DF (P87-P92)	forward	GTY ATT GAC CTY ATG CAV ACY CAC	A	
C1DF (P40)	forward	GTT TCT GCA CTT GAC AAC ACA	C	
As1DF (P74-P77)	forward	GAC ACS ACH CAR RAC CGC CG	Asia-1	

* Y=(C, T); V=(A, G, or C); S=(G or C); H=(A, T, or C); R=(A or G). The expected sizes of amplified products by primer pair combinations: 3DPoF-3DPoR: 422 bp; IRES 2-IRES 3: 197 bp; IRES 2-IRES 4: 251 bp; 2BF-2BR: 131 bp; O1DF-2BR: 402 bp; A1DF-2BR: 732 bp; C1DF-2BR: 596 bp; As1DF - 2BR: 292 bp.

(DEPC)-treated distilled water (DW) containing 2 U/m*l* of RNase inhibitor at 65°C for 5 min. One-tenth of the eluted RNA was used for cDNA synthesis.

RT-PCR and nucleotide sequencing: Different combinations of primers were used for the RT-PCR assay (Table 1). For genetic identification of FMDV, universal primer sequences derived from 3D polymerase (unpublished primer sequence), internal ribosome entry site (IRES) [6] and 2B [24] regions were used. For serotype identification, O, A, C and Asia-1 specific primers derived from 1D/2B regions were used [24]. All of the synthetic oligonucleotides were obtained from the Bioneer Corp., Daejeon, Korea.

Synthesis of cDNA was performed in a final volume of 48 μl/reaction composed of 1× PCR buffer (10 mM Tris, 50 mM KCl, pH 8.3), 1.25 mM $MgCl_2$, 12.5 pmol of reverse primers (3DPoR primer for 3D RT-PCR; IRES 4 primer for IRES RT-PCR; 2BR primer for 2B RT-PCR; 2BR primer for 1D/2B serotyping RT-PCR), 0.2 mM of dNTP mixture, 4 U RNase inhibitor and 20 U MMLV-derived SuperScript reverse transcriptase RNase H- (Gibco BRL, Grand Island, NY, U.S.A.), 250 μg/m*l* trehalose (Sigma Chemical Co., St. Louis, MO, U.S.A.), 25 μg/m*l* cresol red, and RNA from clinical specimens or positive reference RNA for O, A, C, and Asia-1 (kindly supplied by Dr. Michelle Remond, Agence Française de Sécurité Sanitaire des Aliments, Masons-Alfort, France). Reverse transcription was performed at 50°C for 30 min on a DNA thermocycler (Model 9600, Perkin Elmer, Foster City, CA, U.S.A.), followed by inactivation of reverse transcriptase at 95°C for 5 min.

For PCR amplification, 2 μl of PCR reaction mixtures containing 2.5 U AmpliTaq Gold (Perkin-Elmer, Foster City, CA) and 12.5 pmol of forward or paired primers (3DPoF primer for 3D RT-PCR; IRES 2 and IRES 3 primers for IRES RT-PCR; 2BF primer for 2B RT-PCR; serotype-specific forward primers for 1D/2B RT-PCR) were directly added to a cDNA reaction tube. This was followed by 1 cycle for *Taq* polymerase activation at 95°C for 10 min, 45 cycles for amplification reaction at 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, and 1 cycle for final extension at 72°C for 7 min. PCR products were analyzed in 2% 3:1 NuSieve agarose in 1X TBE buffer. The nucleotide sequences of the products were determined using an automated ABI PRISM 377 sequencer (Applied Biosystems Inc., Foster City, CA, U.S.A.), and nucleotide sequence identity was analyzed using the MegAlign program (DNASTAR Inc., Madison, WI, U.S.A.).

ELISA for antigen detection and typing: An indirect sandwich ELISA was performed for the detection and typing of the FMDV structural protein antigen using a kit purchased from Pirbright Laboratory (Institute for Animal Health, Pirbright, UK). Diluted samples (10%), including vesicular fluid or saliva, were subjected to the test according to the instructions provided with the kit. The result was expressed as a mean of corrected optical density (OD) value. For vesicular fluid, a value of over 0.1 (cut-off value) was considered to be a positive result, and the serotype was confirmed, according to the kit instruction. For the saliva samples, a cut-off value was tentatively designated by the mean + 2 standard deviations of corrected OD values obtained from the assay results of all saliva samples with FMDV negative control serum.

