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.

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-mL syringe. Saliva was collected in a 50-mL 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, Paju City, Gyeonggi Province.

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 mL 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 mL 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-mL 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 mL of 0.1% diethyl pyrocarbonate.
(DEPC)-treated distilled water (DW) containing 2 U/ml 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] sequences derived from 3D polymerase (unpublished sequence), FMDV negative control serum. For virus isolation from tissue pools of an infected animal, the animals were inoculated intraperitoneally with 20 μl vesicular fluids 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.

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.

**Table 1. FMDV-specific primer sequences**

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Unpublished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

*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.
saged twice using the RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 100 µg/ml of gentamicin. The aliquots of the cells (2 × 10⁷ cells/ml) were stored in liquid nitrogen until later use. One ml 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% CO₂ in a humidified chamber for 2 to 3 days. After three cycles of freezing and thawing, each culture harvest was blind-passage 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.
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
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 etiological 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
obtained from NAT and ELISA, the result of nucleotide isolation in a laboratory where primary cell cultivation is not possible. Liquid nitrogen, may provide a convenient way of FMDV isolation than that of the BHK-21 cell cultures, since we obtained toxoplasmosis, unknown to us, we did not succeed in this endeavor. Kidney cells as well as BGFL cells. However, for reasons unknown to us, we failed to isolate the virus directly from the vesicular fluid of Paju-3341 using BGFL cells. Later on, we questioned why we failed to isolate the virus in the vesicular fluid of Paju-3341, using primary porcine 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. Further study on the molecular epidemiological characteristics of Korean FMD viruses in 2000 was described elsewhere in the same journal.

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