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Identification and antigenic site analysis of foot-and-mouth disease virus from pigs and cattle in Korea

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From May to June 2002, a total of 16 foot-and mouth disease (FMD) outbreaks due to the serotype O virus, Pan Asia strain, were recorded in Korea. The viruses were identified by antigen ELISA, RT-PCR and sequence analysis. The overall nucleotide sequence divergence of the VP1 region among the 4 isolates in 2002 was 0 to 1.4%, but between O/SKR/2002 and O/SKR/2000 isolates was 1.9-4.9%. Phylogenetic analysis with the some known strains from East Asian countries showed that the 4 Korean isolates in 2002 formed one distinct cluster, which different from clusters of Korean isolates in 2000, with in the same lineage of the ME-SA topotype strains. Deduced amino acid sequences around neutralizable antigenic site on VP1 site of O/SKR/2002 isolates were aligned and compared with other strains. At the antigenic site 1, the replacements of the critical amino acid residues at position 144 from V to L and at position 152 from A to T were observed in O/SKR/2002 viruses. For antigenic site 2 and 4, there were not significant variations in general. At the antigenic site 3, the substitutions of amino acid residues were present at positions 54 and 56 in O/SKR/ 2002 isolates and an alternative residue I at position 54 are observed only at the sequence of O/SKR/AS/2002 (cow) virus. And the substitution (L → P) of significant residue at position 144 was detected at the amino acid sequence of the O/SKR/2002 (cow) virus.

Key words: antigenic site, foot-and-mouth disease virus, FMD, O/SKR/2002

Introduction

Korea had been free from foot-and-mouth disease (FMD) for 66 years before the first FMD suspect case was reported to the Office International des Epizooties (OIE) in March 2000. A total of 15 cattle farms were affected by FMD virus (FMDV) in Korea in 2000 [9]. Molecular epidemiological studies on the viral protein (VP1) gene sequences of the causative viruses showed that they belong to a new lineage, Pan Asia strain, of type O that originated from India in 1990 [10]. Due to the rapid detection of FMDV, swift stamping-out and movement restriction, Korea regained the previous status of FMD free country where vaccination is not practised on 19 September 2001 from OIE [13]. However, Korea was again struck with FMD on 2 May 2002. Between 2 May and 23 June 2002, 16 domestic animal farms (15 pig farms and 1 dairy cattle farm) were diagnosed as FMDV positive using the reverse transcription-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) methods. To further confirm the diagnosis, we isolated FMDV on all outbreak farms diagnosed as FMDV positive.

The FMDV causes one of the most economically important vesicular diseases of livestock [1]. It has a single stranded positive sense RNA genome that undergoes very high levels of mutational change in nature and is composed of four structural proteins, VP1, VP2, VP3 and VP4 [11]. Three of these proteins, VP1, VP2 and VP3, contribute to the formation of five known antigenic sites of FMDV type O1 [3,4]. Three of these sites are located on VP1, one is on VP2, and the other is on VP3. The G-H loop and carboxy terminus of VP1 contribute to site 1, the critical residues located at the position of 144, 148,154 and 208. Amino acids at positions 31, 70-73, 75 and 77 of VP2 contribute to site 2. Site 3 is formed in part by residues 43 and 44 of the B-C loop of VP1. Only one critical residue, at position 58 of VP3, has so far been identified for site 4. The fifth site, characterized by an amino acid at position 149 of VP1, is probably formed by interaction of the VP1 loop region with other surface amino acids. Site 1 is linear and trypsin-sensitive, whereas all other identified sites are conformational and trypsin-resistant [5,6].

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A detailed knowledge of the molecular characteristics of the major antigenic sites of FMDV and the epidemiological study on the origin of outbreak will be helpful to the effective quarantine measures against reintroduction [8] and the development of specific diagnostic tests and protective vaccine.

In the present study, we have isolated FMDV from clinical samples of the 2002 FMD outbreaks in Korea and then analyzed full sequences of VP1 gene and antigenic sites of O/SKR/2002.

