FULL PAPER

Pathology

Pathogenesis of the attenuated foot-and-mouth disease virus O/JPN/2000 in experimentally infected pigs

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ABSTRACT. We examined the pathogenesis of the attenuated foot-and-mouth disease virus (FMDV) O/JPN/2000 in pigs. The virus used in this study was passaged three times in primary bovine kidney (BK) cells and once in baby hamster kidney-21 (BHK-21) cells after isolation. A plaque assay demonstrated that this virus exhibited the small plaque (SP) phenotype. There was no clinical or histological evidence of vesicular lesions in pigs intraorally inoculated with 10^6 50% tissue culture infectious dose ($TCID_{50}$)/ml of the SP virus (SPV) of FMDV O/JPN/2000. Although fever was detected from 2 or 3 days post inoculation (dpi), there was no other prominent clinical sign up to 6 dpi. Virus shedding from saliva and nasal swab samples was not observed in any pigs inoculated with the SPV of FMDV O/JPN/2000. In the foot, mild lamellar degeneration of prickle cells in the upper layer of the stratum spinosum was histologically observed without development into vesicular or necrotic lesions. Immunohistochemical virus antigen- and terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling (TUNEL)-positive reactions observed in the foot at 1 dpi seemed to disappear after 3 and 6 dpi. Our findings suggest that the SPV of FMDV O/JPN/2000 had low pathogenicity against pigs by intraoral inoculation.

KEY WORDS: foot-and-mouth disease virus, histopathology, immunohistochemistry, pig

Foot-and-mouth disease (FMD) is a highly contagious and economically devastating transboundary viral disease that affects cloven-hooved animals such as cattle and pigs [1]. FMD is caused by the foot-and-mouth disease virus (FMDV) of the *Aphthovirus* genus and *Picornaviridae* family, and has seven distinct serotypes [1]. The FMDV serotypes O, A and Asia1 are currently epidemic in Southeast Asia (SEA) [15]. FMDV serotype O is the most prevalent and is divided into several topotypes according to molecular analysis of the structural protein VP1 [15].

In Japan, there have been two FMD outbreaks in the past 100 years, in 2000 and 2010. In 2010, FMD epidemics caused by the FMDV serotype O SEA topotype (FMDV O/JPN/2010) devastated the livestock industry in many Asian countries [15], including Japan [21]. In 2010, a total of 292 outbreaks were confirmed with about 290,000 culled animals, including vaccinated animals [21]. In contrast, in 2000, a total of 4 outbreaks with 740 culled animals were reported in Japan [23]. Further, only Japanese black cattle showed atypical clinical signs, characterized by no vesicle formation in the mouth, nostrils or feet but development of pyrexia, salivation, erosion and ulcers in the mouth and nose [23]. Holstein cattle exhibited no clinical signs, and sheep and goats were not susceptible [25]. The FMD epidemics in 2000 were caused by the FMDV serotype O Middle East-South Asia (ME-SA) topotype (FMDV O/JPN/2000) [14, 18, 23]. A previous study suggested that FMDV O/JPN/2000 has low pathogenesis against host animals [18]. This virus exhibits two plaque biotypes, small plaque (SP) and large plaque (LP), at the second passage stage after isolation [18]. The LP virus (LPV) shows high virulence, while the SP virus (SPV) shows low virulence against suckling mice [18]. The SPV of FMDV O/JPN/2000 is considered an attenuated virus, which may explain the low pathogenicity of FMDV O/JPN/2000 in host animals [18]. It is important to understand the mechanism governing attenuation and the pathogenicity of an attenuated virus against a host animal because improved knowledge of the functional genomics of FMDV may contribute to the development of next-generation countermeasures such as live-attenuated vaccines to improve FMD control and eradication [5]. However, there are currently no experimental studies to have examined the pathogenicity of the attenuated SPV of FMDV O/JPN/2000 in a host

animal.

Experimental models have been developed and validated by natural inoculation to study the infection dynamics of FMDV in pigs [27]. We previously examined the characteristics of FMD lesions in pigs infected with FMDV O/JPN/2010 by intraoral inoculation [29]. Characteristic lesions commonly observed in pigs with FMD arise following inoculation with 10^6 TCID$_{50}$/ml of FMDV O/JPN/2010 in pigs at 3 days post inoculation (dpi) [29].