Animal inoculation: Four to five suckling Balb/c mice were inoculated intraperitoneally with 20 μl vesicular fluids or 10% suspension of clarified epithelial tissue homogenates. Control mice were inoculated with RPMI-1640 medium and maintained in a separate cage. The animals were examined twice daily for 7 days.

Virus isolation: Tertiary black goat fetal lung (BGFL) cells and established cell lines, including BHK-21 and IB-RS-2 cells, were used for virus isolation from clinical specimens. For virus isolation from tissue pools of an infected mouse, BGFL and BHK-21 cells were used. The BGFL cells were prepared by collagenase treatment, and were pas-

saged twice using the RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 100 μg/m*l* of gentamicin. The aliquots of the cells (2×10^7 cells/m*l*) were stored in liquid nitrogen until later use. One m*l* of 10% suspension of vesicular fluid or tissue homogenate was inoculated to the cell monolayers prepared in a 24-well microplate following clarification and filtration through 0.2 μm pores. The microplate was incubated with intermittent rocking at 37°C for 1 hr. The microplate wells were then washed twice with a serum-free medium and replenished with a maintenance medium containing 2% FBS. The microplate was maintained at 37°C with 5% CO_2 in a humidified chamber for 2 to 3 days. After three cycles of freezing and thawing, each culture harvest was blind-passaged twice further. A cytopathic effect (CPE) was examined daily, and an indirect immunofluorescence assay (IFA) was performed using positive reference serum specific for type O provided in FMD ELISA kits (Pirbright Laboratory) and monoclonal antibody specific for O1 Manisa (kindly supplied by Dr. Michelle Remond).

RESULTS

Genetic identification of FMDV by RT-PCR and sequencing: Clinical specimens, including vesicular fluids and saliva, were subjected to FMDV RT-PCR assays using 2 different sets of oligonucleotide primers of which sequences had originated from the conserved regions of the 3D polymerase and IRES genes of FMDV. All samples tested (vesicular fluids from Cow ID Nos. Paju-11 and -3341, saliva from Cow ID Nos. Paju-7 and -3348), as well as positive control RNA (O1 Manisa), resulted in the expected band sizes of amplified products by both the 3D and IRES RT-PCR assays, respectively (Fig. 1). RT-PCR products with the expected sizes of 251-bp and 197-bp were produced because 3 different primers (i.e., IRES 2, 3, and 4) were added in a single reaction tube from cDNA synthesis to PCR amplification steps.

A further RT-PCR assay was then performed to determine the serotype of FMDV using oligonucleotide primers of which sequences had originated from the 1D/2B gene regions. The vesicular fluid of Cow ID Nos. Paju-11 and -3341, as well as 4 different positive control RNA samples including O1 Manisa, A22 IRQ/24/64, C Noville, and Asia-1 CAM/9/80, were subjected to the assay. The overall results of the assay indicated that the causative agent would be FMDV type O, since amplified products with the expected size of 402-bp were observed in the reaction with the O type specific primer pair, O1DF-2BR, in both the clinical specimens and the reference RNA of O1 Manisa (Fig. 2). The same result was obtained for the vesicular fluid of Paju-11 (data not shown).

Next, we determined the nucleotide sequence of the amplified 402-bp products (1D/2B region) from Paju-3341. The partial 1D/2B sequences of Paju-3341 showed 97.9% identity to the O Kinmen strain of FMDV in Taiwan (GenBank accession no. AF167307, Lin *et al.*, unpublished).

Antigenic identification of FMDV by ELISA: Six samples of saliva and 1 sample of vesicular fluid from 6 suspected cases were tested for the presence of the serotype-specific antigen by an indirect sandwich ELISA; the results are summarized in Table 2. Three out of 7 clinical samples showed highly positive results for serotype O: saliva from Cow ID No. 12, saliva and vesicular fluid from Cow ID No. 3341