Materials and Methods

Diagnosis of FMD

National Veterinary and Quarantine Services (NVRQS) personnel had collected clinical samples between 2 May and 23 June 2002 at the infected sites. All clinical specimens were subjected to RT-PCR, virus isolation and antibody demonstration in the Maximum Containment Research Laboratory (MCRL) in NVRQS. RT-PCR assays using universal primers [13] and type O specific primers, ELISA for the detection of type O antigen and liquid phase blocking (LPB)-ELISA for the detection of type O antibody were performed. An antigen ELISA was performed for the detection of FMDV structural protein antigen using a kit purchased from Pirbright Laboratory (Institute for Animal Health, UK). Diluted samples (10%), including vesicular fluid or epithelial suspension, were subjected to the test according to the instructions provided with the kit. The LPB-ELISA was also carried out for antibody demonstration according to the instructions provided with the kit.

Identification of FMDV

Virus isolation and immunofluorescence assay (IFA) were also performed to identify viruses. IB-RS-2 cells, BHK-21 cells and black goat fetal lung (BGFL) cells were cultured at 37°C in cell culture flask with Eagle's essential medium (MEM) containing 3% fetal bovine serum (FBS). Suspected vesicular fluids and epithelial suspension were centrifuged at 2500 rpm for 5 minutes. Before the inoculation onto cells, the supernatant was filtrated through a 0.2 μm filter. Cells were washed with phosphate buffered saline (PBS) twice and filtered samples (0.2 ml/sample) were inoculated onto the cells and incubated at 37°C for 1 hour. After 1 hour, the cells were washed with PBS three times and fresh medium containing 2% FBS was added and then incubated for 3-4 days. If the cytopathic effect (CPE) was not observed, further passage of inoculated cells was performed at the fourth day and incubated for another 4-5 days.

IFA was peformed using monoclonal antibody (Mab) specific for FMDV O1 Manisa. Briefly, BHK-21 cells grown on cover slip were infected with FMDV isolates and then incubated for one day before being fixed with pre-chilled 1 : 3 acetone and methanol for 10 min at room temperature. The cells were reacted with dilutions of FMDV specific Mab for 1 hour at 37°C, followed by washing with PBS. FITC conjugated anti-mouse IgG (KPL, USA) was used for fluorescent studies.

RNA extraction

Total RNA was extracted from infected cell culture supernatant using a slightly modified guanidium isothiocynate (GTC)/Silica method [2]. About 0.9 ml of lysis buffer (5 M GTC, 0.05 M Tris, 25 mM EDTA, 2% Triton-X 100, pH 6.2-6.4) was mixed with 0.1 ml of infected cell culture supernatant and 40 μl of size-fractionated silica particle was added to the mixture. After a ten -minute incubation at room temperature, the tube was short centrifuged at 12,000 g. The supernatant was discarded and the silica-nucleic acid pellet was then washed with washing buffer (5M GTC, 0.05 M Tris, pH 6.2-6.4), 70% ethanol and acetone. After the disposal of the acetone, the pellet was dried at 55°C for 10 minutes. RNA was finally eluted with 0.1 ml of Rnase- and Dnase- free distilled water containing 2 U/ml of Rnase inhibitor at 65°C for 5 minutes. The eluted RNA was stored at –70°C until it was used.

RT-PCR and nucleotide sequencing

The Thermoscript Reverse Transcriptase (Invitrogen, CA, USA) and AmpliTaq Gold DNA polymerase (Perkin-Elmer, USA) were used to perform the reverse transcription (RT) and the subsequent PCR in a single reaction tube. The specific oligonucleotide primers used in the reactions are presented in Table 1. Reverse transcription was done at 50°C

Table 1. Primers used for the amplification and sequencing of FMDV genomes

Primers	Sequence 5'-3'	Direction	Location
F1F	tttgagctgcgtctgccacttga	Sense	VP3
F1R	gaagggcccagggttggactc	Antisense	2A
F2F	gctgcctacctccttcaa	Sense	VP1
F2R	agcttgtaccagggtttggc	Antisense	2B
F3F	cctggtctttccaggtctag	Sense	VP4
F3R	tcaccaagctgtgtgttccat	Antisense	VP3
F4F	tcaacaattactacatgcagc	Sense	VP3
F4R	gtgccactgtactgtgttgtagt	Antisense	VP1