Here, we describe the characteristics of FMD lesions in pigs infected with attenuated SPV of FMDV O/JPN/2000 (serotype O ME-SA topotype) by intraoral inoculation. We also compare the viral shedding, pathological findings and distribution of FMDV across the entire body of pigs due to attenuated SPV of FMDV O/JPN/2000 with those due to FMDV serotype O SEA topotype O/JPN/2010 published in our previous study [29].

MATERIALS AND METHODS

Virus

FMDV O/JPN/2000 was isolated using primary bovine kidney (BK) cells in Japan in 2000 [23] and passaged three times in BK cells and once in baby hamster kidney-21 (BHK-21) cells. The SPV used in this study was confirmed by plaque assay as described in our previous study [18]. The amino acid sequence of the capsid-coding region (VP1-VP4) [18] showed that the 56th amino acid in VP3 was an Arg (data not shown).

Experimental design

Pigs aged 4 weeks old were anesthetized with 2.0 mg of xylazine (Celactal, Bayer Yakuhin, Osaka, Japan) and 20 mg of pentobarbital (Somnopentyl, Kyoritsu Seiyaku, Tokyo, Japan) per kg before inoculation with FMDV. Six pigs (numbers 1–6) were intraorally inoculated with a syringe containing 1 ml of a 10^6 50% tissue culture infectious dose (TCID$_{50}$)/ml of the SPV of FMDV O/JPN/2000. Another pig (number 7) was necropsied before inoculation as a non-infected control.

Pigs 1 and 2 were examined clinically at 1 dpi. Pigs 3 and 4 were examined clinically at 3 dpi. Pigs 5 and 6 were examined clinically at 6 dpi. Clinical samples of sera, saliva and nasal swabs were collected daily from the pigs. Detailed methods of the collection and preparation of clinical samples used in this study were described previously [9]. Rectal temperatures were taken daily. After clinical assessment, the pigs were euthanized by an injection of sodium pentobarbital and subjected to necropsy examination. These methods were conducted in animal rooms and laboratories in a high-containment facility at our institute in Kodaira, Tokyo. The experiments were approved by the Animal Ethics Committee of the National Institute of Animal Health (NIAH), Japan (authorization number: 13-084).

Histopathology and immunohistochemistry

Tissue samples for microscopy examination were collected from the lip; tongue; soft palate; tonsil of the soft palate; lingual tonsil; oropharynx mucosa including the paraepiglottic tonsil; nasopharynx mucosa including the pharyngeal tonsil; larynx; trachea; lung; esophagus; stomach; ileum; rectum; liver; spleen; kidney; heart; pancreas; gallbladder; bladder; thyroid gland; thymus; mandibular lymph node; parotid lymph node; retropharyngeal lymph node; cervical lymph node; inguinal lymph node; popliteal lymph node; mesenteric lymph node; mandibular gland; parotid gland; diaphragm; brachial skeletal muscle; femoral skeletal muscle; and skin of the snout, shoulder, leg, coronet, bulb of the heel and accessory digit of each pig. Tissues were fixed in 10% neutral phosphate buffered formalin, processed according to routine procedures and embedded in paraffin wax. Sections were stained with hematoxylin and eosin (H&E).

For immunohistochemistry (IHC), dewaxed sections were processed using the Universal Immuno-enzyme Polymer method with a HISTFINE simple stain Max PO (M) kit (Nichirei, Tokyo, Japan) according to the manufacturer's instructions. Sections were labeled using a monoclonal antibody specific for FMDV serotype O (72C1, diluted 1 in 8; NIAH, Japan) [19, 29] and counterstained with hematoxylin.

Detection of cells with a DNA strand break associated with apoptosis

To identify cells with a DNA strand break (characteristic of apoptosis), paraffin wax-embedded sections of the tongue and skin of the coronet, bulb and snout from all pigs were subjected to terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling (TUNEL) [7] using an Apoptag Kit (Chemicon International, Tokyo, Japan) according to the manufacturer's instructions.