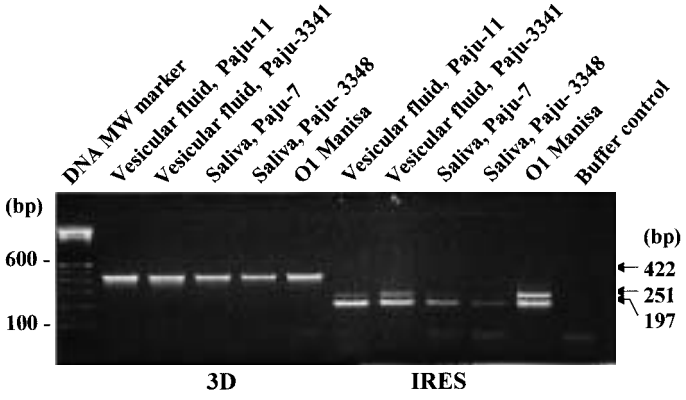


Fig. 1. Detection of FMDV genome by RT-PCR assay. Vesicular fluid and saliva were obtained from the suspected animals, Paju-11, -3341, -7, and -3348. The reference RNA derived from O1 Manisa culture supernatant was included as a positive control. The 3D region was amplified using a primer set, 3DPoF and 3DPoR (422-bp), following cDNA synthesis with the reverse primer 3DPoR. The IRES region was amplified using a primer set, IRES 2 and IRES 3 (197-bp), following cDNA synthesis with the reverse primer IRES 4. Since the undiluted cDNA reaction was used for PCR amplification, a primer pair combination of IRES 2 and IRES 4 produced an additional band with the expected size of 251-bp.

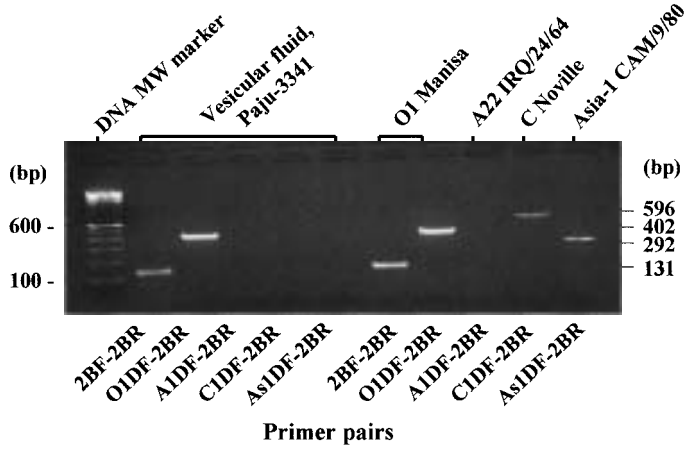


Fig. 2. Serotype identification of FMDV by RT-PCR assay. The 2B region was amplified using universal primers (2BF-2BR), and the 1D/2B regions using primer pair combinations specific for serotypes O (O1DF-2BR), A (A1DF-2BR), C (C1DF-2BR), and Asia-1 (As1DF-2BR). RNA isolated from the vesicular fluid of Paju-3341 and the positive reference RNA of O1 Manisa, A22 IRQ/24/64, C Noville, and Asia-1 CAM/9/80 were subjected simultaneously to the assay.

Table 2. FMDV antigen detection and serotyping by ELISA

Cow ID	Specimen	Serotype							
		O	A	C	SAT 1	SAT 2	SAT 3	Asia 1	NC
2	Saliva	0.11*	0.09	0.10	0.09	0.12	0.11	0.12	0.13
7	Saliva	0.11	0.09	0.10	0.12	0.11	0.10	0.13	0.15
11	Saliva	0.11	0.10	0.15	0.11	0.11	0.13	0.15	0.15
12	Saliva	0.58	0.04	0.04	0.07	0.04	0.01	0.10	0.07
3341	Saliva	0.65	-0.06	-0.02	-0.03	-0.02	-0.05	-0.01	0.02
3341	Vesicular fluid	0.80	-0.05	-0.02	0.00	-0.03	-0.05	0.06	0.00
3348	Saliva	0.04	0.04	0.08	0.09	0.07	0.13	0.17	0.05

* The numerical value indicates a mean of corrected OD values (a cut-off value: 0.22 for saliva; 0.1 for vesicular fluid). NC: negative control serum.

with the mean corrected OD values of 0.58, 0.65 and 0.80, respectively. The cut-off value of 0.1, while well suited for vesicular fluid, was not applicable for saliva. The saliva samples of Cow ID Nos. 2, 7, 11 and 3348 produced relatively higher mean corrected OD values for other serotypes, ranging from 0.11 to 0.17. However, these samples were not considered to be positive since the mean corrected OD values for negative control serum in 3 out of 4 saliva samples showed over 0.1 (0.13 to 0.15). From the values of 6 saliva samples for the negative control serum, the approximated cut-off value for saliva samples was 0.22.