Table 2. Sources of FMD type O virus isolates and nucleotide sequences examined in this study

Virus designation	Year	Species of origin	Gene Bank accession No.
O/JPN/2000	2000	Cattle	AB079061
O/MOG/2000	2000	Cattle	AJ318847
O/CHINA/99(TIBET)	1999	NK	AJ318830
O/RUSSIA/2000	2000	Pig	AJ318850
O/IRAQ/2000	2000	Cattle	AJ303499
O/IRAN/2000	2000	NK	AJ318840
O/SAUDI/2000	1999	NK	AJ318853
O/MANISA	1969	Cattle	AJ004658
O/TAW/97	1997	Pig	AF026168
O/TAW/YUN/97	1997	Pig	AF095874
O/TAW/PEN/97	1997	Pig	AJ294928
O/TAW/KM1/99	1999	Cow	AF162277
O/Kaufeuren/66	1966	NK	X00871
O/Campos	1958	NK	K01201
O/UK/3/2001	2002	Pig	AJ311720
O/KUW/1/96	1996	Cattle	AJ318847
O/VIT/17/99	1999	NK	AJ318858
O/SAR/15/2000	2000	Pig	AF306647
O/SKR/PJ/2000	2000	Cattle	AJ318854
O/SKR/CJ/2000	2000	Cattle	AF428246
O/SKR/BR/2000	2000	Cattle	NA
O/SKR/HS/2000	2000	Cattle	NA
O/SKR/AS(COW)/2002	2002	Cattle	NA
O/SKR/AS(PIG)/2002	2002	Pig	AY114146
O/SKR/JC/2002	2002	Pig	NA
O/SKR/PT/2002	2002	Pig	NA

NK: not known; NA: not available

Table 3. Summary of FMD outbreaks in the Republic of Korea, 2002

No.	Location	Date of Outreak	Species	Ag-ELISA[a]	LPB-ELISA	RT-PCR[a]	Virus isolation[b]
1	Anseong	2 May	Swine	+[a]	-	+	+
2	Jinchon	3 May	Swine	+	-	+	+
3	Yongin	10 May	Swine	+	-	+	+
4	Yongin	10 May	Swine	+	-	+	+
5	Aneong	10 May	Swine	+	-	+	+
6	Aneong	10 May	Swine	+	-	+	+
7	Yongin	12 May	Swine	+	-	+	+
8	Jinchon	12 May	Swine	+	+	+	-
9	Anseong	18 May	Swine	+	NT	+	+
10	Anseong	19 May	Swine	+	-	+	+
11	Yongin	19 May	Swine	+	-	+	+
12	Anseong	19 May	Swine	-	-	+	+
13	Pyoungtaek	2 June	Swine	+	+	+	+
14	Anseong	7 June	Bovine	+	-	+	+
15	Anseong	10 June	Swine	+	+	+	+
16	Anseong	23 June	Swine	+	+	+	+

[a] +: FMDV positive; -: FMDV negative
[b] +: FMDV isolated; FMDV not isolated

for 30 minutes on DNA Thermo Cycler 2400 (Perkin Elmer, USA) preheated at 50°C. This was followed by the inactivation of reverse transcriptase at 95°C for 5 minutes and PCR amplification was conducted to amplify viral genomic cDNA fragments by using Taq polymerase mix (Takara, Japan) with oligonucleotide primers. Subsequent PCR was performed for 30 cycles at 94°C for 30 seconds, 55°C for 60 seconds and 72°C for 60 seconds. The amplified PCR product was directly sequenced using an ABI prism 377 automated sequencer (Perkin Elmer, USA).