Cell culture, virus isolation and titration

Clinical samples and macerated tissue samples were subjected to virus isolation and titration. Tissue samples for virus isolation and titration were collected from the tongue, soft palate, tonsil of the soft palate, oropharynx mucosa including the paraepiglottic tonsil, nasopharynx mucosa including the pharyngeal tonsil, larynx, trachea, lung, esophagus, stomach, ileum, rectum, spleen, heart, thymus, mandibular lymph node, parotid lymph node, retropharyngeal lymph node, cervical lymph node, mandibular gland, parotid gland, brachial skeletal muscle, skin of the snout and skin of the coronet of the right forefoot of pigs.

LFBK-αvβ6 cells were used for virus isolation and titration as described previously [8]. Virus isolation was performed according to the OIE Manual as described previously [8, 9].

Table 1. Results of virus isolation, RT-PCR analysis, immunohistochemistry (IHC) and histology from tissue samples (virus isolation/RT-PCR/IHC/histological necrotic lesion)

Pig No.	1	2	3	4	5	6
dpi	1	1	3	3	6	6
Tongue	–/–/–/–	–/–/–/–	4.1/+/+/+	–/–/–/–	–/–/–/–	–/–/–/–
Tonsil of the soft palate	–/–/–/–	–/–/–/–	7.3/+/–/–	–/–/–/–	3.3/+/–/–	–/–/–/–
Soft palate	–/–/–/–	–/–/–/–	3.1/+/–/–	–/–/–/–	–/–/–/–	–/–/–/–
Oropharynx	–/–/–/–	–/–/–/–	3.8/+/–/–	–/–/–/–	–/–/–/–	–/–/–/–
Nasopharynx	–/–/–/–	–/–/–/–	2.6/+/–/–	–/–/–/–	–/–/–/–	–/–/–/–
Larynx	–/–/–/–	–/–/–/–	2.8/+/–/–	–/–/–/–	–/–/–/–	–/–/–/–
Trachea	–/–/–/–	–/–/–/–	3.6/+/–/–	–/–/–/–	–/–/–/–	–/–/–/–
Lung	–/–/–/–	–/–/–/–	2.6/+/–/–	–/–/–/–	–/–/–/–	–/–/–/–
Esophagus	–/–/–/–	–/–/–/–	2.8/+/–/–	–/–/–/–	–/–/–/–	–/–/–/–
Stomach	–/–/–/–	–/–/–/–	–/+/–/–	–/–/–/–	–/–/–/–	–/–/–/–
Ileum	–/–/–/–	–/–/–/–	2.6/+/–/–	–/–/–/–	–/–/–/–	–/–/–/–
Rectum	–/–/–/–	–/–/–/–	2.8/+/–/–	–/–/–/–	–/–/–/–	–/–/–/–
Heart	–/–/–/–	–/–/–/–	–/–/–/–	–/–/–/–	–/–/–/–	–/–/–/–
Spleen	–/–/–/–	–/–/–/–	3.3/+/–/–	–/–/–/–	–/–/–/–	–/–/–/–
Thymus	–/–/–/–	–/–/–/–	–/–/–/–	–/–/–/–	–/–/–/–	–/–/–/–
Mandibular LN	–/–/–/–	–/–/–/–	3.1/+/–/–	–/–/–/–	–/–/–/–	–/–/–/–
Parotid LN	–/–/–/–	–/–/–/–	–/+/–/–	–/–/–/–	–/–/–/–	–/–/–/–
Retropharyngeal LN	–/–/–/–	–/–/–/–	3.1/+/–/–	–/–/–/–	–/–/–/–	–/–/–/–
Cervical LN	–/–/–/–	–/–/–/–	–/+/–/–	–/–/–/–	–/–/–/–	–/–/–/–
Mandibular gland	–/–/–/–	–/–/–/–	3.3/+/–/–	–/–/–/–	–/–/–/–	–/–/–/–
Parotid gland	–/–/–/–	–/–/–/–	–/+/–/–	–/–/–/–	–/–/–/–	–/–/–/–
Brachial skeletal muscle	–/–/–/–	–/–/–/–	–/–/–/–	–/–/–/–	–/–/–/–	–/–/–/–
Skin of the snout	–/–/–/–	–/–/–/–	2.8/+/–/–	–/–/–/–	–/–/–/–	–/–/–/–
Skin of the foot	–/–/+/–	–/–/+/–	2.8/+/+/–	–/–/–/–	–/–/+/–	–/–/–/–

