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Antigenic properties and virulence of foot-and-mouth disease virus rescued from full-length cDNA clone of serotype O, typical vaccine strain

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We cloned the full-length cDNA of O Manisa, the virus for vaccinating against foot-and-mouth disease. The antigenic properties of the virus recovered from the cDNA were similar to those of the parental virus. Pathogenesis did not appear in the pigs, dairy goats or suckling mice, but neutralizing antibodies were raised 5-6 days after the virus challenge. The utilization of O Manisa as a safe vaccine strain will increase if recombinant viruses can be manipulated by inserting or removing a marker gene for differential serology or replacing the protective gene from another serotype.

Keywords: Foot-and-mouth disease, Vaccine, Molecular cloning

Vaccination is a key method in controlling the spread of foot-and-mouth disease (FMD) [1]. Type O FMD is the most frequently occurring virus in the world [2]. The vaccine strain O1/Manisa/Turkey/69 (O Manisa) can be effectively used to control Middle Eastern-South Asian (ME-SA) and South East Asian (SEA) topotypes [3].

At present, the cloning of all full genomes of type O has been completed for O1K, O1/BFS/1860, OH99, IND-R2/75 GD/China/86, O/HN/CHA/93, and O/QYYS/s/06 virus strains [2,4-10]. O Manisa is the representative vaccine strain that is most often used around the world, but there have been no attempts to produce virus through cDNA cloning. Using reverse genetics for FMD virus (FMDV), especially for the O Manisa strain, can be very useful in the development of new tools to obtain a broader antigenic spectrum of a pathogen through genetic manipulation, weaken the virulence of the virus, insert or delete markers for differentiation from field strains, and improve stability [7,11,12]. Therefore, we cloned the full-length cDNA of FMDV and recovered the virus *in vitro* to verify the possibility of using the O Manisa strain, which has already been developed and proven to be the predominant strain for FMD vaccine.

Viral RNA was extracted from a viral suspension of the FMDV vaccine strain (O1/Manisa/Turkey/69) using the RNeasy kit (Qiagen, Hilden, Germany). We received the O Manisa strain through the generosity of the World Reference Laboratory of FMD (WRL-FMD, Pirbright, UK). Full-length genome cloning was tried as shown in Fig. 1A. The restriction enzyme site, *Sal*I was removed with the KOD-Plus-Mutagenesis Kit (Toyobo, Osaka, Japan). Polymerase chain reaction amplification was performed with Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Vantaa, Finland). Finally, the proper full-length genome cloning of FMDV (pO-Manisa-FG) was con-