Sequencing analysis

Analysis on the similarity, divergence and phylogenic relationship of the nucleotide sequences was performed

Table 4. Percent nucleotide identity and divergence of complete VP1 sequence among the Korean isolates of FMDV

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		
1		98.6	100.	99.5	97.7	95.3	98.1	97.2	98.1	98.1	98.1	97.7	98.6	97.2	98.1	94.8	1	O/SKR/AS/2002 (pig)
2	1.4		98.6	98.6	96.7	94.4	97.2	96.2	97.2	97.2	97.2	96.7	97.7	96.2	97.2	93.9	2	O/SKR/AS/2002(cow)
3	0.0	1.4		99.5	97.7	95.3	98.1	97.2	98.1	98.1	98.1	97.7	98.6	97.2	98.1	94.8	3	O/SKR/PT/2002
4	0.5	1.4	0.5		97.2	94.8	97.7	96.7	97.7	97.7	97.7	97.2	98.1	96.7	97.7	94.4	4	O/SKR/CJ/2002
5	2.4	3.4	2.4	2.9		96.7	99.5	99.1	99.5	99.5	99.5	99.1	99.1	98.6	99.5	96.2	5	O/SKR/CJ/2000
6	4.9	5.9	4.9	5.4	3.4		97.2	95.8	97.2	97.2	97.2	96.7	96.7	96.2	97.2	93.0	6	O/SKR/PJ/2000
7	1.9	2.9	1.9	2.4	0.5	2.9		98.6	100.	100.	100.	99.5	99.5	99.1	100.	95.8	7	O/SKR/HS/2000
8	2.9	3.9	2.9	3.4	0.9	4.4	1.4		98.6	98.6	98.6	98.1	98.1	97.7	98.6	95.3	8	O/SKR/BR/2000
9	1.9	2.9	1.9	2.4	0.5	2.9	0.0	1.4		100.	100.	99.5	99.5	99.1	100.	95.8	9	O/JPN/2000
10	1.9	2.9	1.9	2.4	0.5	2.9	0.0	1.4	0.0		100.	99.5	99.5	99.1	100.	95.8	10	O/MOG/2000
11	1.9	2.9	1.9	2.4	0.5	2.9	0.0	1.4	0.0	0.0		99.5	99.5	99.1	100.	95.8	11	O/China(Tibet)/99
12	2.4	3.4	2.4	2.9	0.9	3.4	0.5	1.9	0.5	0.5	0.5		99.1	98.6	99.5	95.3	12	O/Russicia/2000
13	1.4	2.4	1.4	1.9	0.9	3.4	0.5	1.9	0.5	0.5	0.5	0.9		98.6	99.5	96.2	13	O/Iran/2000
14	2.9	3.9	2.9	3.4	1.4	3.9	0.9	2.4	0.9	0.9	0.9	1.4	1.4		99.1	94.8	14	O/Iraq/2000
15	1.9	2.9	1.9	2.4	0.5	2.9	0.0	1.4	0.0	0.0	0.0	0.5	0.5	0.9		95.8	15	O/Saudi/2000
16	5.4	6.4	5.4	5.9	3.9	7.4	4.4	4.9	4.4	4.4	4.4	4.9	3.9	5.4	4.4		16	O1manisa
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		

The nucleotide sequences of exotic type O strains were also included for comparison.

using DNASIS (Hitachi software, Japan) and MegAlign program (Dnastar, USA). The nucleotide sequences of VP1 gene for comparative analysis were obtained from the international DNA databank (Table 2).

Results

Diagnosis of FMD

In 2002, 35 suspect farms of FMD infection were reported to NVRQS. Of them, 16 cases in 4 counties in 2 provinces were diagnosed as FMD. Most of pigs and cattle affected by the O/SKR/2002 virus in the field had shown some clinical signs and were diagnosed as FMD by antigen ELISA and RT-PCR. Fifteen of 16 (93.7%) suspected samples were positive by FMDV O antigen ELISA and all samples were positive by RT-PCR. However, for serological test, most of the affected animals did not seroconvert showing only 25% (4 of 16) were positive by LPB-ELISA (Table 3).