Virus isolation: Using BHK-21 cells or IB-RS-2 cells, initial attempts to isolate the virus directly from clinical samples, such as vesicular fluid, saliva, epithelial tissue, or the buffy coat, were not successful. Instead, three out of five suckling Balb/c mice injected with vesicular fluid (Paju-3341) died after 3 days post-inoculation. Another group of 4 mice given saliva (Paju-12) showed 50% mortality (2/4) within 3 days. Eleven control mice given the culture medium did not show any abnormal signs or death for 2 wks. Secondary attempts were made to isolate the virus from the vesicular fluid of Paju-3341, and the tissue pools of a mouse inoculated with the vesicular fluid of Paju-3341, using BHK-21 and BGFL cells. CPE was observed only in the clarified mouse tissue homogenate passaged in BGFL cells three times. An IFA test with the type O-specific polyclonal antibody showed intense fluorescence in the cytoplasm of infected BGFL cells (Fig 3). The BGFL isolate was further passaged in IB-RS-2 cells and was tested for the presence of viral antigens in cells by an IFA test using the type O-specific monoclonal antibody. The infected IB-RS-2 cells showed positive fluorescence signals in the cytoplasm (Fig. 3).

DISCUSSION

This report is the first to demonstrate the identification and isolation of FMDV in Korea. Most of the diagnostic

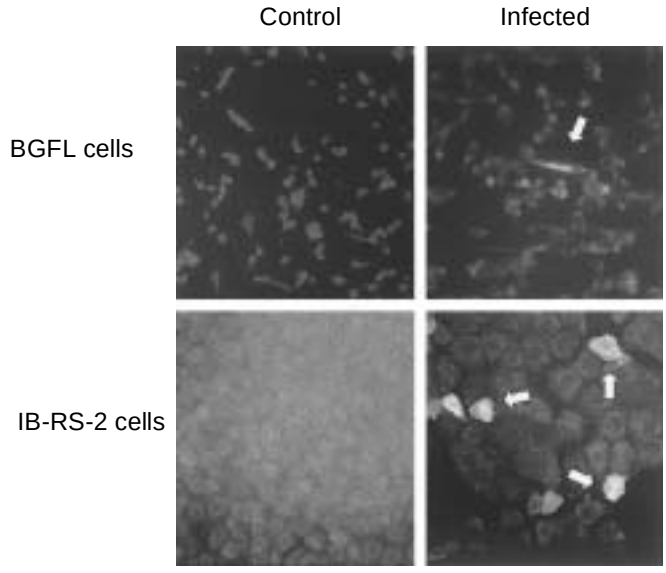


Fig. 3. Indirect immunofluorescence assay of cell culture isolate of FMDV. BGFL and IB-RS-2 cells were fixed at 6 hr post-inoculation with pre-chilled 1:3 acetone and methanol for 10 min at room temperature. An IFA test for BGFL cells was performed using a reference positive serum specific for serotype O included in the FMD ELISA kits. An IFA test for IB-RS-2 cells, a monoclonal antibody specific for O1 Manisa, was used. The photographs were acquired through 4 objective lens for BGFL cells and 10 objective lens for IB-RS-2 cells.

investigations of primary suspect cases for vesicular disease on dairy farms were performed from 25 March to 2 April 2000. From the initial laboratory findings on nucleic acid amplification assays using universal and serotype-specific primers, viral antigen and antibody detection and typing by ELISA, the FMD serotype O was highly suspicious. Our initial findings were subsequently confirmed by nucleotide sequence analysis of the 3D and 1D/2B regions, using clinical samples obtained from affected animals and the virus isolated from the cell culture. In the meantime, clinical specimens from the primary suspect cases were sent to the Pirbright Laboratory, OIE/FAO World Reference Laboratory (WRL) for FMD. On 4 April 2000, the WRLFMD confirmed the etiological agent as the FMDV serotype O, designated as O/SKR/1/2000 and O/SKR/2/2000 by nucleotide sequence analysis of the VP1 gene.

