Isolation of Foot-and-Mouth Disease Virus from Japanese Black Cattle in Miyazaki Prefecture, Japan, 2000

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ABSTRACT. Four outbreaks of foot-and-mouth disease (FMD) occurred from March to May 2000 in Miyazaki and Hokkaido Prefectures, Japan. FMD virus isolation was achieved by sampling probang materials from Japanese Black cattle in the third case found in Miyazaki Prefecture. The probang materials were inoculated to bovine kidney (BK) and bovine thyroid cell cultures. CPE was observed in the BK at two days post-inoculation. Specific amplified DNA segments for FMD virus (FMDV) were detected by reverse transcriptase polymerase chain reaction in the culture fluid. The FMDV was identified as type O by enzyme-linked immunosorbent assay (ELISA) for antigen detection and the nucleotide sequence encoding the VP1 was determined. This FMDV is a strain that is widespread in Pan-Asia and was designated as O/JPN/2000 by the World Reference Laboratory of the Pirbright Institute, England. This report marks the first isolation of FMDV in Japan.

KEY WORDS: FMDV, O/JPN/2000, VP1.

Foot-and-mouth disease (FMD) is recognized as a highly contagious viral disease that affects primarily cloven-hoofed animals. Clinical vesicular signs occur in the mouth, nose, hoofs and udder. This disease is classified under list A of animal diseases to be reported to the Office International des Epizooties (OIE) [1]. An outbreak of FMD causes enormous economical damage to the domestic animal industry and interrupts the international trade of animals and animal products. FMD is enzootic in Africa, Asia, and most of South America. North America, Australia, New Zealand, Japan, and Korea have all been free of the disease for many decades.

However, on 25 March 2000, an initial outbreak of FMD was suspected among Japanese Black beef cattle being reared in Miyazaki Prefecture, Japan [4, 5]. A private veterinarian, finding anorexia and nasal and mouth erosions spreading from some cattle to others, reported the symptoms to the Miyazaki Livestock Hygiene Service Center. Diagnostic materials were taken and sent to the Department of Exotic Diseases at the National Institute of Animal Health. A segment of FMD virus (FMDV) was detected by reverse transcriptase polymerase chain reaction (RT-PCR) in epithelial tissues on the lesions. An ELISA was performed on the serum samples from nine cattle and they showed high antibody titers to FMDV.

After this outbreak, farms epidemiologically related to the initial outbreak and farms that were using imported forages as feedstuff were subjected to nationwide serological surveillance [6, 7]. As a result of the surveillance, two cow-calf operations in Miyazaki Prefecture and one farm in Hokkaido Prefecture were found to be infected with FMDV [8–10]. FMDV isolation was achieved by sampling probang materials from Japanese Black cattle in Miyazaki Prefecture. These cattle possessed high antibody titers against FMDV by this antibody ELISA.

Primary bovine kidney (BK) cells and primary bovine thyroid (BTh) cells were cultured at 37°C in plastic tubes (Nunc Inc., U.S.A.) with Eagle’s essential medium (MEM) (Nissui, Tokyo) containing 5% bovine fetal serum (BFS). The probang samples were centrifuged at 2,000 rpm for 10 min. The supernatants were subjected to three tests, antigen detection ELISA, RT-PCR, and virus isolation. Before the virus isolation, FMDV-specific cDNAs were amplified from two probang fluids in 6 samples. The samples for FMDV isolation were then filtered through 0.45 nm membrane filters (Millipore, U.S.A.). Cells were washed with MEM containing 1.5% sodium carbonate. Filtered sample, 0.4 ml per sample, was inoculated into the cells at 37°C for 1 hr for absorption, and tubes were gently tilted at intervals of 15 to 20 min. The cells were washed twice with the medium. Then 1 ml of MEM with 2% BFS was added and revolved slowly in a 37°C incubator. The cytopathic effects (CPE) were observed in primary BK cells at two days post-inoculation. Among the BK cells, round cells dislodged from the surface of the plastic tubes (Fig. 1).

The virus culture fluids were subjected to RT-PCR for FMDV. Two primer sets (IRES1 and IRES4, FM8 and FM9) were used for RT-PCR. The details of the designed primer sets are shown in Fig. 2. RNAs were extracted from 200 µl of cell culture supernatants using a High Pure™ Viral RNA kit (Roche, Germany) according to the manufacturer’s protocol. The cDNA was synthesized from this RNA with a Titan™ One Tube RT-PCR System (Roche, Germany) and amplified 30 times according to the manufacturer’s protocol. PCR products were separated by electrophoresis in 2% agarose gel, stained with ethidium bromide, and visualized by UV light transillumination. Specific amplified DNA segments of FMDV were detected by the two primers indi-
 individually (Fig. 3).