Identification of FMDV

In the primary isolation from infected pigs, the viruses caused CPE on IB-RS-2 or BHK-21 within 9 days after inoculation. The virus isolated from infected cattle caused CPE on BGFL and BHK-21 cells between the sixth and ninth day of incubation. IFA using FMDV specific Mab was performed to confirm that the isolated virus was FMDV (Fig. 1). A total of 15 virus isolates was obtained in this study and the viruses were serotyped and analyzed by nucleotide sequencing and antigen detection ELISA kit in our lab and in the World Reference Laboratory for FMD at Pirbright (UK). The results indicated that the viruses are FMD serotype O and belong to Pan Asia strain, Middle East-South Asia (ME-SA) topotype. One of those virus was named as O/SKR/2002 and its whole sequence was submitted to GenBank (accession no. AY312588 and AY312589).

VP1 nucleotide and amino acid sequence analysis and comparison with deposited sequences

Complete nucleotide sequences (639 bp) of the VP1 of the Korean isolates were aligned to study the genetic relationship. Analyses of the VP1 nucleotide sequences on

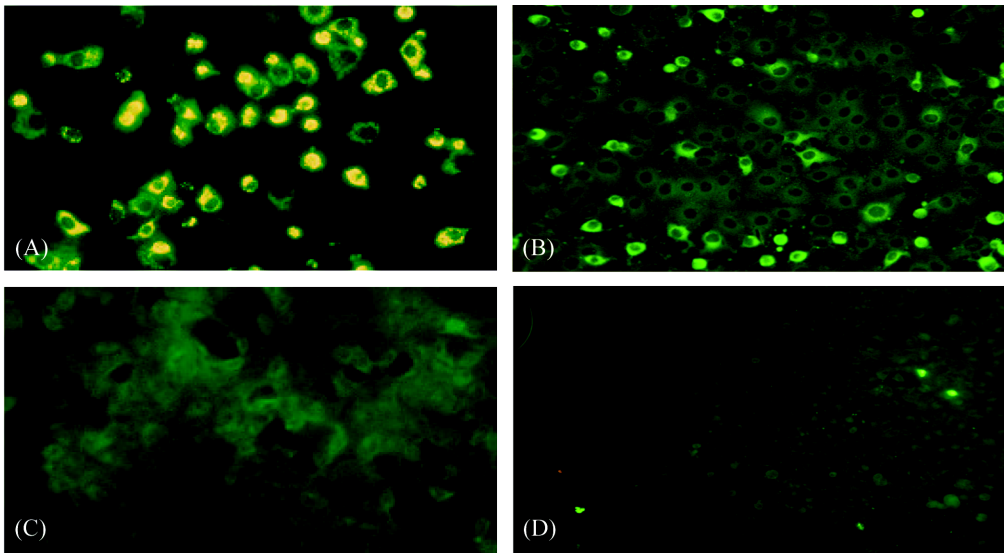

Fig. 1. Indirect immunofluorescence assay of cell culture isolate of FMDV. BHK-21 and IB-RS-2 cells were fixed at 12 hr post-inoculation with pre-chilled 1 : 3 aceton and methanol for 10 min at room temperature. IFA test was performed using a monoclonal antibody specific for O1 Manisa. A; Infected BHK-21 cells, B; Infected IB-RS-2 cells, C; Control BHK-21 cells, D; Control IB-RS-2 cells.

representative viruses isolated in 2002 showed that the viruses share 98.6-100% similarity. Also, the divergence between O/SKR/2002 and O/SKR/2000 isolates was 1.9-4.9%. The VP1 nucleotide sequences of the viruses that we obtained were compared with those of viral strains from the NCBI database using the BLAST program, which revealed the identity ranged from 94.6% to 98.1% (Table 4).

Analysis of known antigenic sites of O/SKR/2002

Deduced amino acid sequences around neutralizable antigenic site on VP1 site [3,4] from O/SKR/2002 isolates were aligned and compared with other strains. At the antigenic site 1, the replacements of the critical amino acid residues at position 144 from V to L and at position 152 from A to T were observed in O/SKR/2002 viruses. Also, when O/SKR/AS/2002 (cow: isolated from a cow) virus was compared with other O/SKR/AS/2002 (pig: isolated from pigs) viruses, it had an impressive mutation of proline from leucine at position 144. However, the rest of the critical residues at the antigenic site 1 were revealed to show no change. In the antigenic site 3, the substitutions of amino acid residues were present at positions 54 and 56 in O/SKR/2002 isolates and only the O/SKR/AS/2002 (cow) virus had an alternative residue I at position 54 (Fig. 2).