RT-PCR: reverse transcriptase-polymerase chain reaction; dpi: day(s) post inoculation; LN: lymph node; 4.1/+/+/+: 4.1 $\log_{10}TCID_{50}$/ml/PCR positive/IHC positive/histological necrotic lesion positive. Samples positive for virus isolation and RT-PCR are colored orange. Samples negative for virus isolation but positive for RT-PCR or IHC are colored yellow.

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

The same samples collected for virus isolation and titration were subjected to RNA extraction and RT-PCR. Viral RNAs were extracted from samples using a High Pure Viral RNA kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions and as described in our previous study [8, 9].

FMDV-specific genes were detected from the extracted RNAs by RT-PCR using FM8 and FM9 primers [23] as described in our previous study [8, 9].

RESULTS

Clinical signs and gross lesions

At 2 dpi, Pig 3 exhibited a fever (39.9°C), and at 3 dpi, three other pigs (Pigs 4–6) exhibited fevers (40.6 to 41.4°C). Fevers in Pigs 5 and 6 continued into 4 dpi (40.1 and 39.7°C, respectively), and their body temperatures returned to normal from 5 dpi. Salivation was not observed in any pigs. No vesicular lesions were clinically observed in any pigs. No gross lesions associated with FMDV infection were observed in any of the six pigs inoculated with the SPV of FMDV O/JPN/2000 at necropsy.

Histological and immunohistochemical examination

The distribution of histological vesicular or necrotic lesions and immunohistochemical viral antigen with virus and virus RNA in multi-systemic organs in the early stages of infection with the SPV of FMDV O/JPN/2000 are summarized in Table 1.

At 1 dpi, no prominent histological lesions were observed in the foot of the two examined pigs. Only mild lamellar degeneration of prickle cells with FMDV antigen and TUNEL-positive reaction was observed in the upper layer of the stratum spinosum in the skin of the coronet, bulb of the heel, interdigital space of the hooves and accessory digits in pigs (Fig. 1a–c). TUNEL-positive reactions in these areas were more prominent than those in non-infected control pigs. There were no histological lesions or FMDV antigen in the other organs including the tongue and heart in the two pigs examined at 1 dpi.