Detection of the FMDV genome by means of RT-PCR has been described previously [3, 4, 7, 8, 11-15, 18-21, 24]. In this study, several primer pairs derived from the 3D, IRES, and 1D/2B regions were chosen for the detection and typing of FMDV by RT-PCR. Primers derived from the 3D region were previously tested in our laboratory, using FMDV 3D cDNA obtained from the Foreign Animal Disease Diagnostic Laboratory (FADDL), Plum Island, New York, USA (unpublished result). Prior to the outbreak, we had an opportunity to test the specificity of IRES primer pairs using all of the 7 serotypes of FMDV and SVDV at the biosecurity facility in the AFSSA, Mason-Alfort, France (unpublished result). Primers derived from the 1D/2B regions had not been tested in our laboratory prior to the outbreak. In the present study, primer pairs of the 1D/2B regions except A1DF-2BR appeared to work well in the given condition of the amplification reaction (Fig. 2). The failure of the amplification reaction with A1DF-2BR did not affect the interpretation of the results in that the type O-specific primer pairs (O1DF-2BR) resulted in positives in both clinical samples and the reference RNA of O1 Manisa. For future reference, it is necessary to establish validated assays for all serotypes.

In our experience, the availability of the validated reliable nucleic acid amplification technique (NAT) for the detection and serotype differentiation of FMDV in a diagnostic laboratory of a FMD-free country (especially where the handling of viral genetic materials and live viruses is strictly restricted) is crucially important for early diagnosis and immediate action for disease control. Furthermore, in addition to the ability to demonstrate the presence of the FMDV genome, the ability to detect the genomes of other pathologic agents producing analogous symptoms would be preferable for an efficient diagnostic procedure [24].

In parallel with NAT, antigenic identification of the etiologic agent provided ample evidence for the disease, since our results of the emergency laboratory investigation using NAT and ELISA for antigen detection and typing showed good agreement. Although saliva samples produced relatively high background OD values for other serotypes, the

overall interpretation of the assay result confirmed the serotype O as the causative agent. Subsequent to the results obtained from NAT and ELISA, the result of nucleotide sequence analysis facilitated further confirmatory diagnosis and guided the choice of appropriate vaccines for the emergency situation and provided valuable information for epidemiological studies.

Currently, both ELISA and the virus neutralization (VN) test are used as the prescribed test methods for international trade. In order to establish the VN test, the cell culture isolate of FMDV is indispensable. Until the time of the outbreak, the VN test was not available because the handling of live FMDV was not allowed in our laboratory. In our previous experience with normal animal sera before the spring 2000 outbreak, liquid phase blocking (LPB)-ELISA for the serotype O resulted in approximately 0.5% false positives per animal. We had to trace the farms having positive reactors determined by LPB-ELISA, and conducted a retest to be certain of our result.

For the isolation of FMDV using cell culture systems, primary bovine thyroid cells and primary pig, calf or lamb kidney cells, as well as established cell lines, such as BHK-21 and IB-RS-2 cells, have traditionally been used [2, 5, 23]. At the time of the emergency, we were not ready for the routine practice in getting primary bovine thyroid cells. Hence, an attempt was made to use tertiary BGFL cells stored in liquid nitrogen for a year. In the previous experience with BGFL cells, it was found to be more efficient than primary black goat kidney cells in terms of the maintenance of cell monolayers. Previously we had used BGFL cells for the isolation of ovine parapoxvirus from scabs, and the result was successful (unpublished). In the present study, we questioned why we failed to isolate the virus directly from the vesicular fluid of Paju-3341 using BGFL cells. Later on, repeated experiments were done to isolate the virus directly from the vesicular fluid of Paju-3341, using primary porcine kidney cells as well as BGFL cells. However, for reasons unknown to us, we did not succeed in this endeavor.

During the 2000 spring FMD outbreaks in Korea, 6 isolates of FMDV were obtained from BGFL cell cultures. The isolation efficiency of BGFL cell cultures was much higher than that of the BHK-21 cell cultures, since we obtained only 2 isolates from BHK-21 cell cultures. BGFL cells with a low level of passage history, which can be recovered from liquid nitrogen, may provide a convenient way of FMDV isolation in a laboratory where primary cell cultivation is not a routine practice.

In conclusion, advances in molecular diagnostic techniques, such as RT-PCR and DNA sequencing, and the availability of ELISA, using inactivated viral antigens or recombinant proteins, allowed us to accomplish the rapid identification and serotyping of FMDV, and to effectively contain the disease as early as possible. In addition, the successful isolation of FMDV using BGFL cells enabled us to establish the VN test, which was found to be an extremely useful tool for serological surveillance. A further study on the molecular epidemiological characteristics of Korean FMD viruses in 2000 was described elsewhere in the same journal.

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