Amino acid replacements at positions 31, 70-73, 75 and 77 of VP2 known as the antigenic site 2 were not observed except one residue changed at position 79 (H to Y). Furthermore, variations were not observed at antigenic site 4 within VP3 region (Fig. 3).

Molecular epidemiological relationships

In the study of the epidemiological relationships of virus strains, the VP1 sequences (165 nts) of the O/SKR/2002, O/SKR/2000 viruses and several reference strains from the Gene Bank databank (NCBI) were aligned and analyzed (Fig. 4). Almost East Asian strains belong to the Middle East-South Asian (ME-SA) topotype, but two Taiwanese strains, O/TAW/YUN/97 and O/TAW/PEN/99, were related to the Cathay topotype [8]. From this analysis, 2002 Korean viral isolates show the greatest similarity at the nucleotide level with O/MOG/2000 strain. Korean viral isolates in 2000 were divided into two clusters, cluster 2 and cluster 3, respectively [9], but 2002 Korean isolates belong to cluster 1 different from them.

Discussion

Sixteen farms (15 pig and 1 cattle farms) were affected by FMDV serotype O between 2 May and 23 June 2002 in the Republic of Korea. Antigen ELISA and RT-PCR for the detection of viral antigen and LPB-ELISA for the measurement of FMD antibodies were performed.

We isolated 15 FMD viruses from pig and cattle herds located in Anseong, Yongin, Jincheon and Pyoungteak counties of two provinces. FMD virus isolated from swine clinical samples showed cytopathic effect in IB-RS-2 and BHK cells but not in BGFL cells. However, FMD virus isolated from bovine clinical samples showed CPE in BHK and BGFL cells but not in IB-RS-2 cells. The results suggest a possibility that FMD viruses from pigs and cattle could reproduce in cells from specific species respectively. In this context, the nucleotide and amino acid sequence of VP1 between FMDV isolates from pig and cattle were aligned and compared. Although no striking differences were