At 3 dpi, a thick layer of lamellar degeneration in the skin of the coronet, bulb of the heel, and interdigital space of the hooves and accessory digits (Figs. 1d and 2a) was observed in the two pigs examined. No FMDV antigen was detected in the skin of the foot (Fig. 1e) except for the skin of the accessory digits in one pig (Pig 3) (Fig. 2b). TUNEL-positive reaction in the skin was lower than that observed at 1 dpi (Fig. 1f). The skin of the accessory digits of Pig 3 developed ballooning degeneration of the
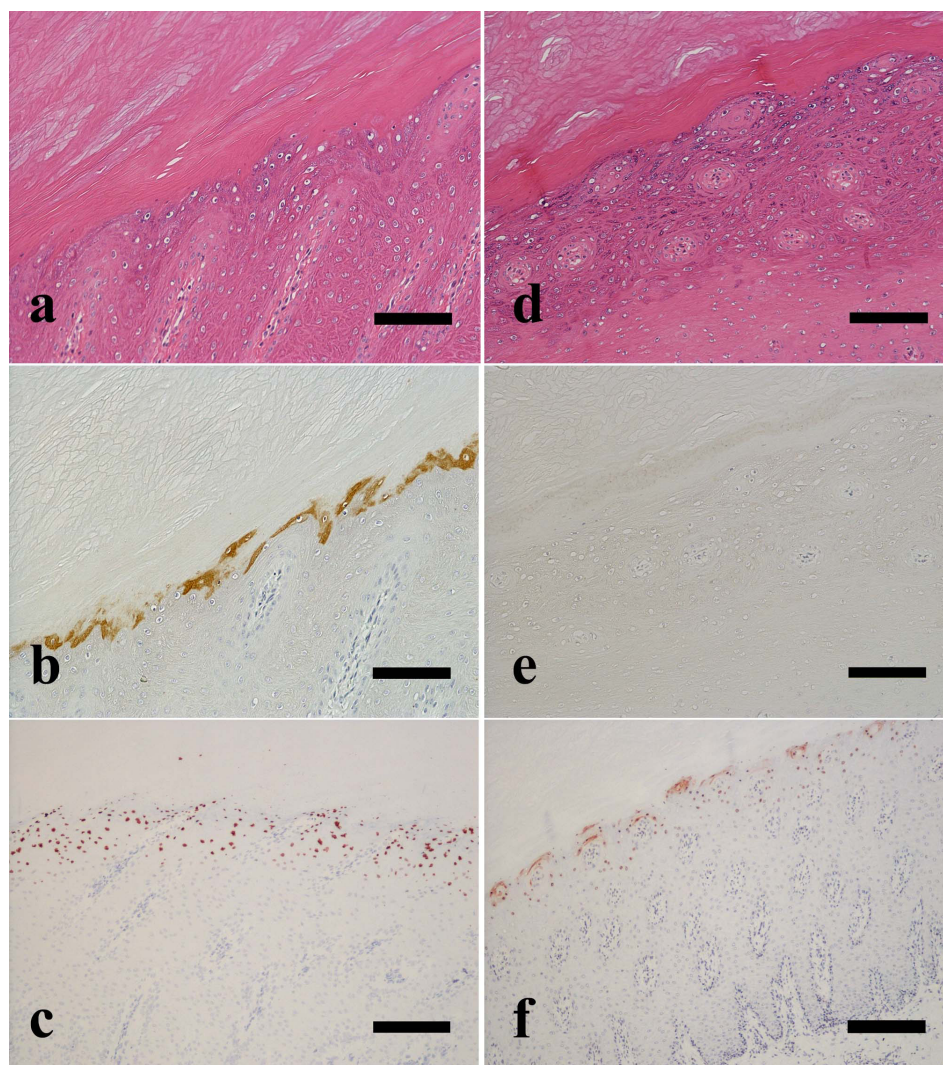
Fig. 1. Histology and immunohistochemical detection of the foot-and-mouth disease virus (FMDV) antigen and terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling (TUNEL) reaction in the skin of the bulb of the heel of pigs inoculated with FMDV O/JPN/2000. (a, b and c) Mild lamellar degeneration of prickle cells with FMDV antigen and TUNEL-positive reaction in the upper layer of the stratum spinosum in Pig 2 at 1 day post inoculation (dpi). a: hematoxylin and eosin (H&E), scale bar=100 μm; b: immunohistochemistry (IHC), scale bar=100 μm; c: TUNEL, scale bar=200 μm. (d, e and f) Lamellar degeneration of prickle cells thickened, but the FMDV antigen disappeared and the number of TUNEL-positive cells decreased and were concentrated in the surface layer of the stratum spinosum in Pig 3 at 3 dpi. d: H&E, scale bar=100 μm; e: IHC, scale bar=100 μm; f: TUNEL, scale bar=200 μm.

follicular epithelium associated with the presence of the viral antigen (Fig. 2a and 2b). However, no TUNEL-positive reaction was detected in serial sections of the ballooning degeneration lesion of the follicular epithelium (Fig. 2c). While no vesicular lesions were detected in the tongue of Pig 3, a small lesion comprising focal necrosis of prickle cells was found in the stratum spinosum in the dorsal epithelium along the midline of the tongue and was associated with the presence of the viral antigen. Within this lesion, epithelial cells in the basal layer were intact and did not contain the viral antigen. Serial sections stained with TUNEL showed that affected prickle cells in the stratum spinosum were weakly labeled. In contrast, epithelial cells in the basal layer were negative for TUNEL. In other areas of the tongue in Pig 3, viral antigen was detected only in prickle cells surrounding the papilla of the connective tissue in the upper layer of the stratum spinosum in the dorsal surface along the midline, without other histological abnormalities. Myositis in the tongue and myocarditis in the heart were not observed in any pigs. Severe bacterial suppurative bronchopneumonia was observed in the lungs of Pig 3. The lung lesion was not associated with FMDV infection and did not contain the viral antigen. No histological lesions or viral antigen were observed in the other organs examined in Pig 3. The other pig examined at 3 dpi did not exhibit any macroscopic or histological lesions or viral antigen.