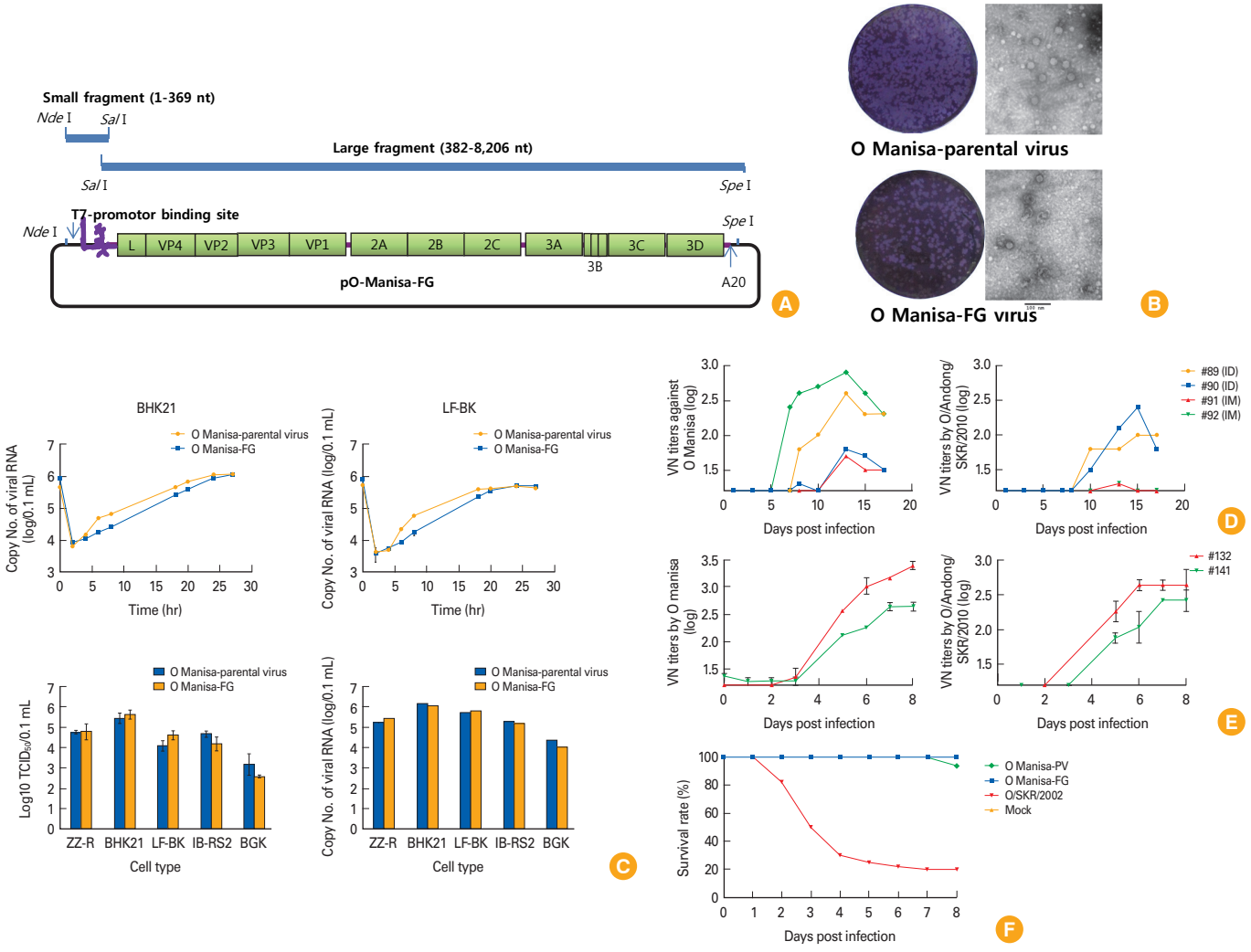

Fig. 1. Recovery of virus from FMDV full-length cDNA clone, and antibody response, pathogenesis in animals after infection of the virus. (A) Recovery of virus from full-length cDNA clone (pO-Manisa-FG) from foot-and-mouth disease virus. The small fragment (1-369 nt), including poly(C) of O Manisa, were cloned into pBluescript II KS (+) vector. The large (382-8,206 nt) were subsequently inserted into plasmid. The redundant restriction enzyme sites were removed (B). The LF-BK cells were overlaid with 2 mL of 1.5% SeaPlaque agarose containing 2% fetal bovine serum in Dulbecco's modified Eagle's medium and cultured at 37°C in 5% CO_2 for three days. The plaques were visualized by staining with a 0.1% crystal violet solution. The recovered virus had a plaque morphology similar to that of the parental virus. For electron microscopy of O Manisa virus, virion purification was performed by sucrose gradient centrifugation. The viruses were concentrated using Amicon ultracentrifuge filters (100 kDa). Samples were observed using electron microscope. The viruses showed size of 28 nm. (C) Growth properties of FMDV, O Manisa-parental virus and O Manisa-FG recovered from pO-Manisa-FG clone. One-step growth kinetics in BHK21 (up-left) or LF-BK cells (up-right). Virus titration by tissue culture (down-left) and quantitative reverse transcription polymerase chain reaction (down-right). There was no significant difference in growth characterization between transfectant virus and the parental virus. (D) VN antibody level after challenge of O Manisa-FG in the pigs. Pigs were challenged by different administration routes (ID injection on the foot-pad with 0.1 mL, and IM injection 1 mL, with $10^{5.0}$ $TCID_{50}$/0.1 mL). The animals did not show clinical signs. The viruses for VN test were O Manisa-parental virus (left) and O/Andong/SKR/2010 strain of SEA topotype (right). (E) VN antibody level after challenge of O Manisa-FG in the dairy goats. Dairy goats were challenged by ID route of the same method as pig injection and the viruses for VN test were O Manisa-parental virus (left) and O/Andong/SKR/2010 (right). (F) Survival in seven-day-old mice after challenge of recovered, parental virus and O/SKR/2002 of $10^{5.0}$ $TCID_{50}$/0.1 mL by intraperitoneal route. FMDV, foot-and-mouth disease virus; VN, virus neutralizing; ID, intradermal; IM, intramuscular; SEA, South East Asian.