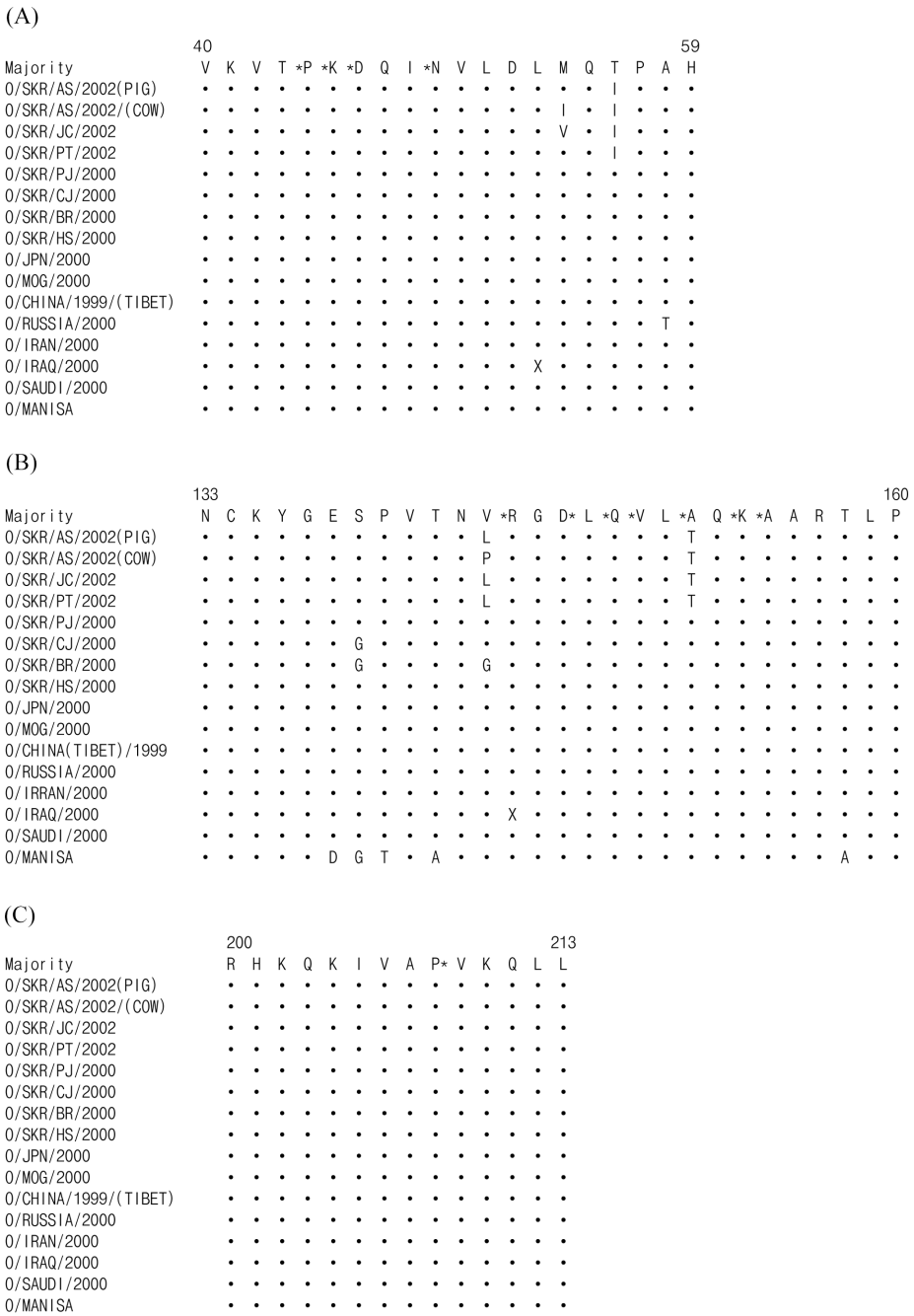

Fig. 2. Comparison of deduced amino acid residues at neutralizable antigenic sites of VP1 polypeptide from Korean viral isolates and several different O-Type FMD virus strains.

observed between these two strains, several replacements of amino acid residues at position 54, 144, and 174 in VP1 were shown. Since the amino acid at position 144 has been known to be the major antigenic site [4], further molecular analysis between these two strains will be of great interest to elucidate species-specific adaptation of FMDV.

The VP1 nucleotide sequence among O/SKR/2002 isolates was compared. Results showed divergence of 0-1.4%. These data indicated that O/SKR/2002 viruses are genetically homogeneous and originated from a single common source. In addition, the study in amino acid sequences of VP1 gene showed that the virus isolates were almost identical. However, when O/SKR/2002 isolates were compared with selected reference viruses, variations at residues 56, 96, 144, and 152 were observed. Thus, it is likely that a host immune response generated against O1/Manisa/Turkey/69 will afford some degree of protection against O/SKR/2002.

	VP2																			VP3									
	28						34		70										80	54									62
Majority	S	S	V	G	*V	T	Y		V	*T	*S	*D	*P	F	*G	R	*C	H	L	F	L	H	F	E	*G	D	V	P
O/SKR/AS/2002(PIG)	•	•	•	•	•	•	•		•	•	•	•	•	•	Y	•	•	•	•	•	•	•	•	•	•	•	•	•	•
O/SKR/PJ/2000	•	•	•	•	•	•	•		•	•	•	•	•	•	•	•	•	Y	•	•	•	•	•	•	•	•	•	•	•
O/JPN/2000	•	•	•	•	•	•	•		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	G	•	•	•	•
O/CHINA/1999(TIBET)	•	•	•	•	•	•	•		•	•	G	•	•	•	•	•	•	Y	•	•	•	•	•	•	•	•	•	•	•
O/TAW/97	•	•	•	•	•	•	•		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	D	•	•	•	•	•
O/Kaufbeuren/66	•	•	•	•	•	•	•		•	•	•	•	S	•	•	•	•	•	•	•	•	R	•	•	•	G	•	•	•
O/Campos	•	•	•	•	•	•	•		•	•	•	•	S	•	•	•	•	•	•	•	•	R	•	•	•	G	•	•	•
O/MANISA	•	•	•	•	•	•	•		•	•	•	•	•	•	•	•	•	•	•	•	•	R	•	•	•	G	•	•	•