The serotype of FMDV was determined by indirect sandwich ELISA for the detection of antigens of FMDV and swine vesicular disease virus (Pirbright Laboratory, UK), according to the given protocol [1]. The virus was identified as FMDV type O by this ELISA (Fig. 4). A PCR product of the VP1 gene amplified with the primers of 1C124 and 2B58 was directly sequenced by a Thermo Sequenase fluorescence-labeled primer cycle sequencing kit (Amersham Pharmacia, U.S.A.), and an ALFexpressII automatic sequencer (Pharmacia Biotech, Sweden). The primers for that sequence (1C609, 1D296 and 2A34) are shown in Fig. 2. The nucleotide sequence of the FMDV VP1 gene was 639bp long (GenBank accession number: AB050978). The sequence data was sent to the Pirbright Institute for the construction of the phylogenic tree, which proceeded by comparison of the sequence from 469 to 639bp with other type O strains.

In Japan, there has not been an FMD outbreak for 92 years. However, at the end of March 2000, an initial outbreak of FMD was suspected among Japanese Black beef cattle being reared in Miyazaki Prefecture, Japan [4, 5]. Coincidental outbreaks occurred in South Korea, Russia, and Mongolia from March to April 2000 [12].

In this particular outbreak, only Japanese Black cattle showed atypical clinical signs. They did not form any vesicles in the mouth, nostrils, or on the feet, but developed clinical signs of pyrexia, salivation, erosion, and ulcers in the mouth and nose. For these reasons, neither the epithelial tissues nor vesicular fluid could be sampled. For the emergency diagnosis of FMD, 1 g of epithelial tissue on the lesions were pooled from several cattle. The samples were subjected to antigen detection ELISA, the standard international method for diagnosis, and also to a complement fixation (CF) test, RT-PCR, and virus isolation. While the antigen ELISA, the CF test and the virus isolation gave negative results, the RT-PCR, by which the specific amplified DNA products for FMD were detected, was positive in the initial FMD suspected case. One of the reasons why only this RT-PCR method was useful for diagnosis at this time was the low quantity of the FMDV in the sample. Another reason was that the cattle had already produced a high titer of antibody against FMDV, thus the virus was masked by the antibody. Therefore, the masked FMDV could be detected by neither the antigen detection ELISA nor the CF test.

Regarding the virus isolation, the CPE was observed in primary BK cells at two days after inoculation, but not in Primary BThy cells. This CPE in BK cells was indistinct and it did not progress day by day. Only some of the round cells among the BK cells peeled off from the surface of the plastic tubes during the first passage. Clear CPE was observed after multiple passages with BK cells (Fig. 1), and the isolated virus was able to propagate well in the cell lines of IBRS-2 cells and the BHK cells. Two isolates from 2 probang fluids in 6 samples were identical on the serotype and on the sequence of the VP1 gene.

The sequence of the VP1 gene has already been determined. The present findings showed that the isolated virus was closely related to Asian strains of the virus referred to as the Pan Asia topotype [3]. The FMDV isolated in Japan was designated as O/JPN/2000 by the Pirbright Institute in England, the World Reference Laboratory (WRL) for FMD. Recently, WRL has also revealed that O/JPN/2000 is closely related to the UK strain which has caused recent pandemic outbreaks of FMD in the UK [2].

The success of the FMDV isolation rendered the emergency diagnosis more reliable. Thus, it was also possible to carry out virus neutralization testing. Recently performed animal experiments using this virus have provided many epidemiological solutions for the control of this disease in Japan.

At the end of September 2000, Japan was recognized as an FMD-free country, without vaccination by the OIE [11].

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ISOLATION OF FMDV IN JAPAN

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Fig. 2. The region of primers in the FMDV genome. Two primer sets (IRES1 (298–318) and IRES4 (651–671), FM8 (6774–6795) and FM9 (7395–7417)) were used for diagnosis of FMD and identification of the virus. VP1 gene exists on the 1D region. Primers of 1C124 and 2B58 were used for amplification, and primers (1C609, 1D296 and 2A34) for VP1 gene sequencing.

Fig. 3. Specific amplified DNA products for FMDV in 2% agarose gel. IRES (internal ribosome entry site) which exists on 5′NCR and the 3D gene which codes RNA polymerase are amplified with the primers of IRES1 and 4, and FM 8 and 9 respectively. M: Size Marker.
Fig. 4. Identification of the serotype of isolated FMDV by antigen-detection ELISA.