firmed by nucleotide sequence analysis. Plasmid pO-Manisa-FG was linearized with *Nde*I and *Spe*I. One day before DNA transfection, baby hamster kidney cells that stably expressed T7 RNA polymerase (BHK/T7-9 cells) [13] were seeded on a 48-well plate. At 80%-90% confluency, the cells were transfected with the linearized DNA using Lipofectamine 2000

(Invitrogen, Grand Island, NY, USA). The plaques in LF-BK cells were visualized by staining with a 0.1% crystal violet solution (Fig. 1B). The recovered virus had a plaque morphology similar to that of the parental virus. Virion purification was performed by sucrose gradient centrifugation. Samples were observed using an electron microscope (Fig. 1B). The virion purified from the recovered virus had a size of 28 nm, the same as that of the parental virus (Fig. 1B). Viral particles were released by three successive cycles of freezing and thawing, and the viral titers were determined (Fig. 1C).

To further compare the growth characteristics of the parental virus and the viruses derived from recombinant plasmids, the growth kinetics of these viruses were examined. We compared the replication in various cells of the field strain of the FMDV and the O Manisa-FG virus produced *in vitro*. Among various cell lines, the best replication was observed in the BHK-21 and LF-BK cells (Fig. 1C). Twenty-four hours after inoculating the cells, the growth potency of most viruses increased to the highest level in the BHK-21 and LF-BK cells. No major difference in growth potency was observed between the two viruses in the BHK-21 cell, which is used as an antigen-producing cell for vaccine manufacture. The O Manisa-FG showed slight low titers compared to the parental virus until 20 hours after infection, but a similar titer was shown at 24 hours (Fig. 1C).

The difference of the genes between the virus recovered from full-length cDNA genome produced *in vitro* (O Manisa-FG) and the parental virus (O Manisa-PV) was that the poly(C) tract and poly(A) tail were longer than that of the field strain (GenBank accession No. AY593823) by three nucleotides (nt) respectively, which was inserted in the O Manisa-FG. The O Manisa-FG in full genome analysis had differences of five amino acids (K765T, E807K, S858C, and K934E in VP1, T1565E in 3A of polyprotein) or twenty-one nucleotides (T21C, T124C, C545T, A580C, T585A, G586A, and C804T in 5´ untranslated region [UTR], G2178A, C2520T, A3392C, G3517A, A3670T, T3831C, A3898G, A4170G, A5791T, T6585C, T6873C, G6993A, and G7464A in coding region, A8169G in 3´ UTR) with those (Genbank accession No. AY593823) of the parental virus (O Manisa-PV) except poly(C) tract and poly(A) tails.

FMDV strains, O Manisa, O/SKR/PJ/2000, O/SKR/AS/2002 for ME-SA topotype, O/Andong/SKR/2010 for SEA topotype, and O/ASP/Cathay for Cathay topotype were used for the cross virus neutralization test (VNT) (Fig. 1D, E). The VNT was performed according to the manual of diagnostic tests and vaccines for FMD by the World Organization for Animal Health (OIE). Serological relationships between the O Manisa parental and recovered virus showed a high r1 value (0.85-1.11) (Table 1). The result from the matching test showed that O Manisa vaccine might protect against ME-SA and SEA topotypes, in case of use of high potency vaccine, even though r1 accuracy is substantially low. O Manisa has been widely used for protection against the ME-SA and SEA topotype. The 2010-2011 outbreaks of the SEA topotype in Japan and South Korea occurred on a large scale and inflicted considerable economic damage by infecting many animals. During this time, the O Manisa vaccine was used to rapidly control the outbreak of FMD in Korea and Japan.

The most important genetic difference between the field strain and the recombinant virus prepared by this study is the

Table 1. Antigenic relationships found in cross virus neutralization test of parental (O Manisa) and the cDNA-recovered virus (O Manisa-FG) against FMD viruses originated in East Asia

Sera from vaccination groups in pigs	Weeks after first vaccination[b]	Mean r_1 value[a] of type O strains for VNT					
		ME-SA topotype				SEA topotype	Cathay topotype
		O Manisa	O Manisa-FG	O/SKR/PJ/2000	O/SKR/2002	O/Andong/SKR/2010	O/ASP/Cathay[c]
Commercial vaccine O Manisa (n = 2)	4	ID	1.11	0.45	0.09	0.34	0.20
	6	ID	0.89	0.30	0.05	0.12	0.04
O Manisa-FG (n = 3)	5	1.08	ID	0.36	0.02	0.10	0.02
	6	0.85	ID	0.32	0.02	0.11	0.02