* Indicated sequences of critical residues

• Represent consensus residue.

Fig. 3. Comparison of deduced amino acid residues at the antigenic sites VP2 and VP3 from O/SKR/2002 and several different O-Type FMD virus strains.

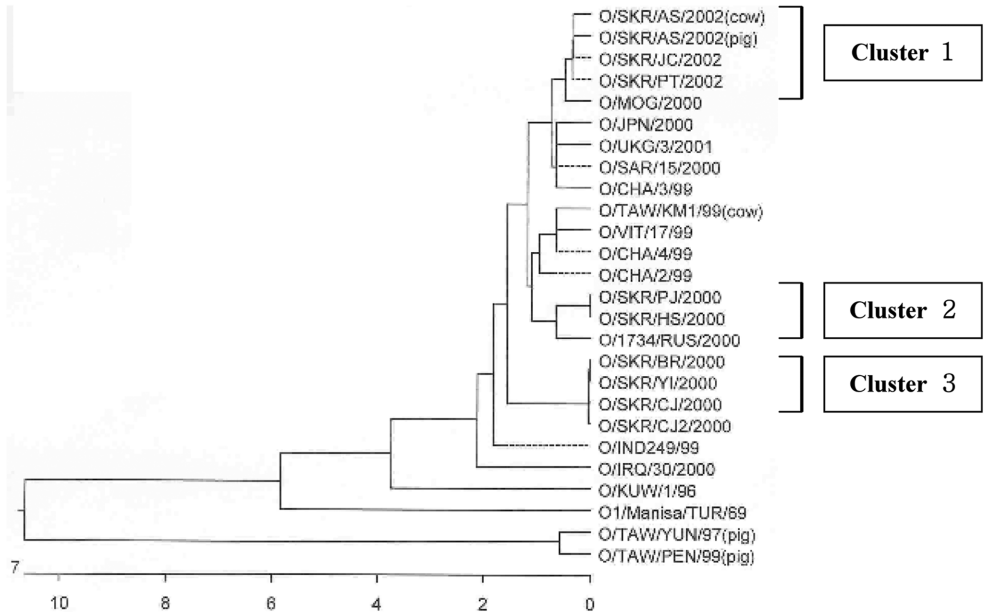

Fig. 4. Phylogenic tree showing genetic relationship among the FMDV type O strain isolates in Korea and other countries at partial VP1 gene.

The tissue culture adaptation of O1 Campos selects viruses which have positively charged Arg residue in viral protein VP3 at residue 56. These can bind with heparin and grow in CHO cells. These are virulent in cattle [7]. In contrast, a bovine-virulent O1 Campos virus had a His in place of this Arg and was unable to replicate on CHO cells [7]. O/SKR/2002 isolate had a histidine at position 56 in VP3, indicating the isolate was a virulent virus.

Phylogenetic analysis showed that the 2002 Korean viral isolates and the O/MOG/2000 were clustered in Cluster 1. This indicated that the O/SKR/2002 isolates and O/MOG/2000 were closely related to each other. However, the O/SKR/2000 isolates were clustered in other two cluster, Cluster 2 and Cluster 3, on this analysis. We assume that O/SKR/2002 isolates were introduced from outside the country rather than it being originated from O/SKR/2000.

In conclusion, advances in molecular diagnostic techniques allowed us to accomplish the rapid identification and to effectively control the disease as early as possible. Also, these studies and further extensive epidemiological analyses would be helpful to determine the exact source of the virus introduction.

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