At 6 dpi, a normally developed epidermal layer had formed under the layer of lamellar degeneration of prickle cells in the skin of the coronet, bulb of the heel, interdigital space of the hooves and accessory digits in the two pigs examined (Fig. 2d). Residual viral antigen was detected only in the lesion comprising the lamellar degeneration between the stratum corneum and normally
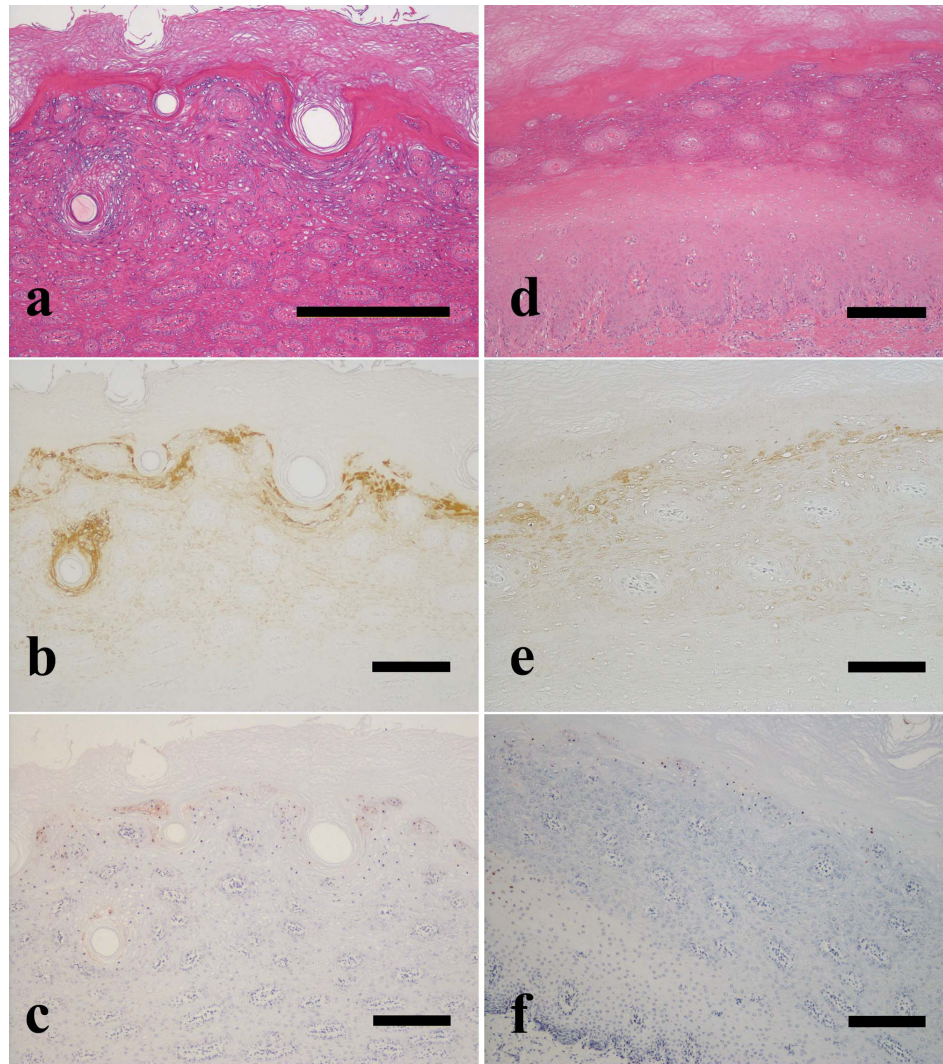
Fig. 2. Histology and immunohistochemical detection of the FMDV antigen and TUNEL reaction in the skin of the foot of pigs inoculated with FMDV O/JPN/2000. (a, b and c) Thickened lamellar degeneration of prickle cells with FMDV antigen was evident, and TUNEL-positive cells were few and had weaker signal in the skin around the accessory digit in Pig 3 at 3 dpi. a: H&E, scale bar=400 μm; b: IHC, scale bar=200 μm; c: TUNEL, scale bar=200 μm. (d, e and f) Thickened lamellar degeneration with residual FMDV antigen was observed between the stratum corneum and stratum spinosum in the skin at the junction between the coronet and bulb of the heel in Pig 5 at 6 dpi. There were very few TUNEL-positive cells, such that the tissue appeared comparable to control. d: H&E, scale bar=200 μm; e: IHC, scale bar=100 μm; e: TUNEL, scale bar=200 μm.