FMD, foot-and-mouth disease; VNT, virus neutralization test; ME-SA, Middle Eastern-South Asian; SEA, South East Asian; ID, identity.
[a]r_1 values were calculated by division between reciprocal arithmetic titers (serum antibody titer against viruses using for VNT/serum antibody titer against vaccine viruses in the vaccination groups). This experiment was repeatedly tested three times.
[b]Booster at four weeks after first vaccination of commercial monovalent vaccine (high dose of > 6 PD_{50}, Merial Co. Ltd.) and inactivated and oil-adjuvanted O Manisa-FG.
[c]O/ASP/Cathay (phenotype of Genbank accession No. HQ412603).

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Table 2. Pathogenesis in dairy goats and pigs infected with O Manisa-FG virus rescued from cDNA of FMDV, O Manisa strain

Species	Animal No.	Administration routes (injected volume)	Clinical sign		Virus detection in sera and nasal swab after virus challenge	
			Clinical score[a]	Lesions in the injected foot site	Virus isolation	qRT-PCR
Dairy goats	#132	ID (0.1 mL)	0	nd	nd	nd
	#141	ID (0.1 mL)	0	nd	nd	nd
Pigs	#89	ID (0.1 mL)	1[b]	Yes	nd	nd
	#90	ID (0.1 mL)	0	nd	nd	nd
	#91	IM (1 mL)	0	nd	nd	nd
	#92	IM (1 mL)	0	nd	nd	nd

FMDV, foot-and-mouth disease virus; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; ID, intradermal; IM, intramuscular; nd, not detected.
[a]Clinical score was determined by adding the following points: an elevated body temperature of 40°C (1 point), 40.5° (2 points), or 41° (3 points); reduced appetite (1 point) or no food intake and food left over from the day before (2 points); lameness (1 point) or reluctance to stand (2 points); presence of heat and pain after palpation of the coronary band (1 point) or not standing on the affected foot (2 points); vesicles on the feet dependent on the number of feet affected and with a maximum of 4 points; visible mouth lesions on the tongue (1 point), gums or lips (1 point), or snout (1 point), for a maximum of 3 points.
[b]Lesion detected only on the injected foot site.

length of the poly(C) tract. The natural poly(C) tract of the field strain FMDV is 100-420 nt [14], which is very long and difficult to artificially reconstruct and insert. This suggests that the poly(C) tract has very diverse effects on the replication, and that the virus with a large number of poly(C) generally shows higher replication. The length of the poly(A) tail also affects the stability of viral RNA [15]. The length of the poly(A) tail can influence infectivity, but similar results were obtained with 10 nt and 40 nt [16]. Therefore, the addition of over 10 nt would show no big differences in replication. The O Manisa-FG that we manufactured had 15 nt for the poly(C) tract, and 20 nt for the poly(A) tail, and the virus had a titer of about $10^{7.0}$ $TCID_{50}$/mL. If virus titer were to later decrease due to the insertion or removal of marker genes, we could overcome this problem by adjusting the length of the poly(C) tract or the poly(A) tail or replacing 3D polymerase.

Until now, FMD vaccines have been produced by inactivating virulent viruses. However, the trend taken in recent developments of the FMD vaccine is to insert or remove markers through recombination of the vaccine seed virus to differentiate it from the field strain, to improve the stability of the capsid antigen, and to develop viruses with the virulence removed [14]. Through animal experiments conducted according to the guidelines of the Animal and Plant Quarantine Agency (QIA) in Korea, we found no pathogenesis of O Manisa-FG in mice, pigs or dairy goats escaping via the leaking problem in mass culture (Table 2). However, neutralizing antibodies in the animals was raised 5-6 days after challenge by intradermal (0.1 mL) or intramuscular routes (1 mL) of $10^{5.0}$ $TCID_{50}$/0.1 mL. The formation of antibodies in the challenged animals seems to show ability of the virus to replicate without virulence (Fig. 1D-F).

In the future, the utilization of O Manisa as a safe vaccine strain will increase if recombinant viruses can be manipulated by inserting or removing a marker gene for differential serology or replacing the protective gene from another serotype.

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