developed stratum spinosum in the epidermis at the junction between the coronet and bulb of the heel in one pig examined at 6 dpi (Pig 5) (Fig. 2e). In the other epidermis of the foot of Pig 5, no viral antigen was detected in the lesions with lamellar degeneration. There was no difference in TUNEL-positive labeling in the foot between the non-infected control pig and infected pigs at 6 dpi (Fig. 2f). No histological lesions or viral antigens were observed in the other organs of the two pigs examined at 6 dpi.

Virus isolation and RT-PCR

Virus and virus RNA was only detected in serum from Pig 3 at 2 and 3 dpi ($10^{4.3}$ TCID$_{50}$/m*l* and $10^{7.8}$ TCID$_{50}$/m*l*, respectively). Virus isolation and RT-PCR analysis were negative for the other clinical samples examined.

Virus isolation and RT-PCR analysis of tissue samples are summarized in Table 1. At 1 dpi, virus isolation and RT-PCR analysis were negative for all tissue samples from the two pigs examined (Table 1). At 3 dpi, virus isolation and RT-PCR analysis were positive from multi-systemic organs from Pig 3, which had severe bronchopneumonia (Table 1). In contrast, virus and virus RNA were not detected in any tissue samples from Pig 4. At 6 dpi, virus isolation and RT-PCR analysis was only positive in the tonsil of the soft palate from Pig 5 (Table 1).

DISCUSSION

There were no clinical or histological vesicular lesions in pigs intraorally inoculated with 10^6 TCID$_{50}$/ml of the SPV of FMDV O/JPN/2000. Fever was observed after 2 or 3 dpi; however, no other prominent clinical signs were apparent up to 6 dpi. Virus shedding from saliva or nasal swab samples was not observed in any pigs inoculated with the SPV of FMDV O/JPN/2000. In the foot, only mild lamellar degeneration of prickle cells in the upper layer of the stratum spinosum was observed following inoculation with the SPV of FMDV O/JPN/2000, and did not develop into vesicular or necrotic lesions. Virus antigen in the foot seemed to disappear after 3 dpi and residual antigen was only observed in a restricted area of the foot of one pig at 6 dpi.

Previously, we reported that characteristic vesicular lesions commonly observed in pigs with FMD [3, 30] arise following intraoral inoculation with 10^6 TCID$_{50}$/ml of FMDV O/JPN/2010 [29]. We also confirmed virus shedding from saliva and nasal swab samples from all six pigs inoculated with FMDV O/JPN/2010 by RT-PCR analysis [29]. At 3 dpi, large vesicles in the skin of the foot, severe ulceration or erosion in the tongue and myocarditis were observed in all three pigs inoculated with O/JPN/2010, and prominent virus antigen was detected concurrently with these lesions [29]. Compared to these results, our present findings revealed that the SPV of FMDV O/JPN/2000 had low pathogenicity against pigs following intraoral inoculation.

FMDV O/JPN/2000 exhibits two plaque phenotypes, LP and SP, during the second passage in BK cells after isolation [18]. Further, SPV shows low pathogenicity, while LPV has high pathogenicity against suckling mice [18]. Typical vesicular lesions were appeared in the foot of pigs which experimentally inoculated with 10^6TCID$_{50}$/ml of FMDV O/JPN/2000 isolate by intraepidermal injection at 2 dpi and the horizontal infection to co-housed pigs was appeared at 2 and 3 dpi [24]. That virulence of FMDV O/JPN/2000 may be due to LPV of O/JPN/2000 (Morioka *et al*., unpublished). The virus used in this study was passaged three times in BK cells and once in BHK-21 cells and showed low pathogenicity against pigs. The amino acid sequence of the capsid-coding region (VP1–VP4) of SPV and LPV have two substitutions, one in the 133rd amino acid in VP2 and the other in the 56th amino acid in VP3 [18]. The 56th amino acid in VP3 is reportedly a heparin sulfate-binding site and affects plaque size and pathogenicity against cattle [6, 11–13, 22]. In this study, the SPV of O/JPN/2000 was attenuated in pigs, a host animal of FMDV, suggesting that the 56th amino acid in VP3 might play an important role in the virus' pathogenicity against pigs, as in cattle.

Previous reports have suggested that FMDV may primarily infect the oropharyngeal tonsil [2, 4, 26–29]. At 1 dpi, we did not detect the SPV of FMDV O/JPN/2000 in serum or the oropharyngeal tonsil, but detected it in the skin of the foot of pigs. In the diseased pig (Pig 3), the virus was isolated from serum and multi-systemic organs at 3 dpi, as the O/JPN/2010 virus was in our previous study. However, no vesicular lesions were observed in the foot at 3 dpi in this study. The distribution of lesions and the virus in pigs in the early stages of infection in this study was atypical compared to those of previous studies [2, 4, 17, 20, 26–28]. We speculate that SPV of FMDV O/JPN/2000 was unable to replicate sufficiently in the oropharyngeal tonsil, which is considered the primary infection site of FMDV. Alternatively, in the case that the virus was able to sufficiently replicate in the oropharyngeal tonsil and cause viremia in a pig that was in poor condition, the replication may not have been sufficient to induce vesicular lesions in the secondary infection site: the skin of the foot. It is possible that prickle cells infected with SPV of FMDV O/JPN/2000 were removed from the epidermis by physiological cellular turn over before the virus was able to sufficiently replicate. Further studies are needed to clarify the detailed mechanism underlying the replication of the attenuated virus and virulent virus of FMD in primary and secondary infection sites and the development of viremia in pigs.

Previous reports indicate that FMD infection induces cell death by apoptosis in susceptible animals [10, 16]. In our previous study, we observed prominent and widely distributed TUNEL-positive labeling indicating apoptosis in the lamellar of the upper layer of the stratum spinosum in the tongue and skin from the coronet to heel before the appearance of cellular injury in FMDV-infected pigs [29]. TUNEL-positive labeling in pigs inoculated with the SPV of O/JPN/2000 was lower and weaker than that in pigs inoculated with O/JPN/2010 [29]. This indicates that the SPV of O/JPN/2000 exhibited low severity virulence for cellular injury by apoptosis. Our findings suggest that the apoptotic process after cellular infection with FMDV might play an important role in vesicular lesion formation in FMDV-infected pigs.

The skin of the foot was negative for viral RNA by RT-PCR but was positive for the viral antigen by IHC in both pigs examined on 1 dpi. This discrepancy may have resulted from the isolation of samples, which we later discovered contained no or minimal antigen. In this study, samples were collected from the interdigital space between the hooves of the foot because our previous study indicated that lesions on the feet first appeared in the interdigital space between the hooves [29]. While we detected some virus antigen in the skin from the interdigital space between the hooves, higher levels were observed in the skin of the coronet in this study. The relationship between the pathogenicity of these two topotypes and the difference in antigen distribution in the skin of the foot is unclear and should be examined in future studies.

We showed that oral inoculation of SPV of FMDV O/JPN/2000 induced an asymptomatic infection, demonstrating the value of this experimental infection model for studying FMD pathogenesis in the continuum of attenuation and virulence of FMDV. Further studies are needed to clarify the mechanism underlying the attenuation of FMDV and the pathogenicity of the attenuated virus against the host animal to improve FMD control and eradication through the development of next-generation countermeasures such as live-attenuated vaccines.

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